2010

Hemin Acquisition in Bartonella quintana

Nermi Lee Parrow
The University of Montana

Hemin Acquisition in *Bartonella quintana*

By

Nermi Lee Parrow

B.S., The University of Montana, Missoula, MT, 2002

B.A., The University of Montana, Missoula, MT, 2002

Dissertation

presented in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in Integrated Microbiology/Biochemistry

The University of Montana
Missoula, MT

January 2010

Approved by:

Perry Brown, Associate Provost for Graduate Education
Graduate School

Michael F. Minnick, Chair
Division of Biological Sciences

J. Stephen Lodmell
Division of Biological Sciences

D. Scott Samuels
Division of Biological Sciences

Willard Granath Jr.
Division of Biological Sciences

Keith K. Parker
Biomedical and Pharmaceutical Sciences

Parrow, Nermi, PhD, Fall 2009 Microbiology and Biochemistry

Hemin Acquisition in *Bartonella quintana*

Chairperson: Dr. Michael F. Minnick

Bartonella quintana, a Gram-negative bacterial pathogen, causes Trench fever, bacillary angiomatosis and endocarditis. Transmitted by the human body louse (*Pediculus humanus corporis*), the agent has a tropism for erythrocytes in humans. *In vitro* growth requires an extraordinary concentration of hemin, and genomic analyses indicate several potential uptake systems and iron-responsive regulators. Transcription of the *hbp* genes (hemin binding protein genes) is responsive to alterations in available hemin and an HbpA homolog in *B. henselae* reportedly functions as a hemin receptor in *E. coli hemA* strain EB53. *B. quintana hbpA* was not able to complement EB53, indicating that it is not a hemin receptor. A functional hemin receptor and coordinate uptake system is encoded by the hemin utilization (*hut*) locus. *B. quintana hutA* was able to complement a *hemA* mutation in *E. coli* EB53 and was shown to be TonB-dependent using an isogenic *E. coli hemA tonB* strain.

Fur (ferric uptake regulator) has been described as a global iron-responsive regulator in γ-proteobacteria. If expression is forced, *B. quintana fur* is able to complement an *E. coli fur* mutant, but an endogenous promoter for the gene could not be located and native expression in *B. quintana* was not detected. Overexpression of the iron response regulator (Irr), a Fur family member, in *B. quintana* repressed *hut* locus transcription. Previous studies showed that Irr interacted with a consensus motif, the H-box, in the promoter of the *hbp* genes. A region with homology to the H-box consensus is present in the divergent promoter between *hutA* and *tonB* and in the promoter region of *hemS*.

The fate of hemin in the bacterial cytoplasm is not well understood. HemS is a potential hemin storage/degradation enzyme. Initial characterization indicates that HemS is able to bind hemin in a 1:1 fashion with an estimated dissociation constant (Kd) of 5.9 $\pm$ 1.7 μM. Complementation analyses using *Corynebacterium ulcerans* CU712*hmuO*Δ strain have not been successful but future experiments plan to use an *E. coli chuS* strain. These studies have characterized the principal hemin uptake system of *B. quintana*, identified its transcriptional regulator, and initiated investigation of a potential heme oxygenase.

ACKNOWLEDGEMENTS

I wish to thank my committee members, Scott Samuels, Steve Lodmell, Bill Granath and Keith Parker, as well as those who served on my comprehensive exam committee, Scott Wetzel and Jesse Hay, for their support, guidance, and expertise. I would be remiss in not acknowledging Michele McGuirl for providing both assistance and support in all of my scientific endeavors since my undergraduate years. I am indebted to Gretchen McCaffrey for assistance with editing this manuscript. I am grateful to Jane Koehler, Christopher Elkins, Michael Schmitt, and Christine Martin for providing bacterial strains and advice. Many thanks to past and present members of the Minnick lab, Jim Battisti, Kate Sappington, Laura Hall, and Linda Hicks, for sharing their knowledge. I especially appreciate Jessica Clarke and Julie Callison for all of the laughs and Rahul Raghavan for also working weekends. I wish to thank Guy and Whitney Leibenguth and Mike and Matt Grunow for supplementing my funding. Most importantly, I wish to thank my mentor, Mike Minnick. Words are completely inadequate, but I am nonetheless grateful for the inspiration, knowledge, patience and generosity. This work is dedicated to Anne Elaine Beal and Leroy Weston Beal II.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

Introduction to *Bartonella quintana* and bacterial hemin acquisition

I. *Bartonella quintana*

A. INTRODUCTION

The genus *Bartonella* encompasses 31 species and 3 subspecies of Gram-negative facultative intracellular bacteria that belongs to the α2-proteobacterial subclass (See Table 1.1, p.18). To date, eighteen of these species have been solely associated with nonhuman mammalian hosts, to date. Another 10 *Bartonella* species are implicated in occasional reports of human illness suggesting incidental infection. In contrast, *B. quintana* and *B. bacilliformis* utilize the human host as the primary mammalian reservoir and have been recognized as human pathogens for a long time (89). Together with *B. henselae*, these three species are the primary *Bartonella* agents of human disease (122).

Alternative mammalian hosts have not been identified for either *B. quintana* or *B. bacilliformis*, but cats provide an alternative reservoir for *B. henselae* (121). Although *B. henselae* has been detected in cat fleas (*Ctenocephalides felis*), transmission to humans is believed to be mediated by the scratch or bite of a cat (189). Typical *B. henselae* infections in humans result in cat-scratch disease, but complications can include endocarditis, bacillary angiomatosis, bacillary peliosis, neuroretinitis and chronic bacteremia (56, 121). Due to the restricted geographical range of its sandfly vector (*Lutzomyia* spp.), infection with *B. bacilliformis*, which results in Carrion's disease, is less common but more serious than other *Bartonella* infections (89). Carrion's disease presents itself in two distinct phases. The initial phase, Oroya fever, is characterized by severe hemolysis-driven anemia and fever. The next phase, verruga peruana, is characterized by the development of vasoproliferative skin lesions (41, 89). Like *B. bacilliformis, B. quintana* has been described as a 'specialist' based on its strict body louse vector-human host cycle that lacks an alternative mammalian reservoir (4). Transmission to humans is mediated by the human body louse (*Pediculus humanus corporis*), which can also transmit the bacterial pathogens that cause relapsing fever and typhus, *Borrelia recurrentis* and *Rickettsia prowazekii*, respectively (3). *B. quintana*

infections cause trench fever and are currently re-emerging as 'urban trench fever'. Complications arising from *B. quintana* infection, like *B. henselae*, include chronic bacteremia, endocarditis, and bacillary angiomatosis (57).

B. GENOME AND EVOLUTION

The genomes of four *Bartonella* species, *B. bacilliformis, B. henselae, B. quintana*, and *B. tribocorum* have been sequenced. Common themes among all four genomes include a core of 959 genes, a G+C content of 38.2 to 38.8%, and a low-coding density of 72.3 to 81.6% (52). In addition to being the first recognized pathogenic member of the *Bartonella* genus and the most virulent, *B. bacilliformis* is thought to represent an ancient lineage while the remaining two major *Bartonella* pathogens, *B. henselae* and *B. quintana*, along with *B. tribocorum* appear to have evolved more recently. The crux of this argument is that the 'modern' lineage, including *B. henselae*, *B. quintana*, and *B. tribocorum*, have genetically acquired factors leading to attenuated virulence and improved host adaption. Genomic analyses indicate that 66 genes required to establish intraerythrocytic infection in a rat model are common to all four *Bartonella* species examined. An additional 15 pathogenicity genes are shared by the representatives of the 'modern' lineage but are absent in *B. bacilliformis*. Fourteen of these 15 genes are components of type IV secretion systems, which allows for the delivery of effector molecules into host cells resulting in enhanced host adaptation (151). The acquisition of these factors is a characteristic of the 'modern' lineage that may contribute to the establishment and maintenance of chronic infections rather than the severe acute phase of *B. bacilliformis* infection.

Direct genomic comparisons between *B. henselae* and *B. quintana* indicate that the *B. quintana* genome is a reduced version of the *B. henselae* genome. Furthermore, both genomes appear themselves to be reduced versions of *Brucella melitensis* chromosome I. In particular, *B. henselae* has a 1.93 megabase pair (Mb) genome containing 1,491 protein coding genes, 301 of which are unique to *B. henselae.* *B. quintana* has a 1.58Mb genome encoding 1,143 proteins, 26 of which are unique to *B. quintana* (4, 32). Like *B. quintana*, *B. bacilliformis* has a small genome consisting of 1.44Mb encoding 1,283 genes (52). Reductive genome evolution is consistent with an intracellular lifestyle and

vector-borne transmission (32). In the case of *B. quintana* and *B. bacilliformis*, it may reflect the utilization by both species of restricted host-range insect vectors and the human host. Alternatively, the genomic expansion observed in *B. henselae* and *B. tribocorum* may reflect selective pressures unique to the cat and rat reservoirs, respectively.

C. HISTORICAL BACKGROUND

Paleomicrobiological methods have been used to amplify *B. quintana* DNA recovered from dental pulp removed from human remains that are estimated to be more than 4000 years old by radiocarbon dating (45). Likewise, *B. quintana* DNA has been recovered and amplified from the remains of 7 Napoleonic soldiers and 3 associated body lice found in a mass grave in Vilnius, Lithuania. Local records date the grave to the period of Napoleon's retreat from Russia in 1812 (20, 137). Despite this evidence of its longevity, the first published reports describing Trench fever occurred during World War I (WWI) (57, 129). Second only to influenza as a leading cause of illness during WWI, Trench fever affected an estimated 1 million soldiers (57, 122). Signs and symptoms were somewhat variable, but typically consisted of possibly relapsing febrile episodes accompanied by chills, headache, and body aches with a focus on the shin (177). The end of the war brought with it improved hygienic conditions resulting in decreased occurrence of trench fever. However, World War II (WWII) initiated another round of epidemics resulting in continued interest in the causative organism of the disease (119). Undoubtedly due to difficulties encountered during attempted *in vitro* cultivation of the organism, *B. quintana* (then *Rickettsia quintana* later *Rochalimaea quintana*) was initially thought to be a *Rickettsial* species (180). Nonetheless, *B. quintana* was definitively identified as the agent of Trench fever with the fulfillment of Koch's postulates by 1969 (177, 181). The end of the WWII again brought improvements in hygienic conditions that were not conducive to louse infestation or the transmission of Trench fever resulting in diminished incidence of and interest in *B. quintana* that would last for more than 2 decades.

Interest in *B. quintana* infections would be renewed again in the 1980s. Although the authors were unaware of it at the time, a 1981 report of *Pneumocystis carinii* infections in

five previously healthy homosexual men marks the first report of AIDS, which is caused by the human immunodeficiency virus (HIV) (67). By 1990, reports of opportunistic *Bartonella* infections in immunocompromised individuals resulting in the development of angioproliferative skin lesions similar to the verruga peruana produced by *B. bacilliformis* began to surface. The condition was called bacillary angiomatosis (BA) (109, 142). *B. quintana* was isolated from the BA lesions of three HIV-positive individuals in 1992 (95). Within a few years of this report, *B. quintana* was simultaneously reported as a cause of bacteremia and blood culture negative endocarditis (BCNE) in homeless populations in Seattle, WA, USA and Marseilles, France (44, 163). More than a decade later, *B. quintana* is widely recognized as a significant re-emerging agent of disease especially in inner city homeless populations and immunocompromised individuals.

D. TRANSMISSION

B. quintana is found extracellularly in the gut lumen of *P. humanus corporis* and is able to replicate and survive there. Infection does not affect the longevity of the body louse and is not transmitted to louse offspring. *B. quintana* is excreted by the louse in a biofilm-like matrix in feces where it survives for up to a year. Lice feed up to five times per day on humans and release factors when biting that produce irritation and itchiness. Transmission to humans is thought to occur when scratching generates breaks in the skin that allow inoculation with the bacteria-laden louse feces (36, 82). Although uptake occurs via an unknown receptor, *B. quintana* has been identified intracellularly in erythrocytes in the human host. However, only a small percentage of red blood cells were found to contain *B. quintana* in the blood of patients with chronic bacteremia. No evidence of *B. quintana*-mediated hemolysis or anemia has been found. (145). *B. quintana* also has a tropism for endothelial cells and is rapidly endocytosed into a vacuole where it survives and replicates (26). The ability of *B. quintana* to infect erythrocytes undoubtedly promotes immune evasion and transmission to the body louse. Likewise, the ability of *B. quintana* to remain infectious in louse feces for long periods of time undoubtedly enhances it transmission to humans. Thus the transmission cycle is a good example of the adaptation of *B. quintana* to its unique and limited host-vector range.

E. DISEASE AND EPIDEMIOLOGY

Re-emerging urban trench fever in HIV-negative individuals was described almost simultaneously in the U.S. and France. Although somewhat variable, signs and symptoms similar to classical trench fever were described in primarily homeless, alcoholic, male populations. Chronic bacteremia and endocarditis were also noted in the initial reports (44, 163). Although there were some issues with the species specificity of the assay used, followup studies at a downtown Seattle, WA clinic for homeless and indigent persons reported that 20% of its subjects were seropositive for *B. quintana* (83). Since that time, numerous studies have been undertaken in order to estimate the prevalence of *B. quintana* in homeless populations. Of note, the population at-risk for urban trench fever and a variety of other infectious diseases, namely the homeless, is by its very nature dynamic and difficult to identify, treat, and conduct followup exams on (138). These facts may account for some variability in results. Alternatively, the variability may be the result of environmental factors favoring epidemics at particular time points with lower prevalence rates observed between epidemics. Finally, the studies are significantly different from one another in terms of the diagnostic measures and target populations. Collectively these issues result in a fairly wide range of prevalence. For example, a 1997 study of seroprevalence rates with 221 samples taken from eight charitable institutions dedicated to the homeless over the course of five months reports positive serology in 1.8% of the population (147). A later study in the same area focused on 71 homeless emergency room patients and reported that 14% had positive blood cultures for *B. quintana* and 30% had high antibody titers (25). During an epidemic typhus outbreak in a refugee camp in the East African Republic of Burundi, up to 12% of suspected typhoid cases also had increased antibody titers to *B. quintana* (139). In a 2005 Marseilles, France study of 930 subjects from two homeless shelters over the course of four years, 22% were infested with body louse and 5.3% were blood-culture positive for *B. quintana* (27). A wide range of seroprevalence rates have also been obtained in U.S. studies. A 1996 study from Baltimore, MD reported elevated *B. quintana* antibodies in 10% of 630 subjects, while a 2001 study of 204 subjects from New York City found only 2% were seroreactive to *B. quintana* (39, 40). Yet another study examined 200

samples obtained from patients at a downtown Los Angeles clinic and reported 9.5% seroprevalence of antibodies to *B. quintana* (162). High titers of *B. quintana* antibodies were also reported in 11% of 151 blood samples from homeless people in Tokyo, Japan (158). One study focused solely on immunocompromised individuals and found that among 382 HIV-positive individuals with a fever of unknown origin, *B. quintana* or *B. henselae* was the cause in 18% of cases. The precise number of fevers attributable to *B. quintana* could not be determined due to cross reactivity between the two species in the assay (97). Again, the differences in reported prevalence of *B. quintana* undoubtedly reflect the differences in diagnostic technique, target population, and study design.

Another series of studies has examined the prevalence of *B. quintana* in body lice. *B. quintana* DNA was amplified by polymerase chain reaction (PCR) in 33% of body lice collected from homeless people in Russia in 1996 and 1997 (150). A recent San Francisco, CA study amplified *B. quintana* DNA from 33% of louse-infested individuals and reported an overall rate of 23.9% body louse infestation in 138 participants. Interestingly, these authors also amplified *B. quintana* DNA from head lice obtained in the same study even though *Bartonella* is not usually associated with this subspecies of *P. humanus* (22, 63). Like seroprevalence rates, a review of a number of these studies shows wide variation in the percentage of lice infected with *B. quintana*. For example, *B. quintana* was detected in 4-26% of lice from Marseilles, 36% of lice examined in the Netherlands, 2.3-93.9% of lice examined in Burundi and only 1.4% of lice in Peru (60). Again, the variability observed in different studies may reflect environmental factors favoring high rates of infection and transmission.

In addition to urban trench fever and chronic bacteremia, *B. quintana* and *B. henselae* are the primary species responsible for *Bartonella*-mediated infective endocarditis (IE) and are thought to account for approximately 1-3% of all infective endocarditis (16, 59). Similarly, one study indicated that *Bartonella* species account for more than one-fourth of blood-culture negative endocarditis cases (BCNE) with *B. quintana* responsible for 53 of the 70 cases (79). Serological testing for *Bartonella* has been suggested as a modification to the original Duke criteria used for the diagnosis of IE (111). Not surprisingly, epidemiologic analyses indicate that risk factors for *B. quintana*-mediated endocarditis include exposure to body lice, alcoholism, and homelessness. This

investigation also found that patients with *Bartonella*-mediated endocarditis were at least twice as likely as controls with endocarditis caused by other organisms to require valvular surgery and were as likely as controls to have experienced embolic complications (59). Additional life-threatening complications associated with IE include congestive heart failure and neurological problems secondary to embolic events (126).

Although isolated reports of the disease in immunocompetent individuals exist, bacillary angiomatosis is almost entirely associated with immunodeficiences (64, 174). Characterized by vasoproliferative lesions containing bacilli observable by Warthin-Starry staining that can occur in a number of different organs, BA has an estimated overall prevalence of 1.2 cases per 1000 HIV-positive individuals (121, 133). Although infection with other *Bartonella* species can also result in BA, reports suggest that *B. quintana* is the causative agent in just under half of the cases examined. Furthermore, *B. quintana*, rather than *B. henselae*, is more likely to be involved in the development of subcutaneous and bone lesions (65, 96).

F. DIAGNOSIS AND TREATMENT

Given the extraordinary range of clinical manifestations of *B. quintana* infection and the fastidious nature of the organism, diagnosis and isolation can both be challenging. In cases of suspected urban trench fever and chronic bacteremia, diagnosis is typically made by blood culture and serological testing. Blood culture techniques have been marginally successful, but basically consist of either direct plating onto solid media or co-cultivation of the sample with cell culture (57, 88). Even when successful, primary isolation of *Bartonella* can take up to two weeks. However, refined protocols specific to species and tissue sample can enhance *Bartonella* recovery (57). Numerous serological tests are available including one that was developed and characterized by the Centers for Disease Control (CDC). Problems exist with respect to cross reactivity with other species, including *Chlamydia pneumoniae* and *Coxiella burnetti*, a lack of anti-*Bartonella* antibody production in up to 25% of blood culture positive HIV-infected individuals, and slow development of antibodies to *Bartonella* in some immunocompetent individuals. In suspected cases of endocarditis and bacillary angiomatosis, additional diagnostic tests include PCR and histopathological examination of tissue. As previously mentioned, BA

lesions contain bacilli that can be seen by Warthin-Starry silver staining and clusters of bacteria are visible in valvular tissue from *Bartonella*-mediated endocarditis (57, 88). Various PCR targets have been used to differentiate *Bartonella* species, including but not limited to 16S rDNA, the heat shock protein gene *htrA*, the citrate synthase gene *gltA*, and the 16S-23S rDNA intergenic spacer region (ITS) (121).

Although trench fever can be self-limiting, the occurrence of chronic bacteremia in homeless people has led to the recommendation that the disease should be treated with 200 mg of doxycycline by mouth daily for 28 days combined with a 14-day regimen of intravenous administration of gentamicin (3 mg/kg body weight). In the case of bacillary angiomatosis, erythromycin or doxycycline (500 mg 4 times daily or 100 mg twice daily, respectively) are the drugs of choice and treatment should be maintained for at least 3 months. Treatment recommendations for *Bartonella*-mediated endocarditis consist of intravenous administration of gentamicin (3 mg/kg body weight) daily for 14 days (88, 144). Risk factors for *B. quintana* infection include homelessness, alcoholism and exposure to body lice. Providing complete clothing changes, boiling infested clothing and sheets, as well as dusting with insecticides, can be used to counter louse infestation. Additionally, oral treatment with ivermectin can result in short term decreases in both number of lice per infested individual and in prevalence of louse infestation (58, 138). With respect to immunocompromised populations and associated *B. quintana*-mediated disease, prophylactic antibiotic treatment is not recommended. Not surprisingly, prophylactic treatment for other infections with macrolides or rifamycin is also effective against *B. quintana* (88).

II. HEMIN

A. HEMIN REQUIREMENTS

The earliest attempts of *in vitro* cultivation of *B. quintana* required media supplemented with erythrocytes. Based on the fact that the red blood cells retained their growth enhancing properties even after being subject to freeze/thaw cycles or autoclaved, hemoglobin was suspected as the critical factor provided by them. Examination of *B. quintana* growth as a function of hemoglobin concentration in a blood-agar baser

indicated 4 mg/ml was sufficient provided the medium was also supplemented with serum (179). Further analyses indicated that the serum requirement could be fulfilled with bovine serum albumin (BSA), which could in turn be replaced with charcoal or starch. Under these conditions, either hemoglobin or high concentrations of hemin (20-40 μg/ml) were sufficient to support growth. The authors propose a detoxification role for serum and suggest that hemin may be required for hydrogen peroxide breakdown (125). *In vitro* studies in the closely related species, *B. henselae*, served to further define the precise components of hemin required for growth of *Bartonella*. Lactoferrin, transferrin, and iron, alone or in combination with protoporphyrin IX, were unable to satisfy the hemin requirement suggesting that hemin itself is the essential component of erythrocyte lysates for supplying *Bartonella* with both iron and protoporphyrin IX (152). These observations were validated by genomic analyses, which indicate that *Bartonella* species lack most of the enzymes required to synthesize porphyrins. Ferrochelatase catalyzes the addition of iron (II) to protoporphyrin IX to generate heme and is among the missing enzymes (4). The importance of hemin to survival and pathogenesis of *Bartonella* is underscored by *in vivo* studies that indicate mutagenesis of any of a number of proteins involved in hemin acquisition interferes with the establishment of chronic intracellular infection of erythrocytes in a rat model (151). Furthermore, *Bartonella* species have one of the highest reported hemin requirements amongst bacterial pathogens. Notably, a similar concentration of hemin is required for growth of some *Haemophilus* species that also lack porphyrin biosynthesis pathways (172).

B. HEMIN AVAILABILITY

Heme is a required component of a number of proteins vital to cellular function in pathogen, vector and host. For example, it is a key component of cytochrome c, myoglobin, nitric oxide synthase, prostaglandin synthase, and hemoglobin. In fact, hemoglobin is the most abundant protein in blood and hemoglobin-bound heme concentrations are approximately 10 mM (68). However, free hemin is toxic in excess due in part to its hydrophobic nature that allows it to disrupt membrane and cytoskeletal interactions. Additionally, the iron moiety of heme is able to participate in Fenton chemistry resulting in the generation of reactive oxygen species (ROS). Accordingly,

incubation with free hemin can result in protein oxidation leading to cross-linking and aggregation, lipid peroxidation, single-strand DNA scission or nicking of DNA and subsequent degradation. Likewise, bloodstream hemoglobin can be oxidized to methemoglobin by activated inflammatory cells resulting in an enhanced release of hemin and all of its associated toxicities (11). A number of strategies exist to limit heme-mediated cytotoxicity. One strategy, employed by bacteria, humans and number of other organisms, is the breakdown of heme into biliverdin, free iron (Fe^{2+}) and carbon monoxide (CO) by heme oxygenases. Another strategy is the production of high-affinity scavenger proteins such as haptoglobin, which binds hemoglobin, and hemopexin, which binds heme, thereby neutralizing their ability to cause cellular damage (103). Serum proteins such as transferrin and lactoferrin are also produced to sequester free iron and prevent its participation in the generation of damaging reactive oxygen species (103). Exacerbating the iron and hemin limitation in the human host and underscoring its importance to the survival and pathogenesis of bacteria is the fact that iron sequestration is an innate immune response to infection. Mechanisms include decreasing absorption of dietary iron, increasing serum concentrations of transferrin, increased production of hemoglobin, haptoglobin and hemopexin, and localized release of lactoferrin by neutrophils (7, 112). The hemoglobin/hemopexin scavenger receptor, CD163, is released from the cell surface as a soluble molecule and cell surface expression is subsequently upregulated in response to lipopolysaccharide- and bacterial flagellin-mediated activation of Toll-like receptors (185).

In contrast to the human host, little is known about the fate of hemoglobin in the louse gut during digestion of a blood meal. It is generally accepted that blood-sucking arthropods face a unique challenge with respect to hemin detoxification as either free or bound hemin is thought to exceed 5 mM during digestion of a blood meal (68). Although the mechanisms employed by *P. humanus* are not known, several adaptations have been reported in other hematophagous insects. Several reports suggest that certain ticks digest hemoglobin intracellularly (1, 107). In contrast, some mosquitoes and other insects sequester heme in the gut lumen by surrounding it with extracellular matrix proteins or phospholipids membranes. Alternatively, they may surround the gut cells with a protective matrix (107).

C. HEMIN UPTAKE SYSTEMS

Utilization of host hemin-containing proteins as a source of both hemin and iron is a common strategy employed by successful pathogens. In fact, recent studies in *Staphylococcus aureus* indicate that hemin is preferred over transferrin as an iron source (161). Both classical and unique heme uptake systems have been characterized in several bacterial pathogens. At least two common solutions to the heme uptake problem exist in Gram-negative bacteria. The first is the use of outer membrane receptors that directly bind heme or heme-containing proteins (See Fig. 1-1A, p. 19). Heme receptors are further subclassified on the basis of substrate specificity. For example, the BhuR receptor of *Bordetella avium* has been classified in the 'heme scavenger' receptor category based on its ability to use hemin, hemoglobin, myoglobin and catalase while the HmbR receptor of *Neisseria meningitidis* is in the more specific hemoglobin subclass based on its limited ability to obtain heme from other hemoproteins (124, 132).

The second common solution combines the utilization of outer membrane receptors with secreted high affinity hemophores. Hemophores obtain heme from hemoproteins in the vicinity and deliver it to the appropriate receptor. A classic example of this solution is the recently crystallized HasA/HasR system of *Serratia marcescens* (See Fig. 1-1B, p. 20). Crystallographic data indicate that the transfer of heme from the high affinity hemophore, HasA, to the lower affinity receptor, HasR, is mediated by a small steric hinderance upon completion of docking that dislodges the heme from HasA (102).

In either scenario, once heme is bound to the outer membrane receptor it is transported into the cell by virtue of a periplasmic binding protein and an ATP binding cassette (ABC) transport system. Heme is slightly larger than the upper threshold size of molecules able to diffuse across membranes but has been shown to transverse model lipid bilayers (66). Regardless, heme transport across the outer membrane is energy-dependent and that energy is obtained from the proton motive force of the inner membrane through the TonB/ExbB/ExbD complex. TonB is in direct contact with receptors through its C-terminal domain and its N-terminal domain is in contact with ExbB and ExbD. The complex is thought to bind protons used to energize TonB through

conformational changes, which are then transduced to the outer membrane through interaction with a weakly conserved amino acid consensus sequence found in TonB-dependent receptors termed the TonB box (172). TonB-dependent receptors share structural similarity; they consist of 22 anti-parallel β-strands and share an N-terminal plug (102). Additionally, heme receptors share a certain amount of amino acid sequence similarity including conservation of the characteristic FRAP/NPNL domain (183).

The fate of heme upon entering the cytosol is unclear. Most heme uptake loci from bacterial pathogens include a putative heme degrading/storage enzyme. In some species, e.g. *E. coli* O157:H7, these enzymes have been shown to function as heme oxygenases while homologous proteins in other bacterial pathogens have been described as heme chaperones or storage molecules (90, 169, 193).

Genomic analyses of *B. quintana* indicate the presence of at least three systems with the potential for involvement in heme or iron uptake (4) . One of these is the *hut* locus, encoding a potential hemin receptor and a coordinate ABC transporter system (discussed in detail in Chapter 3). A second locus, designated *yfe* (A-D), encodes a putative ABC transporter implicated in manganese, zinc, and possibly ferric iron acquisition, but lacks a homolog to *yfeE*. YfeE is an essential component of the system in *Yersinia pestis*, as evidenced by its requirement for complementation of an *E. coli* strain unable to produce the enterobactin siderophore (15). The final locus, *fatB-D*, encodes a putative siderophore transporter system similar to that described in *Vibrio* species (113). Notably, *B. quintana* also encodes a five member family, HbpA-E, of porin-like proteins that have been shown experimentally to bind hemin despite the absence of structural or sequence similarity to classical hemin receptors (discussed in detail in Chapter 2) (31, 123).

D. REGULATION OF HEMIN UPTAKE

Like mammalian cells, bacteria are also sensitive to iron- and hemin-mediated oxidative damage. Therefore, survival of bacterial pathogens requires a balance between obtaining sufficient iron and heme in the limiting environment of the human host such that required levels are available and preventing the accumulation of intracellular concentrations high enough to cause injury. Furthermore, *B. quintana* must be able to rapidly shift between the iron-replete louse gut and the iron-deficient human host.

Numerous examples of iron-responsive transcriptional regulation exist in the literature. Although homologs exist in many bacterial species, the ferric uptake regulator (Fur) of *E. coli* is the most well studied example. Under iron-replete conditions, dimeric Fur binds ferrous iron (Fe^{2+}), which causes a conformational shift that allows binding to a consensus sequence (Fur box) and repression of target genes (28). Reports confirming the role of Fur as a "global" iron responsive regulator are primarily associated with other members of the γ-proteobacterial subclass. A Fur homolog exists in all pathogenic *Bartonella* species, and its role will be discussed in detail in Chapter 2 (131).

In species more closely related to *Bartonella*, such as *Agrobacterium* and *Rhizobium*, the function of Fur has been largely delegated to novel iron response proteins, namely the iron response regulator (Irr) and/or the rhizobial iron regulator A (RirA) (71, 148, 190). RirA has primarily been characterized in α-proteobacteria and appears to be an iron-sulfur cluster regulator (IscR). Mutational analyses suggest that it can function as either a transcriptional activator or a repressor and target genes include transport systems involved in hemin and iron acquisition (148, 171, 178). The *B. quintana* RirA homologue has 85% amino acid similarity to *R. leguminosarum* RirA.

Irr is a member of the Fur family, but responds directly to intracellular hemin concentrations rather than iron and can either transcriptionally activate or repress genes depending on the location of its cognate DNA consensus motif (71, 149). In *Bradyrhizobium japonicum,* Irr is transcriptionally regulated by Fur and post-translationally regulated by hemin, which rapidly degrades the protein upon binding to it via two heme response motifs (HRM) (194). In contrast, the *Brucella abortus* Irr homolog remains stable even upon heme binding (117). Irr has been shown to repress a number of genes, including those involved in protoporphyrin biosynthesis under iron-limiting conditions. Positive regulation of both heme and iron uptake systems has been reported (117, 149). The *B. quintana* Irr protein has 72% amino acid similarity to *B. japonicum* Irr. The precision and speed required for *B. quintana* to react to shifts in available hemin as well as the number of systems dedicated to the uptake of this critical factor suggest a complex regulatory network requiring one or more of the aforementioned regulators to fine tune uptake and detoxification systems.

E. IRON/HEMIN-MEDIATED REGULATION OF VIRULENCE

Iron and hemin limitation in the human host serve as environmental triggers for the expression of virulence factors in many bacterial pathogens. For example, the expression of Shiga-like toxin (SLT-1), which interferes with eukaryotic ribosomal function, is repressed by Fur in enterohemorrhagic *E. coli* strains. Exotoxin A of *Pseudomonas aeruginosa* is also under Fur control, albeit indirectly through the Fur-regulated alternative sigma factor, PvdS (80). A classic example of iron-regulated expression of virulence factors is the diphtheria toxin from *Corynebacterium diptheriae*, which is under control of the DtxR regulator (112). DtxR also controls siderophore biosynthesis and hemin uptake in *Corynebacterium* (188). Although the implications are not clear, a recent study examining lipid A heterogeneity in *Porphyromonas gingivalis* indicates increased production of monophosphorylated tetra-acylated and diphosphorylated penta-acylated lipid A when the organism was grown in media containing high (10 μg/ml) concentrations of hemin compared to the production of only monophosphoryl penta-acylated lipid A when grown in the presence of 1 μg/ml. Of note, lipopolysaccharide (LPS) containing penta-acylated lipid A has been shown to act as a Toll-like receptor 4 (TLR4) agonist (2). Another recent study examining alterations in gene expression in response to iron limitation in *Bacillus anthracis* reported the induction of two putative internalin genes. Although the function of these genes is not precisely defined, a homolog in *Listeria monocytogenes* has been implicated in epithelial cell invasion. The role of these genes in *B. anthracis* virulence was established when deletion of either or both internalin genes resulted in a substantial increase in the number of spores required to kill half of the mice tested (LD_{50}) (30). These examples indicate that the shift to low iron and hemin is frequently an indicator of host invasion to pathogens and results in coordinate control of factors required for virulence.

III. RESEARCH SIGNIFICANCE AND GOALS

A. SIGNIFICANCE

Hemin is a critical factor for the growth of *Bartonella* but the mechanisms employed to obtain it, the regulatory networks controlling those mechanisms and the basis of the extraordinary requirement remain elusive. Reports describing the Hbp family members as potential heme receptors combined with identification of a Fur homolog in *Bartonella* provided much of the impetus for the current studies (123). The lack of structural similarity to known heme receptors makes the role of the Hbps in heme acquisition truly intriguing. Identification of HbpE as one of the predominant proteins recognized by convalescent sera from patients infected with *B. quintana* indicates a role virulence and pathogenesis (23). *Bartonella* species are currently recognized as emerging agents of a number of human diseases ranging from self-resolving flu-like diseases to life threatening endocarditis, but very little is known about mechanisms of pathogenicity. Furthermore, Hbp homologs exist in several bacterial species, including *Brucella* and *Neisseria* (123). Therefore, any further definition of Hbp function has the potential to be generalized to a number of important pathogens. Likewise, not only do *Bartonella* species exhibit an absolute requirement for heme in order to grow *in vitro,* one species has been used to show that both HbpB and several components of the *hut* locus are required to establish intracellular infection *in vivo* (151). Delineation of the roles of the Hbps and the *hut* locus proteins is significant because it will either expand or validate the current paradigm of hemin acquisition in Gram-negative bacterial pathogens. Bacterial hemin acquisition systems are an attractive target for rational drug design because inhibition of these systems is likely to be synergistic with the innate immune response and lethal for the pathogen.

Regulatory networks in *Bartonella* are largely undefined. Regulation of heme acquisition is significant in and of itself, but as argued previously many virulence factors are under transcriptional control of iron-responsive regulators. Given the enormous differences in heme availability thought to be encountered by *B. quintana* in the human host and body louse vector, environmental heme concentrations may serve as an even more potent cue indicative of the host environment in this species than it is in others. Iron-responsive regulation in Gram-negative bacteria has primarily been attributed to the Fur protein owing to the fact that the majority of studies have been done in *E. coli*. Identifying Fur targets in *Bartonella* and examining the activity of the less well known α-

proteobacterial regulators Irr and RirA will be an important first step in defining the regulatory network of this hemotrophic bacterium. On a more concrete and practical level, these studies may begin to characterize inducible species-specific promoters with potential utility in genetic manipulation.

Finally, the fate of heme upon delivery to the cytoplasm by classical heme uptake systems is not precisely known. Heme oxygenases (HO) have been identified in mammalian cells; bacterial HOs have various levels of homology to them (62, 156). Recent reports suggest that the HemS homolog in *E. coli*, ChuS, is a unique HO (169). This is in direct contrast to reports in other bacterial species that suggest that this protein stores, protects, or transports heme. While these multiple functions are not necessarily exclusive, no reports of overlapping function exist. The function of this protein is of particular interest in *B. quintana* because examination of the genome suggests that this organism does not possess any sort of iron storage protein, e.g. bacterioferritin (Minnick and Battisti, unpublished data). Iron acquisition per se has not been described in *Bartonella* beyond reports indicating that iron alone is insufficient for *in vitro* growth (152). Even if independent iron uptake systems exist, free iron outside of the heme molecule, or perhaps hemosiderin, is unlikely to be abundant in an erythrocyte. It seems likely that heme serves as both the porphyrin source and the iron source in *B. quintana*, suggesting the necessity of a heme oxygenase. Of additional importance, ChuS is notably absent from avirulent strains of *E. coli* and homologous proteins are present in the genomes of many bacterial pathogens (169). Regardless of the mechanism used by these proteins to neutralize heme, it undoubtedly contributes to the pathogens' ability to colonize the mammalian host. Like other components of bacterial heme uptake systems, ChuS-like enzymes represent an attractive target for the rational design of antimicrobial peptides in part due to the absence of similarity to mammalian heme oxygenases.

B. RESEARCH GOALS

These studies were undertaken to elucidate the mechanisms and regulation of heme acquisition in *B. quintana*. Initial experiments focused on the Hbps and Fur. Based on descriptions of Fur as a 'global' iron-responsive regulator, the first goal was to determine the function of *B. quintana* Fur by complementation of an *E. coli fur* strain. The ability

of the Hbps to bind hemin and confer a hemin-binding type on *E. coli* was well established, but expression profiles in response to alterations in available heme were not defined. Therefore, the second goal was to examine hemin-mediated regulation of *B. quintana* HbpA. The third goal was to examine the effect of *B. quintana* Fur on *hbp* transcription. However, difficulties in identifying natively expressed Fur led to the modification of this goal such that it became an attempt to identify expression of *B. quintana* Fur by either *in vitro* transcription/ translation (IVTT) or Western blotting. The HbpA homolog, Pap31, in *B. henselae* was shown to function as a hemin receptor by complementation analyses in an *E. coli hemA* strain (199). The fourth goal was to examine the ability of HbpA to similarly function as a hemin receptor by complementation analyses.

The second set of experiments focused on characterizing the function and regulation of the *B. quintana hut* locus. The primary goal of this set of experiments was to examine the ability of HutA to function as a hemin receptor by complementation analysis of the *E. coli hemA* strain EB53. Classical heme receptors are typically TonB-dependent. The ability of HutA to function as a heme receptor in an otherwise isogenic *E. coli* strain with a second mutation in *tonB* was also tested by complementation analyses. A secondary goal of these studies was to establish heme-mediated transcriptional regulation of the *hut* locus and to examine the effects of each of the potential iron-responsive regulators, namely Fur, Irr, and RirA, on the transcriptional profile of the *hut* locus genes. Additionally, these experiments were performed with RNA isolated in parallel from three different hemin concentrations in order to account for cofactor availability. This was accomplished by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). A third goal was to define the transcriptional organization of the *hut* locus and this was accomplished by RT-PCR. The final goal of the experiments was to map the transcriptional start sites (TSSs) of the transcriptional units in order to identify putative promoter and regulatory binding sequences.

The final set of experiments undertaken has focused on characterization of the *B. quintana* HemS protein. The first goal of these experiments was to generate and purify a natively-folded His-tagged HemS. The second goal of these experiments was to examine the ability of the purified recombinant HemS protein to bind hemin. This goal was

achieved spectrophotometrically and by hemin blotting. The third goal of these studies was to examine the ability of *B. quintana* HemS to function as a HO in a *Corynebacterium ulcerans hmuO* mutant by complementation. The final goal of these experiments was to examine the ability of recombinant HemS to catalyze the breakdown of hemin *in vitro*. This goal was approached spectrophotometrically.

Table 1.1. *Bartonella* species and their ability to cause human disease.

Species	Vector	Reservoir	Reference(s)	Disease	Reference(s)
alsatica	Unknown	Rabbit	(75)	Isolated	(85)
australis	Unknown	Kangaroo	(61)	No	
bacilliformis	Sandfly	Human	(36)	Oroya fever	(89)
birtlesii	Unknown	Mouse	(18)	No	
bovis	Horn fly (?)	Cattle	(17, 38)	No	
capreoli	Tick (?)	Roe Deer	(17, 21)	No	
chomelii	Unknown	Cattle	(114)	No	
clarridgeiae	Flea (?)	Cat	(110, 144)	Isolated	(98, 115)
coopersplainsensis	Unknown	Rodent	(70)	No	
doshiae	Flea	Rodent	(20, 170)	No	
elizabethae	Flea (?)	Rodent, Dog	(49, 50, 116)	Isolated	(42, 130)
grahamii	Flea	Cat	(20, 170)	Isolated	(93)
henselae	Flea, Cat	Cat	(89)	Cat Scratch	(6, 89)
japonica	Unknown	Rodent	(81)	No	
kohlerae	Flea, Cat	Cat	(47, 146)	Isolated	(9)
peromysci	Unknown	Rodent	(20)	No	
phoceensis	Unknown	Rodent	(69)	No	
queenslandensis	Unknown	Rodent	(70)	No	
quintana	Body louse	Human	(89)	Trench fever	(89)
rattaustraliani	Unknown	Rodent	(70)	No	
rattimassiliensis	Unknown	Rodent	(69)	No	
rochalimae	Unknown	Human (?)	(53)	Isolated	(53)
schoenbuchensis	Deer ked	Deer	(118)	No	
silvatica	Unknown	Rodent	(81)	No	
tamiae	Unknown	Rodents (?)	(100)	Yes	(100)
taylorii	Flea	Rodent	(20, 170)	No	
tribocorum	Flea	Rodent	(76, 141)	No	
talpae	Unknown	Moles	(20)	No	
vinsonii sub sp. a*urepensis*	Unknown	Rodent	(187)	Isolated	(54)
sub sp. *berkhoffi*	Ticks (?)	Dog, Coyote	(34, 77, 99)	Isolated	(24)
sub sp. *visonii*	Unknown	Vole	(99)	No	
washoensis	Ticks (?)	Dog, Squirrel	(34, 37, 101)	Isolated	(134)
weissi	Unknown	Cat	(196)	No	

A)

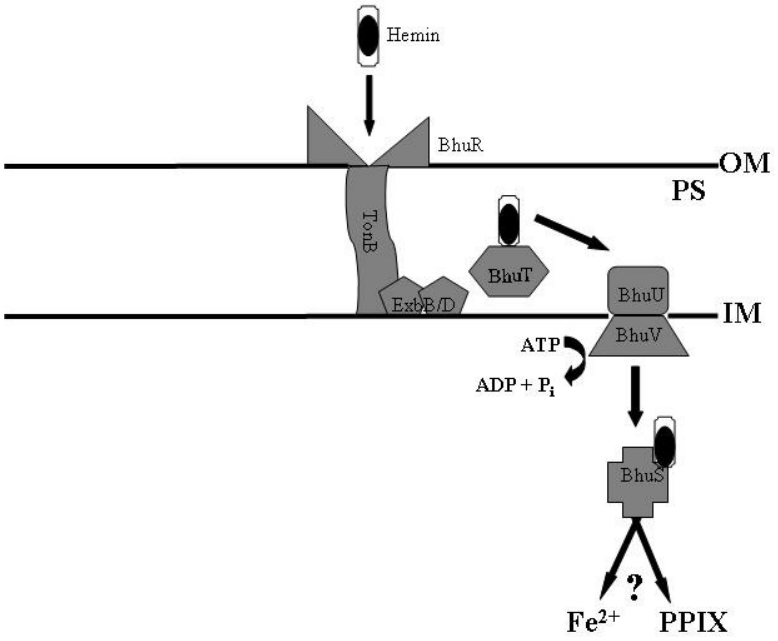
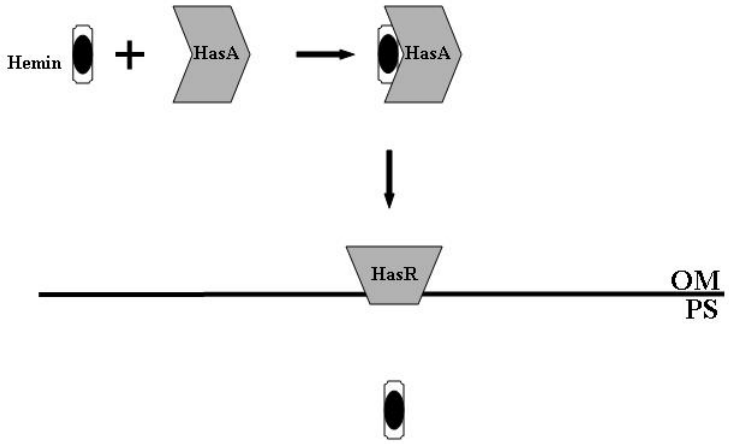


Figure 1-1. Schematic of bacterial hemin acquisition systems. A) Model of hemin acquisition via an outer membrane (OM) hemin receptor and a coordinate transport system as described in *Bordetella* (175). BhuR is a hemin receptor that uses energy transduced by TonB and ExbB/D to bring hemin into the periplasmic space (PS). BhuT is a periplasmic binding protein that shuttles hemin to the membrane-spanning permease, BhuU. Energy for hemin transport across the inner membrane (IM) is provided by the ATPase activity of BhuV. The fate of hemin in the cytoplasm is uncertain. Predicted functions for BhuS and its homologs include storage or degradation of hemin. B) Model of hemin acquisition via a hemophore as described in *Serratia marcescens (33)*. The hemophore HasA is secreted and binds hemin with high affinity. The hemin-HasA complex docks onto the hemin receptor, HasR, and releases hemin for transport into the cytoplasm as described in A.

B)



CHAPTER TWO

Hemin Uptake Is Not Mediated by Hemin Binding Protein A and the Ferric Uptake Regulator Is Not Central to Iron-Responsive Regulation in *Bartonella quintana*

A. INTRODUCTION

Bartonella quintana has an extraordinary heme requirement that is thought to fulfill both iron and porphyrin needs. *B. quintana* is expected to have evolved specialized systems adept at satisfying this requirement despite the heme-limiting nature of the human host environment. In addition to its success as a human pathogen, *B. quintana* is also able to rapidly shift between the human host and the heme-replete gut of the body louse. Thus, *B.* quintana must not only be able to acquire heme when it is scarce, but must also be able to protect itself from heme-mediated toxicities when heme is abundant. Therefore, the expression of heme uptake systems in *B. quintana* is expected to be under tight iron- and/or heme-responsive control. The disparity in heme availability between these two environments suggests that heme concentration may also serve as an indicator of the shift into either the human host or the body louse. Consequently it would be logical to co-regulate heme acquisition and virulence, as described in numerous other bacterial pathogens. Despite the pivotal role of heme to the survival and pathogenesis of *B. quintana*, little is known about either the mechanisms or the regulation of heme acquisition in this versatile species.

Iron-responsive regulation in Gram-negative bacteria is primarily attributed to the ferric uptake regulator (Fur) and has been most widely studied in *E. coli*. In the presence of excess iron, the dimeric Fur protein binds the ferrous form (Fe^{2+}) and undergoes a conformational shift. The Fur dimer then binds DNA at a consensus motif, GATAATGATAATCATTATC, in the promoter region termed the Fur Box and represses transcription (164). Fur has also been implicated in the activation of genes, but the mechanisms may involve the regulation of intermediate regulators and/or post-transcriptional control. Fur-targeted genes encode iron and heme uptake systems, superoxide dismutase, and several virulence factors. In fact, more than 90 genes are regulated by Fur in *E. coli* (72). Fur homologues have been identified in several Gram-

negative pathogenic genera including *Yersinia, Vibrio, Neisseria, Salmonella* and *Haemophilus* (143). Although it only has 38% amino acid identity to *E. coli* Fur, a Fur homologue was also identified in *B. henselae* (131). Corresponding *fur* genes were also identified in *B. bacilliformis* and *B. quintana* (131). Functional analyses of *B. henselae fur* indicate that it was able to complement a *Vibrio cholerae fur* mutant when constitutively expressed by a *ptac* promoter (131). Based on these data, we hypothesized a major role for *B. quintana* Fur in transcriptional control of heme acquisition systems.

The heme acquisition systems of *B. quintana* have not been fully characterized, but studies to date have focused on the genes encoding hemin-binding protein (Hbp) family members as a potential Fur target. This family consists of five porin-like outer membrane proteins (HbpA-E) that share ~ 48% amino acid identity to one another and are able to bind hemin, as implied by the name. Structural predictions suggest that each Hbp forms a β- barrel consisting of eight conserved transmembrane domains connected intracellularly by four small loops and extracellularly by four large loops (123). Out of eight membrane proteins identified by hemin blots, HbpA was chosen for further investigation because it was the only protein that hemin remained bound to after the blot was extensively washed (31). Pretreatment of *B. quintana* with α-HbpA antibody fractions was sufficient to partially inhibit heme binding relative to controls. Although recombinant HbpA was unable to confer a hemin-binding phenotype on *E. coli*, analysis of a *B. quintana hbpA* mutant showed increased hemin binding relative to wild type. Correspondingly, quantitative reverse-transcriptase-PCR (qRT-PCR) examining expression profiles of the remaining *hbp* transcripts in the *B. quintana hbpA* mutant indicated that compensatory expression occurred with all remaining members of the family upregulated to some degree. Of note, a sequence was identified in the promoter region of the *hbp* genes that shares ~50% identity to the Fur Box of *E. coli* suggesting a role for the *B. quintana* Fur protein in transcriptional control.

HbpA shares amino acid similarity with an outer membrane protein from *Brucella*, Omp31, the Opa proteins from *Neisseria*, and the phage-associated protein, Pap31, from *B. henselae* (123). Examination of the hemin-binding capacity of *Brucella suis* or *B. ovis* Omp31 indicates that it also binds hemin. As described with HbpA, pretreatment of *B. ovis* with α-Omp31 antibodies inhibits heme binding. Moreover, Omp31 expression is

increased when *B. suis* is grown on iron-deficient media relative to the amount produced when the strain is grown on iron-replete media (43). The hemin-binding capacity of these proteins is extended in the characterization of Pap31 from *B. henselae*. Investigation of this HbpA homolog focused on its ability to function as a hemin receptor in an *E. coli hemA* mutant, EB53, which is unable to synthesize porphyrin. As the parental strain is relatively impermeable to the heme molecule, there are only two ways for this strain to obtain hemin. The first is to bypass the *hemA* mutation with the addition of δ-aminolevulinic acid (the product of the reaction catalyzed by HemA) and the second is to express a recombinant hemin receptor so that hemin can be imported from an external source. These experiments indicated that expression of recombinant Pap31, consisting of the sequence encoding the mature protein fused to the signal sequence from *ompT* of *E. coli*, was sufficient to allow growth of EB53 in the presence of exogenous hemin (199). Together, these data led to the hypothesis that HbpA functioned as a hemin receptor in *B. quintana*, that Hbp expression was responsive to available hemin and/or iron concentrations, and that this response was mediated at least in part by the Fur protein.

These studies were undertaken to examine these hypotheses and define the major mechanisms and regulation of hemin acquisition in *B. quintana*. Here, we report that although *B. quintana* HbpA expression is responsive to available hemin concentration, expression of the recombinant protein in EB53 is insufficient to reproducibly complement the *hemA* strain. Furthermore, while forced *B. quintana fur* expression is able to complement an *E. coli fur* mutant, we were unable to identify an endogenous promoter in the *B. quintana fur* gene or express the native protein.

B. MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains were routinely grown overnight at 37°C with shaking in either Tryptone-yeast extract (TY) and lysogeny broth (LB) media and standard antibiotic concentrations were added as needed. 25 μM δ-aminolevulinic acid (ALA) (Research Products International, Prospect, IL) was added to the medium for growth of *E. coli hemA* strain EB53 (51). Induction of gene expression

was achieved with isopropyl-β-D-thiogalactopyranoside (IPTG) at a concentration of 2 mM. *B. quintana* strains were cultivated on heart infusion blood agar (HIAB) or on *Brucella* agar (BA) supplemented with 6 μM to 2.5 mM hemin chloride (Becton Dickinson, Sparks, MD). Cultures were grown at 37°C in 100% relative humidity and 5% CO_2 (12). 10 mg/mL stock solutions of hemin chloride in 0.2 M NaOH were filter sterilized prior to use. Table 2.1 (p. 34) lists the strains used in this study.

Preparation and manipulation of DNA. All plasmids used or generated in this study are listed in Table 2.1 (p. 34), and all primers used in this study are listed in Table 2.2 (p. 35). Genomic DNA from *B. quintana* and *E. coli* was obtained with a DNeasy blood and tissue kit (Qiagen, Valencia, CA) per protocol. Plasmids used for restriction analysis and sequencing were purified with a Perfect Prep plasmid minikit (Eppendorf, Hamburg, Germany) and those used for electroporation and *in vitro* transcription/translation were purified with a Qiagen Midi-Prep kit (Valencia, CA). Standard procedures were used for ligations, cloning, restriction endonuclease digestion and analyses, and polymerase chain reaction (PCR) (8). When appropriate, a QIAquick spin kit was used for purification of PCR amplicons and extraction of DNA from excised agarose gel slices (Qiagen). Fusion of the mature *B. quintana hbpA* gene with the *E. coli ompT* signal sequence was generated by overlap extension PCR as previously described (78). The fusion was cloned into the low-copy plasmid pWSK29 (184).

Generation of anti-Fur antisera. A his_6-tagged *B. quintana* Fur protein was generated and purified under denaturing conditions per QIAexpressionist protocol (Qiagen). Separation of purified protein fractions was accomplished with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% (wt/vol) acrylamide. Unfixed gels were washed three times (5 min each) with deionized water (dH_2O), stained with 0.05% Coomassie Brilliant Blue R-250 (Fisher Scientific, Fair Lawn, N.J.), and destained with dH_2O. Purified Fur bands were excised, combined and used to generate polyclonal anti-Fur antiserum in a female New Zealand White rabbit (154).

Hydrogen peroxide treatment of *B. quintana*. In an effort to upregulate Fur expression, *B. quintana* was treated with hydrogen peroxide (H_2O_2) basically as described previously (197)**.** Briefly, three plates of mid-log phase *B. quintana* were harvested, pelleted (2,300 x *g* for 10 min at 4°C) then resuspended in 1 ml *Brucella* broth supplemented with histidine-hematin (BBH-H50) (50 µg/ml) (35). H_2O_2 was added to a final concentration of 50 mM. 200 µl samples were removed at time 0, 10 min, 60 min and 120 min. Cells were pelleted (15,700 x *g* for 20 sec at room temperature (RT)), resuspended in 100 µl sterile dH_2O, and placed in a -80°C ethanol bath until needed.

Complementation analyses. Various plasmids containing *B. quintana fur* were assayed for functional expression using *E. coli fur* strain H1780. Strains were plated onto MacConkey agar supplemented with 100 µM $FeCl_3$ and appropriate antibiotics, then incubated overnight at 37°C. The ability of *B. quintana* Fur to repress β-galactosidase activity was assessed by color: Lac^+ Fur^- strains produced red colonies and Lac^-Fur^+ strains produced clear colonies as previously described (73). Complementation assays of *E. coli hemA* strain EB53 with *B. quintana* (*ompTSS*) *hbpA* were performed basically as described (199). In brief, cultures were grown overnight, then pelleted and grown for ~2 hours in TY media lacking δ-aminolevulinic acid to exhaust intracellular porphyrin stores. Cultures were then used to inoculate TY media alone, supplemented with ALA (50 µM), or hemin (10 µg/ml or 50 µg/ml) to an initial optical density at 600 nm (OD_{600}) of 0.02. Growth curves were generated by measuring the OD_{600} every four hours for 24 hours.

***In vitro* transcription/translation.** The *B. quintana fur* gene and successively larger portions of the upstream *secA* gene were directionally cloned in either the same orientation or the opposite orientation to the *lacZ* promoter of either pCR2.1 TOPO or XL-TOPO in order to map the *fur* promoter. The *E. coli* S30 extract system for circular DNA (Promega, Madison, WI) and a S-35 Express (cysteine-methionine) mix (New England Nuclear-Dupont, Boston, MA) were used for translation and radiolabeling of the proteins. Products were separated by SDS-PAGE using 12.5% (wt/vol) acrylamide gels that were subsequently dried and exposed to X-ray film for up to 96 hours.

Sarkosyl fractionation, proteinase K treatment, and immunoblotting. Overnight cultures of *E. coli* were harvested, washed in phosphate-buffered saline (PBS pH 7.4) and resuspended in dH_2O. Cells were lysed with a FP120 Fast Prep bead homogenizer (45 s at top speed) using 0.1 mm zirconia beads (Qbiogene, Carlsbad, CA). Cell lysates were incubated in 2% (wt/vol) N-lauroyl sarcosinate (Sigma, St. Louis, MO) for 30 min and then centrifuged at 4°C for 60 min at 100,000 x g. The sarkosyl-soluble supernatant was removed and the insoluble pellet was resuspended in 0.2 mM phenylmethanesulfonyl fluoride (PMSF) in dH_2O.

Surface accessibility of recombinant HbpA was also confirmed by proteinase K treatment with slight modifications to previously described methods (120). Briefly, cells were pelleted, washed three times with PBS supplemented with 0.9 mM $MgCl_2$ and 1.0 mM $CaCl_2$ (PBS $MgCl_2/CaCl_2$), and enumerated with a LIVE/DEAD *Bac*Light bacterial viability kit (Invitrogen, Eugene, OR). Live cells were diluted to 10^9 cells/ml and treated with 0-1500 μg/ml proteinase K for 10 min at room temperature. The reaction was stopped by the addition of 200 μg of PMSF in isopropanol. Cells were pelleted and washed two more times with PBS $MgCl_2/CaCl_2$. A bicinchoninic acid kit was used to determine protein concentrations (Pierce, Rockford, IL). Samples were separated by SDS-PAGE and resulting gels were transferred to nitrocellulose (GE Water & Process Technologies, Trevose, PA) for Western blotting (173). Blots were probed overnight with either rabbit anti-HbpA (1:5000) or anti-Fur (1:500) antiserum and developed with hydrogen peroxidase-conjugated secondary antibodies (Sigma), 4-chloronapthol, and hydrogen peroxide. Fur protein expression was also probed with anti-*E. coli* Fur antisera (A generous gift from M. Vasil) at a 1:500 dilution for 2 hours, then developed (as described above).

C. RESULTS

Complementation of *E. coli fur* strain H1780. The *E. coli fur* mutant H1780 has a *lacZ* fusion under control of the *fur* promoter that allows iron-mediated control of β-galactosidase production when a functional Fur protein is provided in *trans* (73). In order

to test the hypothesis that *B. quintana fur* encodes a functional ferric uptake regulator, a construct containing *B. quintana fur* under control of the plasmid-encoded *lac* promoter was generated and used to transform *E. coli* strain H1780. The resulting strain, along with a control consisting of H1780 transformed with vector alone, was tested for its ability to repress β-galactosidase production when grown on iron-rich MacConkey medium. MacConkey medium contains lactose and a pH indicator such that production of β-galactosidase results in the generation of lactic acid which makes colonies appear red, while repression of *lacZ* results in the generation of whitish-yellow colonies. Results from these experiments indicate that *B. quintana fur* is able to repress *lacZ* in H1780 when grown in the presence of 100 μM $FeCl_3$, as evidenced by the production of white colonies on the left half of the plate in Figure 2-1 (p. 37). Likewise, transformation of H1780 with pDS1.1 carrying the *Brucella abortus fur* gene resulted in the generation of white colonies on iron-rich MacConkey agar (data not shown). In contrast, transformation of H1780 with vector alone does not complement the *fur* mutation as evidenced by the continued production of β-galactosidase resulting in red colonies on the right-hand side of the plate despite the presence of a high concentration of iron (See Fig.2-1). These data clearly indicate that the *B. quintana* Fur protein is a functional ferric uptake regulator that is able to recognize and bind the *E. coli* Fur box consensus sequence.

Synthesis of Fur in *E. coli* and *B. quintana.* Initial attempts to identify the Fur protein utilized polyclonal antisera generated against *E. coli* Fur. Western blots probed with this antisera showed reactivity with both uninduced and induced His_6-tagged *B. quintana* Fur protein expressed in JM109 (Figure 2-2A lanes 1 and 2, respectively, p. 38). In contrast, although an ~ 50 kDa protein cross-reacts with the anti-*E. coli* Fur antisera, there is an absence of reactivity with any protein in the appropriate molecular weight range in the *B. quintana* cell lysate (Fig. 2-2A, lane 3). In an effort to provide a more specific and accurate tool for the detection of *B. quintana* Fur, we generated anti-*B. quintana* Fur antisera using purified recombinant his_6-tagged Fur. Immunoblot analyses indicate strong reactivity to the recombinant *B. quintana* Fur protein when induced in *E. coli* (Fig. 2-2B, lane 2), but no reactivity with any protein in the *B. quintana* cell lysate

(Fig. 2-2B, lane 3). Notably, anti-*B. quintana* Fur antisera also reacts with recombinant *Brucella abortus* Fur protein expressed in *E. coli* (data not shown, Table 2.3, p. 36). Together, these data suggest that the both antisera recognize conserved Fur antigens but that the protein is not synthesized in *B. quintana* under the growth conditions used for these experiments. In an effort to increase expression of the Fur protein, *B. quintana* was treated with hydrogen peroxide for up to 2 hours and then assayed for Fur production by Western blot. Again, these experiments failed to yield reactivity with the native Fur protein in *B. quintana* cell lysates (data not shown).

Mapping the *B. quintana fur* promoter by *in vitro* transcription/translation. In the previous study examining *Bartonella henselae* Fur, a fusion construct consisting of the 204 base pairs (bp) *fur* promoter and the gene encoding green fluorescent protein (*gfp*) was used to transform *B. quintana* and *B. henselae*. Promoter activity, as evidenced by the detection of GFP production by flow cytometry, was not detected despite growing the strains on a range of hemoglobin and iron concentrations (131). These data, combined with the inability to detect native Fur from *B. quintana* cell lysates, led us to examine the potential transcriptional arrangement of this gene. Genomic analyses indicate that *Bartonella fur* is separated from its upstream gene, *secA*, by only 17 bp or less in *B. quintana*, *B. henselae*, and *B. bacilliformis* (Fig. 2-3A, p. 39) (4). Although it does not contain the *fur* gene, *secA* is transcribed as part of an operon in *E. coli* (127). In an effort to locate the promoter region of *fur*, we generated a series of *fur* constructs containing portions of the upstream *secA* gene and directionally cloned them such that they were either in the same orientation or flipped with respect to the plasmid-encoded *lac* promoter. Examination of the proteins synthesized from these constructs by *in vitro* transcription/translation (IVTT) indicates strong expression of the Fur protein when the *fur* gene is immediately preceded by the *lac* promoter (Fig. 2-3B lane 3, p. 39). Faint expression of the Fur protein is also evident when the entire *secA-fur* gene fragment is cloned in the same orientation as the *lac* promoter (Fig. 2-3B lane 6). In contrast, Fur expression was not detectable when the *fur* gene was in the opposite orientation to the *lac* promoter (Fig. 2-3B lane 2) regardless of the inclusion of 1600bp of upstream *secA* sequence (Fig. 2-3B lane 4) or the entire *secA* gene (Fig. 2-3B lane 7). Constructs

containing the entire *secA* gene produced SecA regardless of orientation (Fig. 2-3B lanes 6 and 7).

IVTT data were corroborated by complementation and Western blot analysis. Specifically, constructs that produced detectable Fur *in vitro* also produced Fur *in vivo* as evidenced by reactivity with anti-Fur antisera and were able to complement the *E. coli* H1780. All of the constructs containing *fur* in opposite orientation to the *lac* promoter failed to produce detectable Fur *in vivo* and failed to produce functional Fur in the *E. coli fur* strain (See Table 2.3). In total, these data imply that although *secA* contains a functional promoter, *B. quintana fur* is not transcribed as part of an operon originating from *secA*. In fact, *B. quintana fur* does not appear to possess a consensus promoter.

Synthesis and localization of *B. quintana* HbpA in *E. coli*. Although a role for Fur in the transcriptional regulation of the *hbp* genes was not established, the ability of HbpA to function as a hemin receptor was still of interest. In order to ensure proper trafficking of *B. quintana* HbpA in *E. coli*, Hbp's original signal sequence was replaced with the signal sequence from OmpT, a known outer membrane protein of *E. coli*. Outer membrane localization of the recombinant HbpA fusion in *E. coli* was subsequently confirmed by sarkosyl fractionation and immunoblotting. Briefly, selective solubilization of the bacterial cytoplasmic membrane in 0.5-2% (wt/vol) sarkosyl results in the isolation of outer membrane proteins (55). The sarkosyl-insoluble protein profile of pWSKHbpA/EB53 resolved by SDS-PAGE is consistent with that presented in a previous study of *E. coli* using the same methodology (See Fig. 2-4A lane 6, p. 40) (48). A corresponding immunoblot shows the presence of the HbpA fusion in both uninduced and induced cell lysates from pWSKHbpA/EB53 but absent in EB53 containing only the vector (Fig. 2-4B, lanes 3, 4, and 2 respectively). Moreover, the immunoblot indicates that although some HbpA is present in the sarkosyl-soluble fraction (lane 5), the majority of it resides in the sarkosyl-insoluble pellet (lane 6). These results indicate that the majority of the *B. quintana* HbpA fusion protein is properly trafficked to the outer membrane in *E. coli* strain EB53.

Outer membrane localization was confirmed by proteinase K treatment of intact cells to examine the surface accessibility of HbpA in EB53. Immunoblot analyses of cells

lysates from proteinase K-treated cells indicate a progressive decrease in the amount of HbpA that directly corresponds to treatment with successively higher concentrations of proteinase K (See Fig. 2-5B, lanes 2-5, p. 41). Visible changes in HbpA were verified by densitometry (data not shown). A corresponding silver-stained gel (Fig. 2-5A) served as a control for protein loading and showed a lack of non-specific protein degradation. Likewise, a protein slightly larger than HbpA that was cross-reactive with the anti-HbpA antisera served as an internal control and remained constant despite increasing concentrations of proteinase K (Fig. 2-5B, lanes 2-5). These results provide strong evidence for appropriate trafficking of HbpA to the outer membrane of EB53.

Complementation of *E. coli* strain EB53. In order to examine the ability of *B. quintana* HbpA to function as a hemin receptor, complementation analyses of the *E. coli hemA aroB* strain EB53 were undertaken. EB53 is a K-12 derivative with a mutation preventing the generation of δ-aminolevulinic acid (ALA), a key intermediate in the porphyrin biosynthesis pathway. In the absence of exogenously-supplied ALA, this strain is unable to exploit hemin as a porphyrin/hemin source due to the relative impermeability of the membrane to hemin and the absence of a native hemin receptor (153). However, the severe growth defects resulting from this mutation can be corrected if a functional hemin receptor is provided. We used this strain to test the hypothesis that *B. quintana hbpA* functions as a hemin receptor. In short, overnight cultures of pWSKHbpA/EB53 and pWSK29/EB53 were incubated for ~2 hours in unsupplemented media to deplete intracellular porphyrin and ALA stores and were then used to inoculate TY media alone, or supplemented with ALA or hemin. Growth curves were generated by measuring OD_{600} every four hours for 24 hours. As expected, data indicate that both strains fail to grow in the absence of exogenously-supplied hemin or ALA (Fig. 2-6A, p. 42). When the *hemA* mutation was bypassed by the addition of 50 μM ALA, both strains produced normal growth curves (Fig. 2-6B, p. 43). pWSK29/EB53 shows a slightly higher 'leak' rate than pWSKHbpA/EB53 when the strains are grown in media supplemented with 10 μg/ml hemin (Fig. 2-6C, p. 44). This phenomenon is somewhat less pronounced when the hemin concentration is increased to 50 μg/ml (Fig. 2-6D, p.

45). Regardless, the pWSKHbpA/EB53 strain is not able to uptake exogenously-supplied hemin any faster than EB53 containing the vector alone at either hemin concentration.

D. DISCUSSION

These experiments were undertaken to characterize the mechanisms of hemin acquisition and its regulation in *B. quintana*. The first aim of this study was to characterize the role of Fur in iron-responsive transcriptional regulation. Unexpectedly, the data presented herein challenge the hypothesis that Fur is the dominant transcriptional repressor controlling heme and iron uptake in *Bartonella*. Although the *B. quintana fur* gene encodes a functional Fur protein that recognizes the *E. coli* Fur box, there was no evidence of a promoter for the gene even when the search was extended to encompass the entire upstream *secA* gene and its promoter region. It is tempting to speculate that the inability to identify an endogenous promoter by IVTT is due to the existence of a *B. quintana*-specific sigma factor required for transcription of *fur*. However, consistent with the reported inability to detect a promoter in *Bartonella* using a *B. henselae fur-gfp* fusion, we also failed to detect any evidence of native Fur expression in *B. quintana* by immunoblotting (131). A notable exception to the inability to detect Fur in *Bartonella* occurred when overexpression was forced by transformation of *Bartonella* with a construct containing the *fur* gene in the same orientation as a plasmid-encoded *lac* promoter (14). This detail combined with the fact that the antisera reacted well with *Brucella abortus* Fur refute any possibility that the inability to detect Fur is due to weak or nonspecific antisera (data not shown). In contrast, these data collectively suggest that Fur is not expressed in *Bartonella* under any of the conditions used in this study.

The absence of Fur expression in *Bartonella* implies the existence of an alternative iron-responsive regulator. Consistently, several closely-related α-proteobacterial species have reported a diminished or nonexistent function for Fur in iron-mediated transcriptional control despite the almost universal presence of an intact *fur* gene (86). In several of these species, the role of Fur has been entrusted to one of two novel regulators. The first of these is the rhizobial iron regulator, RirA, which resembles the iron-sulfur cluster regulator of *E. coli* and has primarily been characterized in *Rhizobium*

leguminosarum (171). The other is the iron response regulator, Irr, which has been primarily characterized in *Bradyrhizobium japonicum* and is thought to respond directly to intracellular heme concentrations (195). Genomic analyses revealed that genes encoding homologs to both of these proteins are present in *B. quintana.* Of note, the *B. quintana* Fur and Irr proteins share ~ 50% amino acid similarity across 91 residues (of 138 possible residues) (Parrow and Minnick, unpublished observation). Therefore, the focus of our investigation shifted to the role of Irr and RirA in the transcriptional regulation of the *hbp* genes. Subsequent studies found that overexpression of either Irr or RirA in *B. quintana* resulted in the generation of a bloodstream-like transcriptional *hbp* profile, that is, increased transcription of *hbpA, D,* and *E*. These data led to the identification of a conserved consensus sequence in the promoter region of the *hbp* genes, designated the H-box, which directly interacts with Irr. In contrast, forced overexpression of Fur resulted in decreased transcription of only *hbpC* (14). Based on previous data suggesting that HbpC may be important in colonization or survival in the louse gut and the probability that hemin and/or iron are released at toxic levels in this environment, it is possible that Fur is expressed in *B. quintana* in response to hemin concentrations that are toxic *in vitro* or to an alternative environmental trigger (13).

The second major aim of this study was to examine the ability of *B. quintana* HbpA to function as a hemin receptor. Again, the data obtained from this investigation opposed the original hypothesis. Despite replacing the native signal sequence of *B. quintana* HbpA with one certain to be recognized by *E. coli* followed by confirmation of appropriate outer membrane localization of recombinant HbpA in *E. coli*, this protein failed to complement the porphyrin biosynthesis defect in EB53. While these data are in direct opposition to a previously-published report indicating that the *B. henselae* homolog Pap31 was able to function as a hemin receptor in EB53, it is important to note that the *E. coli hemA* strains are somewhat leaky when grown in the presence of hemin (199). This characteristic increases the likelihood of obtaining a false-positive result, especially if growth is ascertained "by eye" rather than quantitatively. The absence of any discernable TonB box in HbpA, as well as both structural and amino acid similarity to known hemin receptors, are consistent with our data (Parrow and Minnick, unpublished observation).

Genomic analyses indicate a more probable hemin receptor, HutA, which possesses these characteristics (discussed in Chapter 3).

Even though HbpA is not a hemin receptor, the strength of the association between the Hbps and hemin is undeniable. Furthermore, the entire Hbp family is transcriptionally responsive to fluctuations in hemin concentration (13). Additionally, HbpA can be specifically retrieved from cell lysates using hemoglobin bound to agarose beads (data not shown). Collectively, these observations suggest that this interaction is biologically relevant. The possibility of a function parallel to that established for leghemoglobin in generating a microaerobic environment for the closely related nitrogen-fixing *Rhizobium* and *Bradyrhizobium* has been suggested (14). However, an oxygen (O_2)-labile nitrogenase is not readily apparent in the *B. quintana* genome (Parrow and Minnick, unpublished observation). If the Hbps have a similar function in *B. quintana*, then the enzyme requiring protection from oxygen is novel or there is an alternative reason for excluding O_2.

It is tempting to speculate that either the Hbps have an important but indirect role in hemin acquisition or that the hemin has a role in the adaptation of *Bartonella* to its radically different growth environments. Previously suggested possibilities include the Hbps serving as a surface reservoir for hemin storage or as detoxifying entities via the intrinsic peroxidase activity of hemin (13). An intriguing alternative to these potential functions is the possibility that surface heme serves as a sensor via its ability to bind biologically relevant molecules such as carbon monoxide (CO), nitric oxide (NO), and oxygen (O_2). A similar function for heme has been reported in mammalian cells, whereby heme binds to and blocks calcium-activated potassium channels but heme bound to CO releases the channel resulting in its activation (84). Under such a model, the Hbps would function to transport an unknown molecule across the outer membrane, which their porin-like structure would facilitate, and heme would serve as a "gatekeeper". The opening and closing of the gate would in turn be dictated by the presence of a messenger molecule. As a starting point for the investigation of this hypothesis, the spectrophotometric profile of the an Hbp-hemin solution could be compared with the profile generated by the same solution following addition of CO (84).

In summary, the data presented herein indicate that HbpA is not able to transport hemin across the outer membrane when expressed in *E. coli*. Likewise, our data indicate that Fur is not a dominant iron-responsive transcriptional repressor because it does not appear to be expressed under the conditions used in this study. These data are significant primarily because they indicate the existence of alternative hemin acquisition systems (discussed in Chapter 3) and transcriptional regulators (14). Furthermore, they indicate a more subtle role for the Hbps in heme acquisition. Despite the inherent difficulties in untangling the interaction of the Hbps with hemin, due to the fact that it is absolutely required for *in vitro* growth of *Bartonella*, further studies of this unique family of proteins are warranted. Elucidation of their function is expected to result in improved appreciation of *Bartonella* pathogenesis and host adaptation. Furthermore, the identification of HbpE as one of the predominant proteins recognized by the human immune system underscores the importance of these proteins *in vivo* and may be exploited as a potential therapeutic target (23).

Table 2.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant Characteristic(s)	Source/Reference
Bartonella quintana		
OK90-268	Human isolate	CDC
LS200	OK90-268 cured of pEST	122
E. coli		
DH5α	Host strain for cloning	Gibco-BRL
JM109	Host strain for expression	Promega
H1780	MC4100 but *fur::λ placMu53*	72
EB53	*hemA aroB rpoB*	152
Plasmid		
pCR2.1 TOPO	TA cloning vector	Invitrogen
BqFur TOPO	pCR2.1 TOPO with *B. quintana fur* insert	130
pNP10	pCR2.1 TOPO with B. quintana *fur* and flanking insert; same orientation as P*lac*	This study
pNP20	As FurB/TOPO but opposite orientation to P*lac*	This study
pNP30	pCR2.1TOPO with 1600 bp of *B. quintana secA* and *fur*; opposite orientation as P*lac*	This study
pQE30	Expression vector	Qiagen
pNP70	pQE30 with *B. quintana fur* insert	This study
pUC 9	Cloning vector	Kovach, M.E.
pDS1.1	pUC 9 with *Brucella abortus fur* insert	Kovach, M.E.
pWSK29	Low-copy expression vector	183
pWSKHbpA	pWSK29 with *B. quintana hbpA(ompTSS)*	This study
XL-TOPO	TA cloning vector for long PCR products	Invitrogen
pNP40	XL-TOPO with 3336 bp *B. quintana secAfur* insert; same orientation as P*lac*	This study
pNP50	As SecAFur 3/ XL-TOPO but opposite orientation to P*lac*	This study
pBBR1MCS	Broad host range vector	Kovach, M.E.
pNP60	FurB but pBBR1MCS; same orientation as P*lac*	This study

Table 2.2 Primers used in this study

Primers	
SacI OmpTSS For	AGAGCTCTGCAAGCCATTGCGAGGCCT
OmpTSS Rev	AGCAATAACATCAGCAGCAAAAGAGCTGATCGCAATAGGGGTTGTCAG
HbpA For	GTCCTGACAACCCCTATTGCGATCAGCTCTTTTGCTGCTGATGTTATTG
HbpA ApaI Rev	GTGGGCCCACAATCACAAAAATAGAGG
pbbrfur For	CACCTAGGATGAATAAAAAACGAAATTATGAG
pbbrfur Rev	ACGGCGCGCCGTTACTAGTGGATCCGAGCTC
BqfullFur ORF For	GTAATGAGCGTTGCCCTTGTGG
BqfullFur ORF Rev	GTATAAAATTCAATTCGCATGAGG
SecA3 For	ATAGGAAACTGTCTGGCATGACGG
SecA full ORF For	GCGCACCAACAAGTATAATGATACC

Table 2.3. Summary of Fur constructs and experimental results for each.

Abbreviations: C'= complementation data; Ab = Anti-Fur antibody recognition; ND = not determined.

Strain	Consists of	IVTT	C'	Ab
pNP60	*B. quintana fur* and flanking sequences in pBBR1MCS	ND	yes	yes
pDS1.1	*Brucella abortus fur*	ND	yes	yes
pNP10	pNP60 but pCR2.1TOPO; same orientation as Plac	yes	yes	yes
pNP20	pNP10 but opposite orientation as Plac	no	no	no
pNP30	1600bp of *B. quintana secA* and *fur;* in opposite orientation to P*lac*	no	no	no
pNP40	3336 bp *B. quintana secA-fur* insert; same orientation as P*lac*	faint	partial	faintly
pNP50	pNP40 but opposite orientation to Plac	no	no	no
BQ Fur 2/ Topo	*B. quintana fur* insert in pCR2.1TOPO	yes	partial	yes
pNP70	*B. quintana fur* insert in pQE30	ND	no	yes

A) **B)**

Figure 2-1. Complementation of *E. coli fur* mutant H1780 with *B. quintana fur*. Overnight growth of H1780 containing A) pNP60 and B) pBBR1MCS on iron-rich MacConkey agar. *B*-galactosidase activity is evident by the production of red colonies in B), while its repression is indicated by the production of whitish-yellow colonies in A).

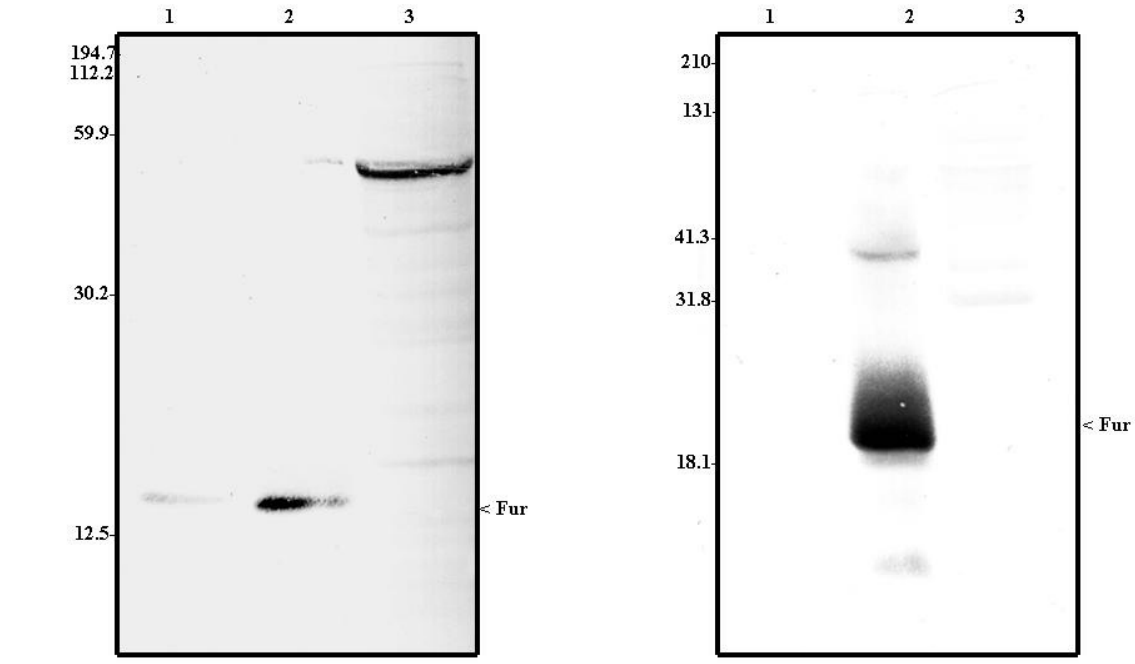
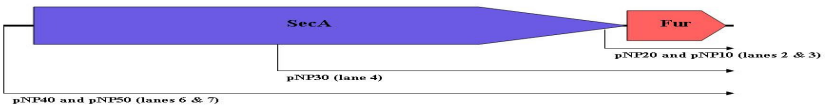


Figure 2-2. Analysis of Fur expression by immunoblotting. Western blot of cell lysates from uninduced JM109/pNP70 (lane 1), induced JM109/pNP70 (lane 2), and *B. quintana* strain LS200 (lane 3) probed with A) anti-Fur antiserum generated with *E .coli* Fur or B) anti-Fur antiserum generated with *B. quintana* Fur. The recombinant His_6-tagged Fur protein is evident in the induced samples when blots are probed with either antiserum. Although cross reactive proteins are present when probed with the *E. coli* antisera, Fur is not apparent in the *B. quintana* lysate in either blot.

A)



B)

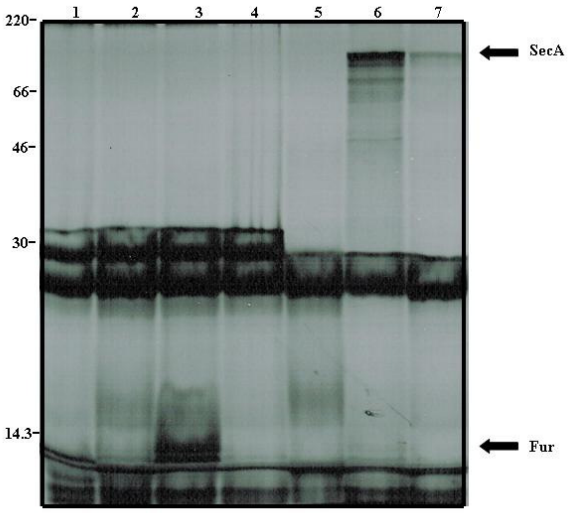


Figure 2-3. Absence of an endogenous *fur* promoter by *in vitro* transcription/ translation. A) Schematic showing the genomic arrangement of the *secA* and *fur* genes in *B. quintana* and the relative sizes of the various constructs used in IVTT. B) IVTT analysis of constructs containing *B. quintana fur* containing portions of the upstream *secA* gene in the same or opposite orientation to the plasmid-encoded *lac* promoter. Lanes: 1, pCR2.1 TOPO; 2, pNP20; 3, pNP10; 4, pNP30; 5, XL-TOPO; 6, pNP40; 7, pNP50. A Fur product is evident in lane 3 and faintly visible in lane 6. Molecular mass standards are given on the left in kilodaltons.

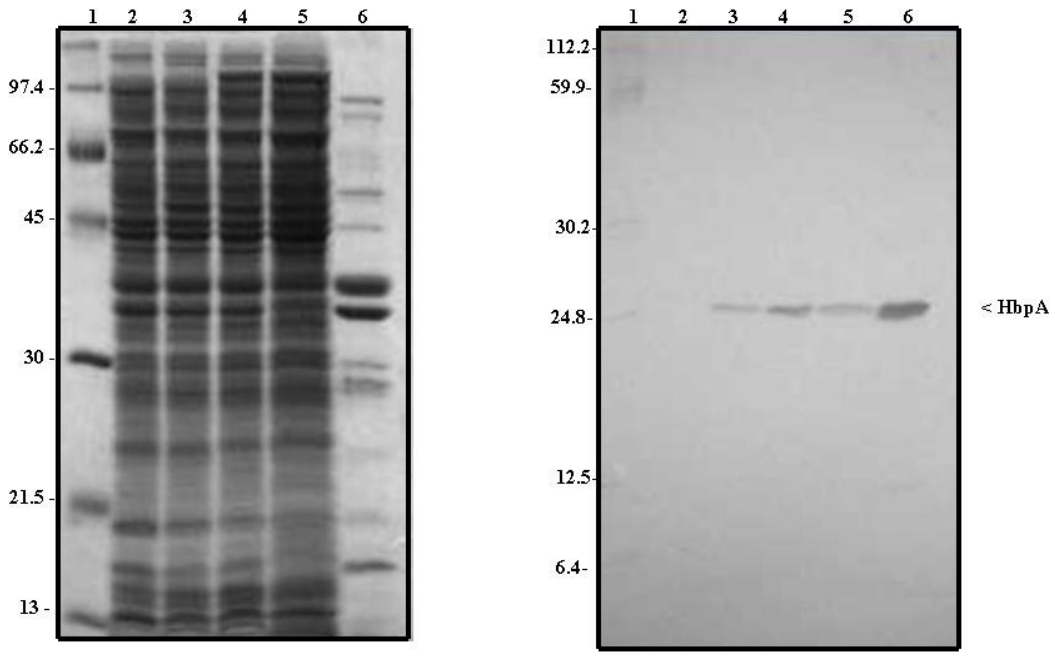


Figure 2-4. Outer membrane localization of HbpA in *E. coli* strain EB53 by sarkosyl fractionation. Lanes: 1, Molecular mass standards; 2, pWSK29/EB53; 3, pWSKHbpA/EB53 (uninduced); 4, pWSKHbpA/EB53 (induced); 5, pWSKHbpA/EB53 sarkosyl-soluble fraction; 6, pWSKHbpA sarkosyl-insoluble fraction. A) Coomassie blue stained SDS-PAGE gel. B) Corresponding immunoblot developed with anti-HbpA showing HbpA is present in uninduced and induced cell lysates, as well as both sarkosyl fractions. The majority of the HbpA is in the outer membrane. Molecular mass standards are indicated on the left in kilodaltons.

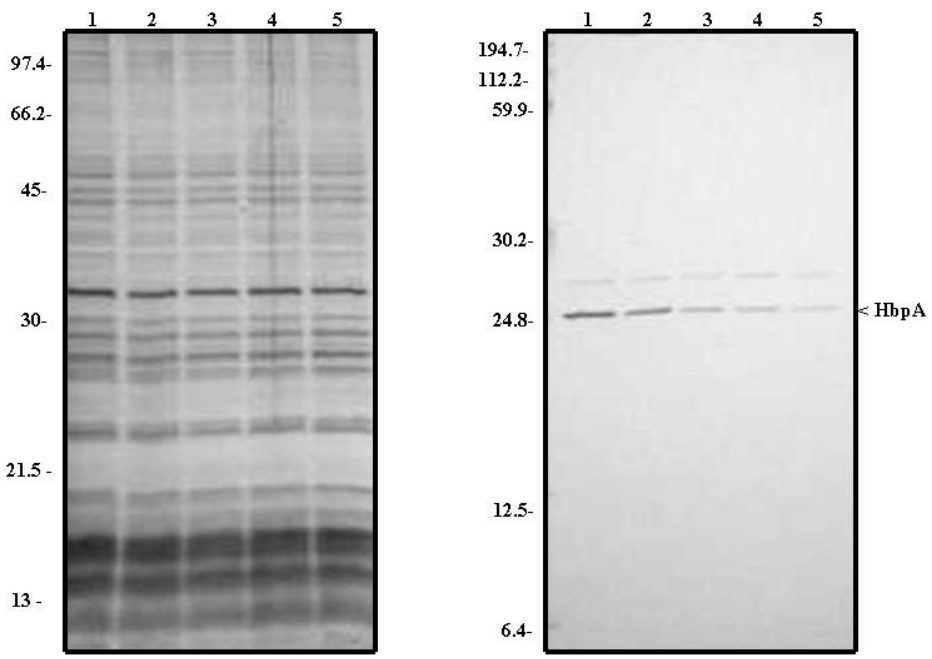


Figure 2-5. Surface accessibility of HbpA in *E. coli* strain EB53 by proteinase K digestion. A) Silver stained SDS-PAGE gel of proteinase K-treated pWSKHbpA/EB53. Lanes: 1, 0µg/ml PK; 2, 100µg/ml PK; 3, 500µg/ml PK; 4, 1000µg/ml PK; 5, 1500µg/ml PK. B) Corresponding immunoblot probed with anti-HbpA indicates surface accessibility as evidenced by dose-dependent proteinase K sensitivity.

A)

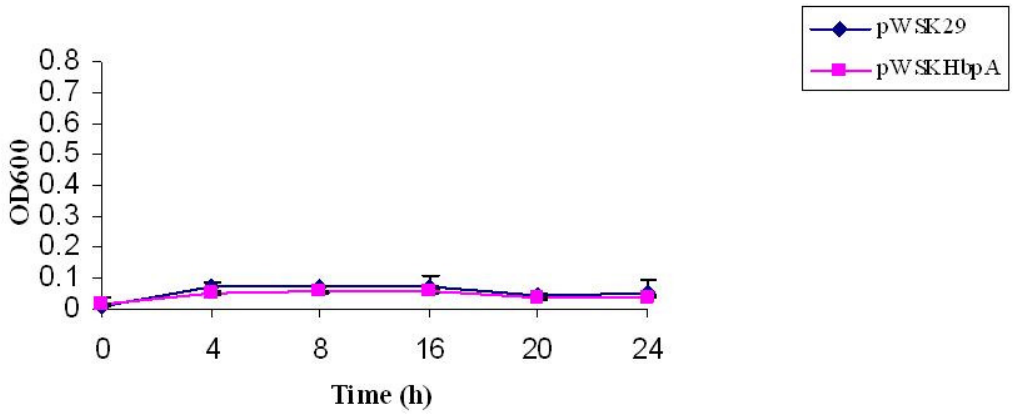
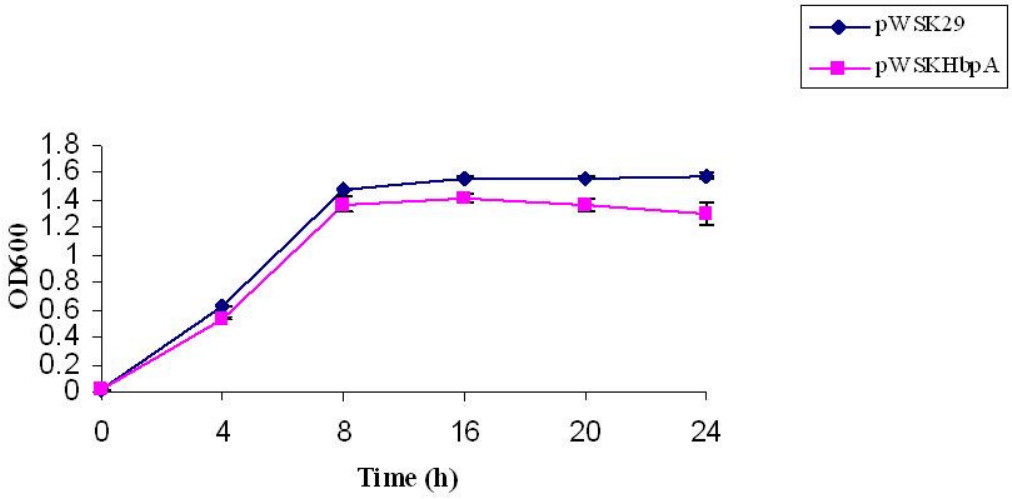
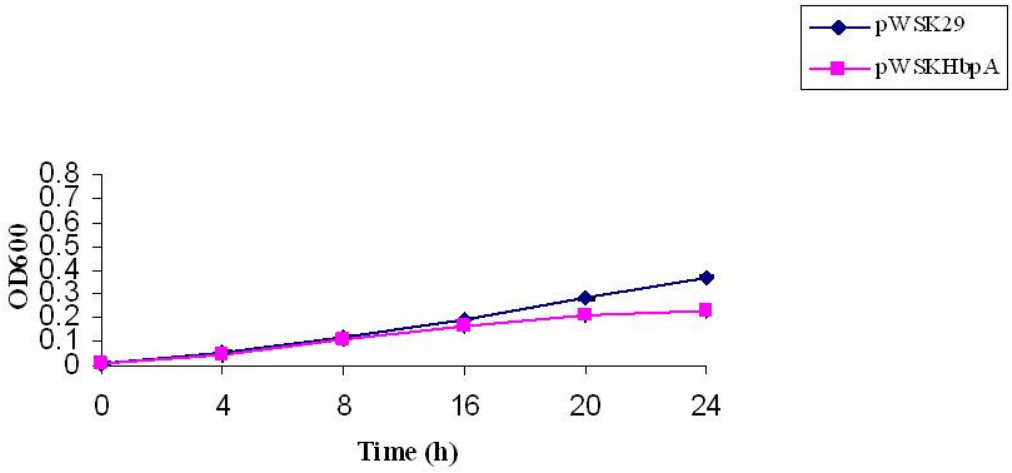


Figure 2-6. Complementation of *E. coli hemA* strain with *B. quintana hbpA*. 24 hour growth curves of *E. coli* EB53 containing vector alone (pWSK29) or pWSKHbpA inoculated into TY media: A) containing neither ALA nor hemin B) supplemented with 50µM ALA C) supplemented with 10µg/ml hemin or D) supplemented with 50µg/ml hemin.

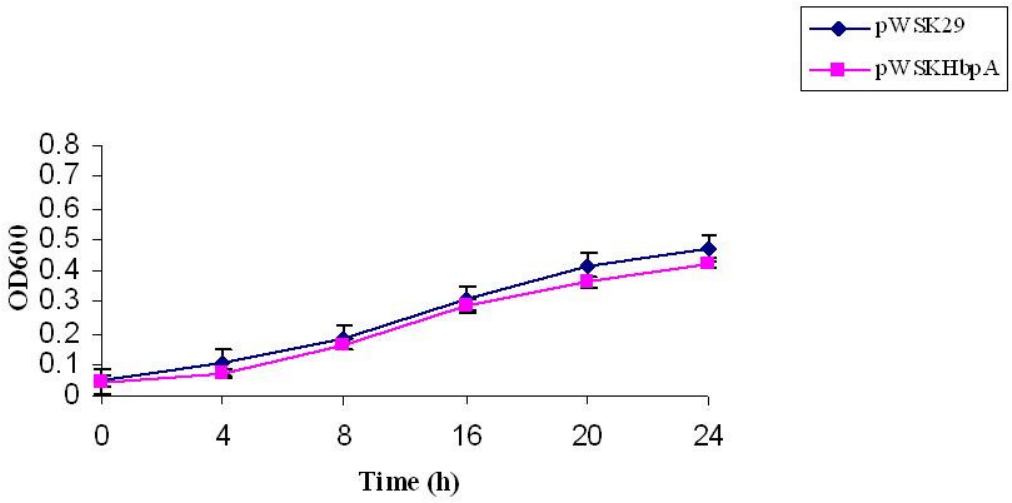
B)


pWSK29
pWSKHbpA
OD600
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
4
8
16
20
24
Time (h)

C)


pWSK29
pWSKHbpA
OD600
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
16
20
24
Time (h)

D)


pWSK29
pWSKHbpA
OD600
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
16
20
24
Time (h)

CHAPTER THREE

Function, Regulation, and Transcriptional Organization of the Hemin Utilization Locus of *Bartonella quintana*

Nermi L. Parrow, Jasmin Abbott, Amanda R. Lockwood, James M. Battisti, and Michael F. Minnick. 2009. Infect. Immun. **77**(1): 307-316

A. ABSTRACT

Bartonella quintana is a Gram-negative agent of trench fever, chronic bacteremia, endocarditis, and bacillary angiomatosis in humans. *B. quintana* has the highest known hemin requirement among bacteria, but the mechanisms of hemin acquisition are poorly defined. Genomic analyses revealed a potential locus dedicated to hemin utilization (*hut*) encoding a putative hemin receptor, HutA, a TonB-like energy transducer, an ABC transport system comprised of three proteins, HutB, HutC, and HmuV, and a hemin degradation/storage enzyme, HemS. Complementation analyses with *E. coli hemA* show that HutA functions as a hemin receptor and in *E. coli hemA tonB* indicate that HutA is TonB-dependent. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses show that *hut* locus transcription is subject to hemin-responsive regulation, which is primarily mediated by the iron response regulator (Irr). Irr functions as a transcriptional repressor of the *hut* locus at all hemin concentrations tested. Overexpression of ferric uptake regulator (*fur*) represses transcription of *tonB* in the presence of excess hemin, whereas overexpression of the rhizobial iron regulator (*rirA*) has no effect on *hut* locus transcription. RT-PCR analyses show that *hutA* and *tonB* are divergently transcribed, and remaining *hut* genes are expressed as a polycistronic mRNA.

Examination of the promoter regions of *hutA/tonB* and *hemS* indicates homology to consensus sequence promoters and encompasses an H-box element previously shown to interact with *B. quintana* Irr.

B. INTRODUCTION

Bartonella quintana, the Gram-negative bacterial agent of epidemic trench fever during World Wars I and II, is one of several *Bartonella* species of current medical relevance (27). *B. quintana* is transmitted to humans via the human body louse (*Pediculus humanus corporis*) and is re-emerging in large metropolitan areas amongst destitute individuals as 'urban trench fever'. Risk factors for urban trench fever include alcoholism, homelessness, and exposure to body lice (33). Unlike classical trench fever, a self-limiting flu-like disease, the re-emerging disease is associated with chronic bacteremia and endocarditis regardless of immune status (33). *B. quintana* infection can also result in bacillary angiomatosis (BA); which is the development of proliferative vascularized lesions of the skin (43). Although BA primarily affects patients infected with human immunodeficiency virus (HIV) or other immunodeficiencies, a limited number of cases have been reported in immunocompetent individuals (46).

HIV infects approximately 0.47% of the general U.S. adult population and there are an estimated 800,000 homeless people in the U.S. on any given day (26,38). Some studies suggest that HIV prevalence is up to five times higher in homeless populations than in the general population (1). Despite the relatively large population at risk for *B. quintana* infection, trench fever is recognized as a "neglected infection of poverty" (19).

Accordingly, insufficient data exist for a general estimate of prevalence in the U. S. and very little is known about the pathogenesis of this bacterium.

Utilization of host heme-containing proteins as a source of iron is a common strategy for bacterial pathogens (16). In addition to using these heme or hemin (the Fe^{3+} oxidation product of heme) sources, *Bartonella* species are unique in their ability to parasitize human erythrocytes (27,37). In the absence of erythrocyte lysates or hemoglobin, i*n vitro* growth of *B. quintana* requires media supplemented with the highest known concentrations of hemin among bacterial species (31). Free heme is toxic in humans due to its lipophilic nature and ability to participate in the generation of reactive oxygen species via Fenton chemistry. Therefore, it is either rapidly catabolized by a heme oxygenase system or neutralized by one of several host heme-binding proteins, maintaining a very low concentration (22). However, complexed heme, primarily hemoglobin, is abundant (16). Acquisition of heme in the limiting environment of the human host is pivotal to the survival and pathogenesis of *B. quintana*. In contrast to the human host, free heme is thought to exceed toxic levels in the gut of blood-sucking arthropods during the initial digestion of a blood meal (34). The ability of *B. quintana* to withstand the heme-limiting environment of the human host and the heme-replete gut of the body louse suggests that its heme acquisition systems are tightly regulated.

Little is known about molecular mechanisms or regulation of heme acquisition by *Bartonella*. Previous studies by our lab focused on the hemin-binding proteins (HbpA-E), a five-member family of outer membrane porin-like proteins (28). In addition to binding hemin, Hbps are transcriptionally regulated in response to variations in ambient temperature, oxygen levels, and hemin concentration (5). This regulation is mediated in

part by Irr (iron response regulator), a member of the ferric uptake regulator (Fur) superfamily first described in *Bradyrhizobium japonicum*, that responds directly to hemin (17). Irr acts as either a transcriptional activator or repressor by binding the iron control element (ICE) of *B. japonicum*, and the effect of Irr on target genes is believed to be a consequence of ICE's location relative to the transcriptional start site (39). In *B. quintana,* Irr operates by binding a unique DNA motif found in the promoter region of all *hbp* genes, termed "H-box" (6). *B. quintana* has at least two additional iron- and/or hemin-responsive regulators, namely Fur and RirA (rhizobial iron regulator A) (2). In γ-proteobacteria, Fur functions as a transcriptional regulator of iron and hemin uptake systems with activation dependent on intracellular iron concentrations (18). In contrast, Fur has been shown to play a diminished or nonexistent role in α-proteobacteria (20). *fur* overexpression in *B. quintana* resulted in decreased *hbpC* transcription but had no effect on other *hbp* genes (6). RirA has primarily been studied in *Rhizobium leguminosarum* and is homologous to the iron-sulfur cluster regulator (IscR) of *E. coli* (48). Overexpression of *rirA* in *B. quintana* resulted in increased expression of *hbpA, hbpD,* and *hbpE* (6).

Hbps lack amino acid sequence similarity and predicted structural similarity to known bacterial hemin receptors, despite their ability to bind hemin and regulation by hemin-responsive transcription factors (10). Therefore, we hypothesized that an alternate hemin uptake and utilization locus was present in *B. quintana* and identified a candidate through analysis of the available genome (2). The current study was undertaken to characterize the function, regulation, and transcriptional organization of this locus.

C. MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* was routinely grown overnight at 37°C in Luria-Bertani (LB) or tryptone-yeast extract (TY) media with standard antibiotic concentrations when required. For induction of gene expression, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to mid-log cultures at a final concentration of 2mM and cultures were grown for an additional 4-5 h. For growth of *E. coli hemA* strains EB53 and IR754, media were supplemented with 25μM δ-aminolevulinic acid (ALA) (Research Products International , Prospect, IL) (12). *B. quintana* strains were grown on chocolate agar or on *Brucella* agar (BA) (Becton Dickinson, Sparks, MD) supplemented hemin chloride (CalBiochem, San Diego, CA) at 37°C in 5% CO_2 and 100% relative humidity. 10mg/ml hemin chloride was dissolved in 0.2M NaOH and filter sterilized for a stock solution. In order to maintain pBBR1MCS and derivatives in *B. quintana*, medium was supplemented with 1μg/ml chloramphenicol. *B. quintana* plates were harvested at mid-log phase [3-5d post-inoculation (6)] and age-matched for individual experiments. Strains used in this study are summarized in Table 3.1 (p. 65).

***In silico* analyses.** Genomic sequences for *B. quintana* strain Toulouse were accessed at the *Rhizo*DB website (http://xbase.bham.ac.uk/rhizodb/) (11) or the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). BLAST was employed for all database searches (3) and ClustalW version 2.0 (23) was used for multiple amino acid sequence alignments. The Protein Homology / Analogy Recognition

Engine (Phyre) program (9) was used to predict three-dimensional protein structures (http://www.sbg.bio.ic.ac.uk/phyre/).

Preparation and manipulation of nucleic acids. *B. quintana* genomic DNA was purified with a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). Plasmids were purified with a QIAprep Spin Miniprep kit (Qiagen), Perfectprep Plasmid Mini kit (Eppendorf, Hamburg, Germany), or Wizard Plus Midiprep DNA Purification System (Promega, Madison, WI). Routine procedures were employed for PCR amplification, ligation, cloning and restriction endonuclease digestion (4). PCR and sequencing primers were synthesized by Operon Biotechnologies (Huntsville, AL).

Total RNA was isolated from *B. quintana* immediately upon harvest with a RiboRure-Bacteria Kit (Ambion, Austin, TX) per protocol except cell lysis was done with an FP120 Fast Prep bead homogenizer (45 sec at top speed) using Zirconia beads supplied (Qbiogene, Carlsbad, CA). DNase treatment was accomplished with a Turbo DNA-free kit (Ambion). Nucleic acids were quantified using a Spectronic Genesys 2 (Milton Roy, Rochester, NY) or a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA) spectrophotometer. Based on published sequence (2), primers for qRT-PCR were synthesized by Integrated DNA Technologies (Coralville, IA) and are listed in Supplementary Table 3.1 (p. 65).

Complementation assays. The ability of *E. coli hemA* strains EB53 or IR754 (containing pNP1 or vector alone) to use hemin was examined as previously described with modifications (47). Briefly, overnight cultures were centrifuged at 3900 x g for 5

min at 4°C and pellets were resuspended in 5ml TY without ALA. Cultures were incubated ~2 h at 37°C with shaking to deplete intracellular ALA and hemin and then used to inoculate 8ml cultures of TY alone or supplemented with either ALA (50μM) or hemin chloride (10μg/ml or 50μg/ml) to an initial optical density of 0.02 at 600 nm (OD_{600}). Cultures were incubated at 37°C with agitation and OD_{600} was measured every 4 h for 24 h.

Generation of anti-HutA antisera. A 6xHis-tagged mature *B. quintana* HutA protein was generated and purified under denaturing conditions using the QIAexpress kit (Qiagen). Purified protein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 12.5% (wt/vol) acrylamide gels (4). Gels were rinsed three times for 5 min in deionized water and then stained with 0.05% (wt/vol) Coomassie blue in deionized water for ~30 min. Following destaining in water, the purified HutA band was excised and used to generate rabbit anti-HutA antiserum as previously described (42).

Sarkosyl fractionation and immunoblotting. Proteins were quantified by a bicinchoninic acid protein kit (Pierce, Rockford, IL). Sarkosyl fractionation was performed essentially as previously described (52). Briefly, overnight cultures of *E. coli* were harvested, washed in phosphate-buffered saline (pH 7.4), and resuspended in sterile distilled H_2O. Cell lysis was done with a Fastprep bead homogenizer as above. Cells were incubated for 30 min in 2% (vol/vol) N-lauroyl sarcosinate (Sigma, St. Louis, MO) at room temperature and then centrifuged for 1 h at 100,000 x g at 4°C in a SW60Ti rotor

(Beckman Coulter, Fullerton, CA). The sarkosyl-insoluble pellet was resuspended in 0.2mM phenylmethylsulfonyl fluoride (PMSF) in deionized water (Sigma). Both the resuspended pellet and sarkosyl-soluble supernatant fraction were stored at -20^{o}C until needed. Samples were resolved by SDS-PAGE and transferred to supported nitrocellulose (GE Water & Process Technologies, Trevose, PA) for immunoblotting (49). Resulting blots were probed overnight with rabbit anti-HutA antiserum and developed with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Sigma), 4-chloronaphthol, and hydrogen peroxide, as previously described (42).

qRT-PCR and RT-PCR. Relative differences in *hut* locus expression were quantified in *B. quintana* grown on BA supplemented with low (0.05mM) or high (2.5mM) hemin relative to an optimal hemin concentration (0.15mM) or for JK31 overexpressing *fur, irr*, or *rirA* relative to JK31 with pBBR1MCS vector alone (6). For each condition, 500ng RNA was reverse transcribed per manufacturer's instructions with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). 0.67 ng template cDNA and 500nM of each primer were used per 25µl reaction with iQ SYBR Green Supermix (Bio-Rad) as recommended. qRT-PCR reactions were incubated for 5 min at 95^{o}C, then 40 cycles at 95^{o}C for 30 sec followed by 55^{o}C for 30 sec. Data were obtained with a MyIQ Real-Time PCR detection system and Optical System software version 1.0 (Bio-Rad). Mean values from each triplicate reaction were used to determine individual fold differences in gene expression by the $2^{-\Delta\Delta Ct}$ method using 16S rRNA as the internal control (24).

Transcriptional organization of the *hut* locus genes was examined by reverse transcribing the *hmuV* transcript and using it for PCR amplification of individual *hut* genes as previously described (29). Briefly, 500-1100ng DNase-treated RNA from JK31 grown on BA containing 0.05mM hemin was reverse transcribed using SuperScript III First Strand Synthesis for RT-PCR (Invitrogen) per protocol. Resulting cDNA was used as a PCR template with primer sets for *hemS, hutA, hutB, hutC* and *hmuV*. A reaction lacking reverse transcriptase was used as a PCR template to control for contaminating DNA.

Transcriptional start site (TSS) mapping. RNA was isolated from *B. quintana* grown on BA supplemented with 0.05 or 0.15mM hemin and used for TSS mapping of *tonB*, *hutA*, and *hemS* with a 5' RACE system for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA). Briefly, RNA was reverse transcribed with Superscript II and the resulting cDNA was RNase treated. Following purification of cDNA with a QIAquick PCR purification kit (Qiagen), a 3' dC tail was added, and tailed cDNA was PCR amplified with the Abridged Anchor Primer (AAP) supplied in the 5'RACE kit and a nested, gene-specific primer. PCR products were cloned into pCR2.1-TOPO and used to transform *E. coli* TOP10F' per TOPO TA Cloning (Invitrogen) protocol. Plasmids were screened for appropriately sized inserts and sequenced.

DNA sequencing. Sequence data were obtained with an automated DNA sequencer (AB3130x1 Genetic Analyzer) and a BigDye Terminator Cycle Sequencing Ready

Reaction kit 3.1 (ABI, Foster City, CA). Sequence data were analyzed with ChromasPro 1.13 (http://www.technelysium.com.au/ChromasPro.html).

Statistical analyses. Three independent determinations were used to calculate the mean and standard deviations for all numerical data. Statistical significance was determined using the Student's *t* test with P values < 0.05 considered significant.

D. RESULTS

***In silico* analyses of *B. quintana hut* locus.** The *hut* locus consists of six genes encoding a potential receptor, HutA, an ABC transport system, HutBC and HmuV, a TonB orthologue, and a hemin storage/degradation enzyme, HemS (Fig. 3-1A, p. 66). The gene arrangement suggested the possibility for divergent transcription of *tonB* and *hutA* (8), while *hemS, hutBC* and *hmuV* appeared polycistronic due to close linkage. BLAST analyses indicated that orthologues exist for each member of the *hut* locus in other α-proteobacteria (Fig. 3-1A). Of note, *B. quintana* TonB lacks an N-terminal domain that facilitates ExbB-ExbD (cytosolic membrane proteins used in energy transduction) contact and cytoplasmic anchorage in *E. coli* (35). Similarly, the predicted TonB box of *B. quintana* HutA shares only ~50% conservation with the consensus sequence of TonB-dependent proteins (data not shown) (30). ClustalW alignment of HutA with hemin/hemoglobin receptors of other pathogenic bacteria shows conservation of characteristic FRAP and NPNL domains (10) (Fig. 3-1B, p. 67). A conserved histidine (HIS 461) essential for hemin utilization by *Yersinia enterocolitica* HemR has been replaced by a tyrosine (TYR 505) in *B. quintana* HutA (10) (Fig. 3-1B). This

substitution is also seen in BhuR, the *Bordetella avium* heme/hemoprotein receptor (30). Like BhuR, HutA shares more homology with the "heme scavenger" subclass of receptors than with the hemoglobin subclass (*e.g.,* HmbR of *Neisseria meningitides*) (45). Three dimensional modeling of *B. quintana* HutA shows structural similarity to the ferric citrate receptor (FecA) of *E. coli* (14), where threading revealed the expected twenty-two antiparallel β-strands and 11 extracellular loops characteristic of TonB-dependent receptors (data not shown) (13). TYR 505 was centrally positioned in one of the extracellular loops of the protein, as were four additional tyrosines (i.e., residues 278, 451, 511, and 512) and histidine 389 (9). *In silico* data strongly suggest the *hut* locus is a system dedicated to hemin acquisition and that HutA functions as the receptor.

Complementation of *E. coli hemA* strains. The outer membrane of *E. coli* K12 is impermeable to hemin, and growth defects from mutations in porphyrin biosynthesis genes cannot be overcome with hemin supplements (41). *E. coli hemA aroB* strain EB53 is a K12 derivative with a mutation in glutamyl-tRNA reductase, required for biosynthesis of ALA and ultimately protoporphyrin IX (7). Exogenously supplied ALA can restore growth of EB53, however, utilization of hemin requires a functional hemin receptor (44). To test the hypothesis that *B. quintana* HutA functions as a hemin receptor, *hutA* was cloned into pWSK29 to produce pNP1 and used to transform EB53. When grown in the absence of ALA and hemin, EB53/pWSK29 and EB53/pNP1 exhibited the characteristic "leaky" growth (maximum OD_{600} of ~ 0.185 in 24 h; data not shown) previously noted in these strains (41). Normal growth curves were obtained for both EB53/pWSK29 and EB53/pNP1 when TY broth was supplemented with 0.05mM

ALA (data not shown). However, when media were supplemented with 10µg/ml hemin, EB53/pNP1 grew to a significantly higher OD_{600} by 24 h ($p<0.029$) than EB53/pWSK29 (Fig. 3-2A, p. 68). This difference was more pronounced when strains were grown in media supplemented with 50µg/ml hemin ($p<0.0002$) (Fig. 3-2B). Interestingly, rescue of the *hemA* mutation in *E. coli* by *B. quintana* HutA is not apparent until ~16 h post-inoculation despite hemin/ALA starvation. Regardless, these data indicate that *B. quintana* HutA functions as a hemin receptor, and its expression in EB53 is sufficient to allow utilization of hemin as a porphyrin source.

In order to examine HutA's potential dependence on TonB for energization, pNP1 was also used to transform *E. coli* strain IR754 (47). As observed in the EB53 strains, both IR754/pWSK29 and IR754/pNP1 exhibited normal growth curves in TY media supplemented with 0.05mM ALA and neither strain surpassed the basal "leaky" growth level in media lacking both ALA and hemin (data not shown). However, expression of *B. quintana* HutA could not restore growth of IR754 in media supplemented with either 10µg/ml or 50µg/ml hemin (Figs. 3-2A and 3-2B, respectively) in direct contrast to EB53. These data clearly indicate that HutA-mediated hemin uptake is dependent on *E. coli* TonB.

Synthesis of HutA in *B. quintana* and *E. coli*. We were able to identify native HutA in *B. quintana* and recombinant His-tagged HutA in *E. coli* strain JM109/pNP3 (data not shown), but were unable to detect HutA in *E. coli* strain EB53/pNP1 by Coomassie blue staining or Western blotting. However, expression and induction of *hutA* mRNA in *E. coli* strain EB53/pNP1 were detectable by qRT-PCR. As expected, cDNA from

EB53/pWSK29 gave results similar to those obtained from a no-template control (data not shown). Recombinant HutA protein is undoubtedly localized to the outer membrane of EB53/pNP1 as deduced from its ability to rescue the *hemA* mutation and restore growth in the presence of hemin. Failure to detect HutA in EB53/pNP1 is possibly due to a combination of low level expression and antiserum cross-reactivity. A similar situation was reported for detection of the recombinant *B. henselae* orthologue (Pap31) in *E. coli* strain M15 until a monoclonal antibody was employed (52).

Transcription of *hut* locus genes is hemin-responsive. *hut* locus genes were expected to be tightly regulated in response to available hemin. To investigate this hypothesis, RNA was isolated from *B. quintana* grown on media supplemented with low (0.05mM), optimal (0.15mM), and high (2.5mM) concentrations of hemin and used to examine differences in *hut* levels by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Data show that expression of *hut* locus genes from JK31 grown on BA-0.05mM hemin is only ~1.5-fold higher than that obtained from JK31 grown on BA-0.15mM hemin, suggesting that this range of hemin concentrations does not substantially alter expression of the *hut* locus. The most pronounced change was observed when transcript levels from JK31 grown on BA-2.5mM hemin were compared to those from JK31 grown on BA-0.15mM, where results show an ~2.2-fold decrease in transcription of *hut* locus genes in response to excess hemin (Fig. 3-3, p. 69). Furthermore, the *hut* locus genes are coordinately regulated as evidenced by the fact that fold differences for each *hut* locus gene are repressed to approximately the same magnitude. These results

show that *hut* locus genes are transcriptionally down-regulated in response to excess hemin.

Hemin-responsive control is mediated by *B. quintana* Irr. To elucidate regulation of *hut* locus genes, JK31 strains that overexpress one of three iron/hemin-responsive regulator genes were used for qRT-PCR experiments to investigate the effects on transcription. RNA was isolated from overexpression and vector-only strains grown in parallel on BA-0.05mM hemin, BA-0.15mM hemin, and BA-2.5mM hemin to control for cofactor availability. Average fold differences between JK31+pBBR-RIRA and JK31+pBBR indicate a 7 to 16-fold increase in *rirA* transcription but less than a 1.8-fold increase in transcription of any *hut* locus gene under optimal or hemin-limiting growth conditions. Likewise, overexpression of *rirA* results in less than a 1.8-fold decrease in transcription of any *hut* locus gene when RNA is isolated from strains grown in the presence of excess hemin (Fig. 3-4A, p. 70). These data suggest that *rirA* overexpression does not appreciably affect transcription of *hut* locus genes regardless of ambient hemin concentration.

Similar results were obtained when *hut* locus transcript levels were compared in JK31+pBBR-FUR relative to JK31+pBBR after growth on BA-0.05mM hemin. These conditions resulted in less than a 1.7-fold increase in any *hut* locus gene, while *fur* overexpression was evident by a 4.4-fold increase (Fig. 3-4B, p. 71). On BA-0.15mM hemin, *fur* transcription was increased ~8-fold in JK31+pBBR-FUR relative to JK31+pBBR, *hemS* levels were almost identical, and the remainder of the *hut* locus genes showed a minor decrease in transcription. *fur* overexpression results in a 2.5-fold

increase in *fur* and a 4-fold decrease in *tonB*, when comparing *hut* locus expression from strains grown in the presence of excess hemin,. The remainder of the *hut* locus shows only a minor decrease in expression as seen in strains grown with optimal hemin concentrations. These data suggest that *fur* overexpression in the presence of excess hemin exerts a repressive effect on *tonB* that is not imposed on other members of the *hut* locus.

irr overexpression results in decreased transcription of the entire *hut* locus (Fig. 3-4C, p. 72). Average fold differences in transcription of *hut* locus genes from JK31+pBBR-IRR relative to JK31+pBBR resulted in a 7 to 12-fold increase in *irr* mRNA and an ~2.5 fold decrease in transcription of all *hut* genes regardless of hemin concentration. Interestingly, the decrease in transcription during *irr* overexpression is similar in magnitude to the decrease in *hut* locus expression in the presence of excess hemin (Fig. 3-3, p. 69). These data suggest that hemin-responsive regulation of *hut* genes is mediated, at least in part, by Irr.

Transcriptional organization of the *hut* locus. Genomic arrangement of the *hut* locus suggested that *hutA* and *tonB* might be divergently transcribed, while *hemS*, *hutBC*, and *hmuV* could be polycistronic (Fig. 3-1A, p. 66). To test these hypotheses, RNA from JK31 was reverse transcribed with a *hmuV* primer. PCR analyses were performed on the resulting cDNA using primers specific to each member of the *hut* locus (except *tonB*) and a separate PCR reaction using genomic DNA template was used as a positive control for each gene. Data indicate that *hemS, hutB, hutC,* and *hmuV* are all present in the *hmuV* transcript as evidenced by the PCR amplicons generated with primers specific to each of

these genes from the *hmuV* cDNA. In contrast, no *hutA* amplicon is generated from the cDNA (Fig. 3-5, p. 73). Furthermore, no PCR amplicons were generated from the reactions using the sample that was not reverse transcribed, which confirms the absence of contaminating DNA. These data show that *hemS, hutB, hutC*, and *hmuV* are co-transcribed as part of a polycistronic transcript from the *hemS* promoter, while *hutA* is transcribed as a separate mRNA.

TSS mapping and identification of "H-box" elements. To further elucidate regulation of the *hut* locus, TSS's were mapped for *tonB*, *hutA,* and *hemS* by 5' RACE. The *hutA* TSS was found 121 bp upstream of its start codon, and the *tonB* TSS was mapped 40 bp upstream of its start codon. Putative -10 and -35 sites were identified in the 78-bp divergent promoter region (Fig. 3-6A, p. 74). Examination of the divergent promoter region between *tonB* and *hutA* showed ~69% identity with a 40-bp consensus sequence, previously identified in *Bartonella hbp* promoter regions (6). The H-box completely encompasses the -10 and -35 sites of *hutA* and is located 1 bp before the potential -35 site of *tonB*. In contrast, no obvious similarity to the Fur-binding motifs of *E. coli* or *B. japonicum* Fur proteins was found upstream of *tonB* (15).

The *hemS* TSS was mapped 70 bp upstream of the start codon and potential -10 and -35 sites were identified relative to the TSS (Fig. 3-6B, p. 75). The *hemS* promoter region also contains a site with ~57% identity to the H-box consensus sequence. This region overlaps the predicted -35 site of *hemS* but does not extend to the -10 site. Identification of motifs similar to the H-box and surrounding consensus sequence in the promoter region of *hutA/tonB* and *hemS* is consistent with qRT-PCR data showing repressive

effects of *irr* overexpression on *hut* locus expression (Fig. 3-4C, p. 72). Together, these results suggest Irr represses transcription of *hut* locus genes by binding at or near RNA polymerase recognition sites.

E. DISCUSSION

Genomic analyses of the *B. quintana* genome show multiple systems possibly involved in hemin and/or iron acquisition. One of these, the *hut* locus, appeared to be the most likely and complete candidate for a hemin uptake system. Based on multiple lines of *in silico* evidence, including amino acid similarity, domain conservation, and structural similarity, we hypothesized that HutA was functioning as a hemin receptor (Fig. 3-1, p. 66).

We tested the hypothesis by functional expression of *B. quintana hutA* in *E. coli hemA* strain EB53 (12, 41). Expression of *hutA trans*-complemented EB53 was tested in the presence of hemin at two concentrations (Fig. 3-2, p. 68). The *E. coli hemA* strains have a leaky phenotype which accounts for the low level increase in optical density over time (41). The results required an ~12-16 h lag before a difference in growth rates was discernible between complemented and control strains. This observation may be due to limited homology between TonB and TonB boxes of *B. quintana* and *E. coli* (30). Nevertheless, sufficient homology in TonB allowed *B. quintana* HutA to function as a hemin receptor in EB53, whereas HutA could not complement an otherwise isogenic *E. coli* strain (IR754) where both *tonB* and *hemA* are mutagenized (Fig. 3-2, p. 68).

The *B. quintana hut* locus is similar to other bacterial hemin acquisition systems in that it is transcriptionally regulated in a hemin-responsive manner (Fig. 3-3, p. 69). Growth on low hemin results in a slight increase in *hut* locus mRNAs relative to the quantity obtained from growth on optimal hemin. Although the difference in hemin concentrations between BA-2.5mM hemin and BA-0.15mM is much greater, the decrease in *hut* gene transcription is fairly modest. Based on these data, it is tempting to speculate that changes in extracellular hemin are buffered in *B. quintana,* possibly by an accessory hemin-binding system, such as the Hbps. Such a system could enhance the ability of *B. quintana* to withstand hemin fluctuations in the divergent environments of the body louse and human bloodstream.

Effects of overexpression of hemin/iron-responsive regulators (*irr*, *rirA*, and *fur*) indicate that hemin-responsive changes in *hut* locus transcription are primarily mediated by Irr. Although Irr homologs have been reported to act as transcriptional activators of hemin/iron genes when hemin is limiting (25, 39), *B. quintana* Irr represses *hut* locus genes. *B. quintana* Irr may also transcriptionally activate *hut* locus genes in the absence of hemin, but the absolute requirement for hemin by *B. quintana* prohibits investigating this possibility (31). Our data suggest that *irr* overexpression results in repression of *hut* locus genes regardless of ambient hemin concentration, despite previous reports suggesting that *B. japonicum* Irr is degraded upon binding hemin (51). Interestingly, the N-terminal heme response motif (amino acids 28-33) of *B. japonicum* Irr is not conserved in *B. quintana* Irr (51). Likewise, only two of three histidine residues implicated in a second hemin-binding site (51) are present in *B. quintana* Irr and both *B. quintana* and *E. coli* Fur have two histidines in this position but are not degraded by hemin (data not

shown). *B. japonicum* Irr represses protoporphyrin biosynthesis genes when heme is present, but the majority of these genes are not present in the *B. quintana* genome suggesting that *B. quintana* Irr plays a distinct role (6, 36). Of specific interest, heme-mediated degradation of Irr in *B. japonicum* requires ferrochelatase (36), but an orthologue is absent in *B. quintana* (2).

RT-PCR analyses show that the *hut* locus is expressed as three transcripts, originating from a divergent promoter region between *hutA* and *tonB* and a polycistronic mRNA transcribed from the region upstream of *hemS* (Fig. 3-5, p. 73). Consistent with qRT-PCR data from the *irr* overexpression strain, both promoters possess regions with considerable identity to a consensus sequence containing H-box (Fig 3-6, p. 74-5) (6). Unlike Hbps, the majority of which were activated by Irr, the consensus sequence encompassing H-box in the *hut* locus promoters either overlaps the -10 and -35 regions (*hutA*) or is located nearby (*tonB* and *hemS*). A similar location for the ICE motif was reported in *B. japonicum* genes repressed by Irr (39, 40). These data strongly suggest that Irr directly represses the *hut* locus.

In contrast to *irr*, *rirA* overexpression showed only minor changes in transcription of *hut* locus genes, regardless of hemin concentration (Fig. 3-4C, p. 72). In the presence of excess hemin, *fur* overexpression resulted in decreased transcription of *tonB* (Fig. 3-4B, p. 71). However, no obvious consensus Fur box sequence is evident in the promoter region of *tonB* (15). The effect of *fur* overexpression on *tonB* may be indirect in *B. quintana*, but this seems unlikely as *tonB* is known to be repressed by Fur in other bacteria (32). Most likely, *Bartonella* Fur recognizes a unique consensus sequence, as described for *B. japonicum* (15).

To our knowledge, this is the first study to characterize a complete system of hemin acquisition in *Bartonella*. Our data indicate that the *hut* locus is surprisingly similar to hemin uptake systems described in other Gram-negative bacterial pathogens and is primarily controlled by Irr. However, given the importance of hemin to the survival and pathogenesis of *Bartonella*, *B. quintana* provides a unique model for studying its acquisition and utilization. Interesting areas of future study include the interplay between the Hut proteins and potential accessory systems including proteins able to bind hemin (*e.g.,* Hbp's), proteins able to remove heme from hemoglobin, and proteins able to function as hemoglobin receptors. Any of these systems would contribute to the success of *Bartonella* pathogenesis by buffering fluctuations in available heme and by allowing *Bartonella* to use the most abundant source of heme in the human host (16).

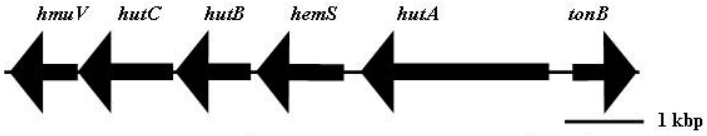
F. ACKNOWLEDGEMENTS

We thank Jane Koehler (UC San Francisco) for *B. quintana* strain JK31 and Christopher Elkins (UNC Chapel Hill) for *E. coli* strains EB53 and IR754 and helpful discussions regarding complementation. We thank Patty McIntire (Murdock Sequence Facility) for providing sequence analyses and Rahul Raghavan for helpful discussions. We are grateful to Kate Sappington, Laura Smitherman, Linda Hicks, Jessica Clark, and Sean Wolf for technical assistance. This work was supported by Public Health Service grant R01 AI053111 from the National Institutes of Health to M.F.M..

Table 3.1. Bacterial strains and plasmids used in this study.

Strains and plamids	Description	Source or reference
Strains		
B. quintana		
JK31	Low-passage human isolate	J. Koehler
JK31+pBBR	JK31 with pBBR1MCS	(6)
JK31+pBBR-FUR	JK31 with pBBR-FUR	(6)
JK31+pBBR-RIRA	JK31 with pBBR-RIRA	(6)
JK31+pBBR-IRR	JK31 with pBBR-IRR	(6)
E. coli		
JM109	Host strain for cloning	Promega
TOP10F'	Host strain for cloning	Invitrogen
EB53	*hemA aroB rpoB*	(47)
IR754	EB53 but *tonB::*Kan	(47)
EB53/pWSK29	EB53 harboring pWSK29	This study
EB53/pNP1	EB53 harboring pNP1	This study
IR754/pWSK29	IR754 harboring pWSK29	This study
IR754/pNP1	IR754 harboring pNP1	This study
Plasmids		
pCR2.1TOPO	TA cloning vector	Invitrogen
pNP2	pCR2.1TOPO containing *B. quintana hutA*	This study
pWSK29	Low copy-number expression vector	(50)
pNP1	pWSK29 containing *B. quintana hutA*	This study
pQE30	Expression vector for 6xHis-tagged fusion proteins	Qiagen
pNP3	pQE30 containing *B. quintana hutA*	This study
pBBR1MCS	Shuttle vector for *Bartonella*	(21)
pBBR-FUR	pBBR1MCS containing *B. quintana fur*	(6)
pBBR-RIRA	pBBR1MCS containing *B. quintana rirA*	(6)
pBBR-IRR	pBBR1MCS containing *B. quintana irr*	(6)

A)



Protein	Hypothetical function	BLAST (%ID:%Positive)	pI	Mass (kDa)
HutA	Hemin receptor	*S. medicae* (39:56)	9.2	82.7
HutB	Periplasmic hemin-binding protein	*M. loti* (49:72)	9.8	32.7
HutC	Hemin ABC transporter, permease	*R. leguminosarum* (63:78)	9.5	38.7
HmuV	Hemin ABC transporter, ATPase	*M. loti* (49:69)	8.6	28.9
HemS	Hemin degradation/storage	*M. loti* (44:62)	6.2	39.4
TonB	Energy transducer	*R. palustris* (38:52 C-terminus only)	6.1	28.9

Figure 3-1. Arrangement and homology of *B. quintana* hemin uptake locus.
A. Genomic arrangement of the *B. quintana hut* locus and BLAST results indicating closest orthologues outside *Bartonellaceae* (percent amino acid identity: percent amino acid similarity), as well as predicted: function, isoelectric point (pI) and mass (kDa).
B. ClustalW alignment of C-terminal region of *B. quintana* HutA with hemin/hemoglobin receptors of *Yersinia enterocolitica* (HemR), *Y. pestis* (HmuR), and *Haemophilus influenzae* (HxuC). Conserved FRAP and NPNL domains are boxed and shaded. The tyrosine 505 substitution aligned with typically conserved histidines is indicated by bold-face type. Fully conserved residues are indicated by a star, a colon shows strongly conserved residues, and a dot indicates weakly conserved residues.

B)

```
HemR    KWS-------------SRGAVSVTPTDWLMLFGSYAQAFRAPTMGEMYNDSKHFSMNIM- 467
HmuR    KWS-------------SRGAISITPTDWLMLFGSYAQAFRAPTMGEMYNDSKHFAIPIRP 468
HxuC    HLS-------------PATKLTWKVTNWLDFTAKYNEAFRAPSMQERFVSGAHFGTSTRV 490
HutA    LISNKYPSERSGSRFSPKLRMEWDFRDQVTFYAQWAQAFRAPRVSELYLS--YIKPPLY- 511
          *           : .   :     : : : ..: :***** : * : .  ::

HemR    GNTLTNYWVPNPNLKPETNETQEYGFGLRFNDLMMAEDDLQFKASYFDTNAKDYISTGVT 527
HmuR    GLTLTNYWVPNPNLKPETNETQEYGFGLRFSDLLMAEDDLQFKVSYFDTKAKDYISTRVD 528
HxuC    GDIIN-SFVANPNLRPETAKNKEITANLHFDSLFKQGDKFKIEATYFRNDVKDLINLKRL 549
HutA    ------YVKGNPDLKSEGSNG--YDIGIQYGNVNFGG-----SLSAFINQYKDFITTVDK 558
                  **:*:.*  :      .::::.:         . : * .. ** *.
```

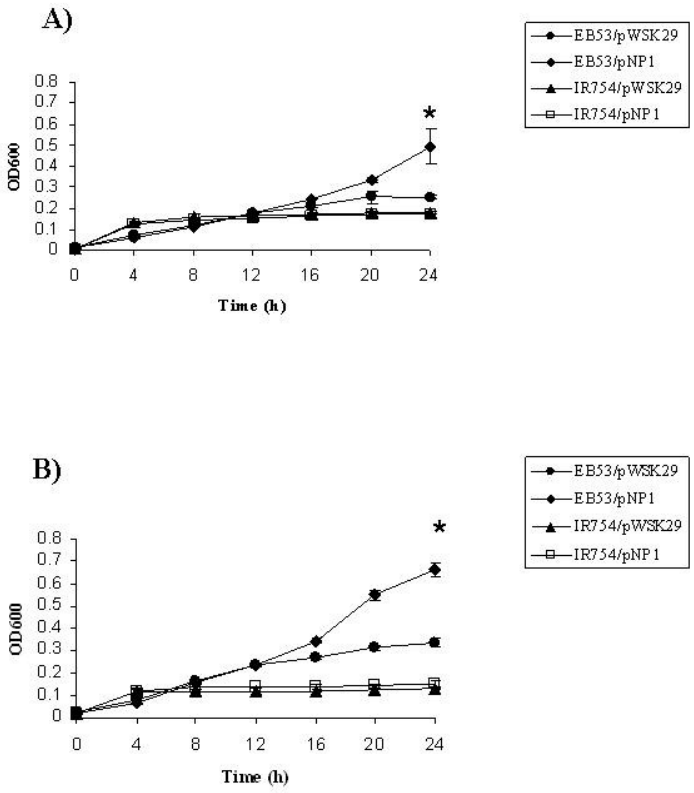


Figure 3-2. Complementation of *E. coli hemA* strains with *B. quintana hutA*. Growth curve of *E. coli* EB53 and IR754 containing pWSK29 (vector control) or pNP1 inoculated into TY supplemented with: A) 10µg/ml or B) 50µg/ml hemin after a brief period of hemin/ALA starvation. The asterisk (*) indicates a statistically significant difference in OD_{600} relative to controls.

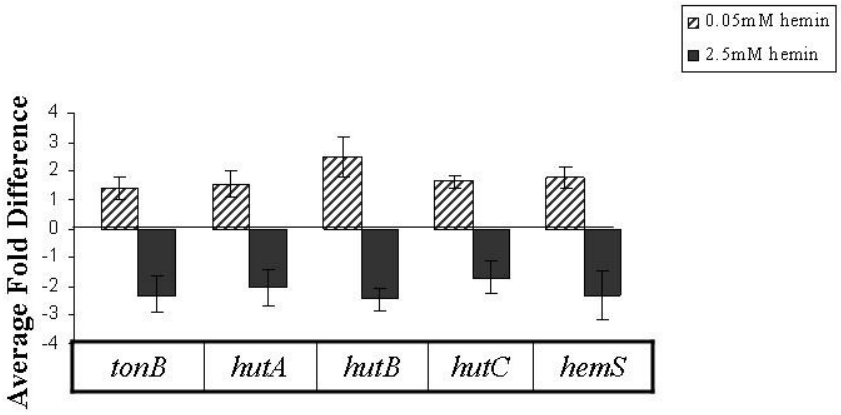


Figure 3-3. qRT-PCR analysis of *B. quintana hut* locus transcription in response to hemin availability. Average fold differences in *hut* locus transcript levels from JK31 grown under hemin-limiting conditions (0.05mM) or excess hemin (2.5mM) relative to optimal levels (0.15mM). Data represent the means of three independent determinations (per gene per condition) +/- standard deviation.

A)

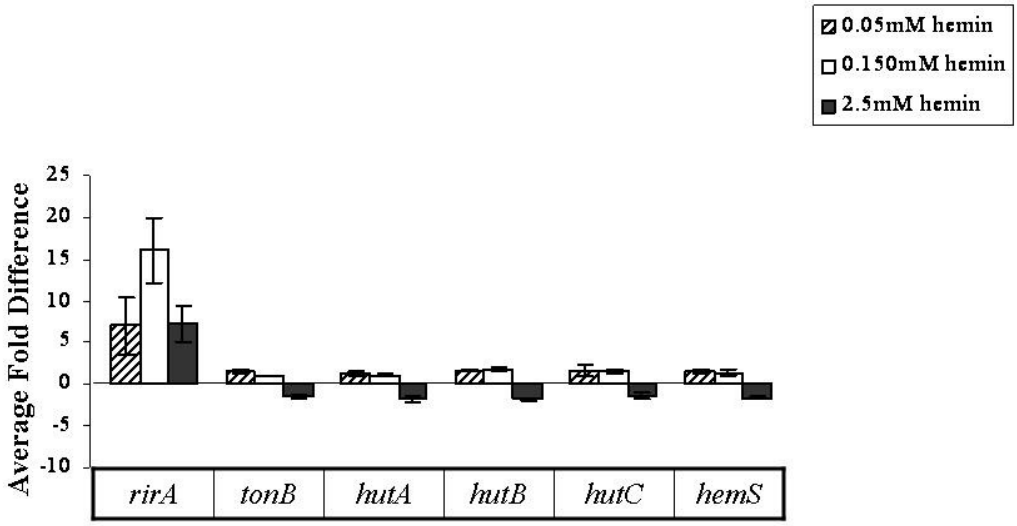
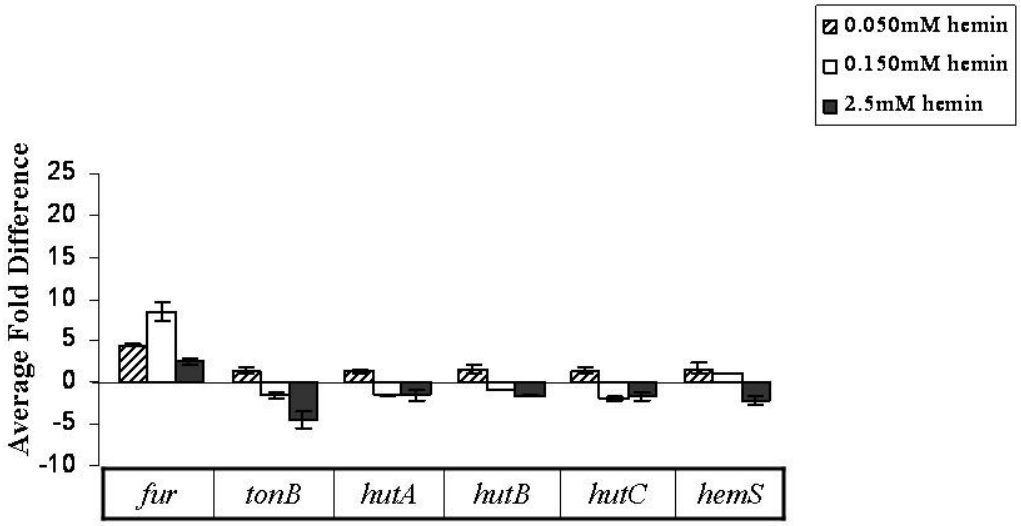
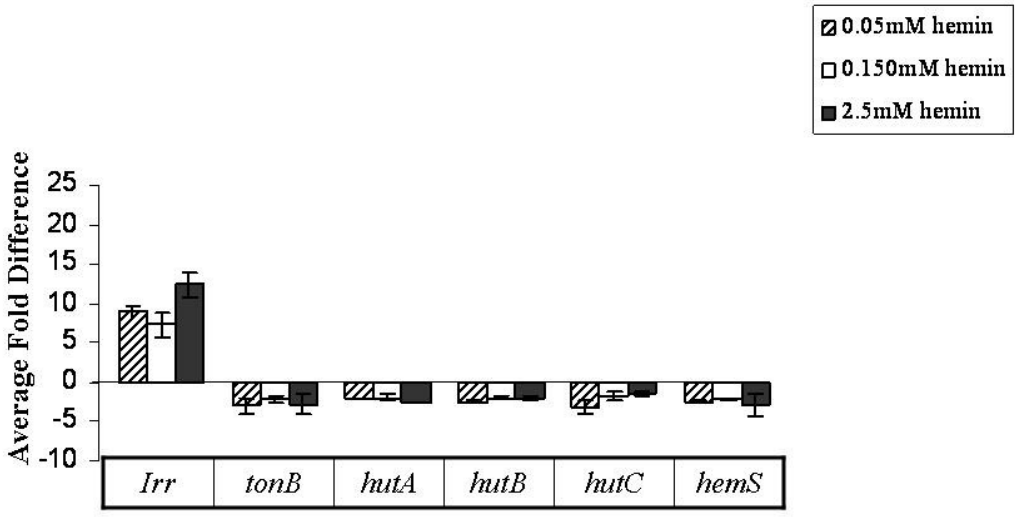


Figure 3-4. qRT-PCR analyses of *B. quintana hut* locus in response to overexpression of various iron response regulators. Average fold difference in *hut* locus transcription from: A) JK31+pBBR-RIRA, B) JK31+pBBR-FUR and C) JK31+pBBR-IRR relative to JK31+pBBR. Strains were grown in parallel on BA-0.05mM hemin, BA-0.15mM hemin, and BA-2.5mM hemin. Data represent the means +/- standard deviation of three independent determinations.

B)


0.050mM hemin
0.150mM hemin
2.5mM hemin
Average Fold Difference
25
20
15
10
5
0
-5
-10
fur
tonB
hutA
hutB
hutC
hemS

C)


0.05mM hemin
0.150mM hemin
2.5mM hemin
Average Fold Difference
25
20
15
10
5
0
-5
-10
Irr
tonB
hutA
hutB
hutC
hemS

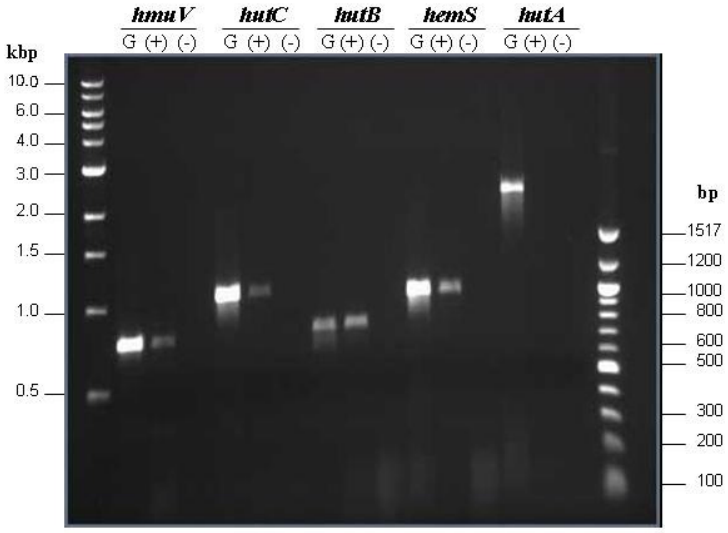


Figure 3-5. RT-PCR analysis verifies polycistronic nature of *hut* mRNA.

PCR analysis of *hmuV* transcript components using gene-specific primers for each member of the *hut* locus except *tonB*. (G), genomic DNA from JK31 used as template; (+), *hmuV* RT product used as template; (-), JK31 RNA without reverse transcription used as template. DNA size standards are indicated in kbp on left and bp on right side of gel.

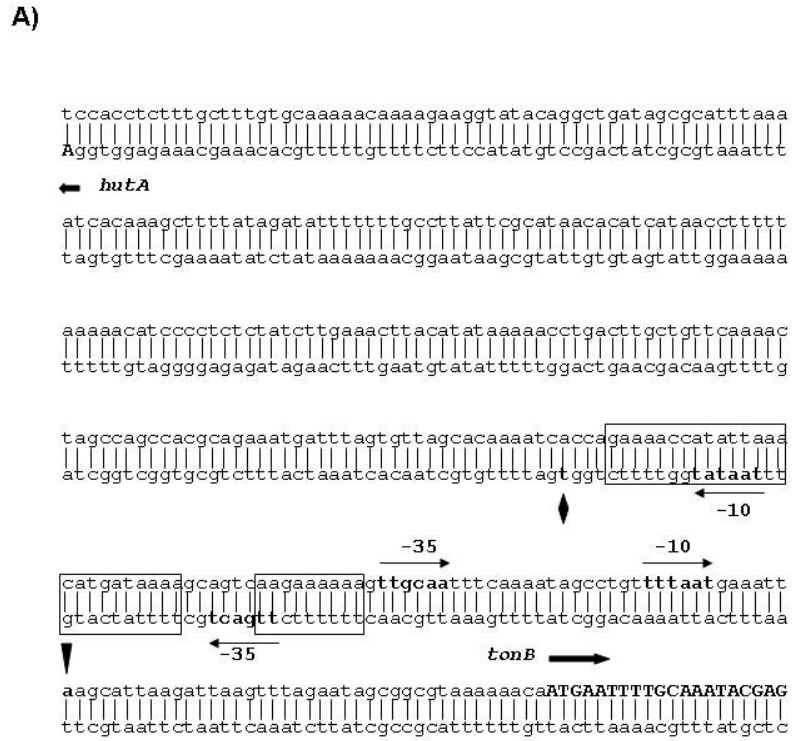
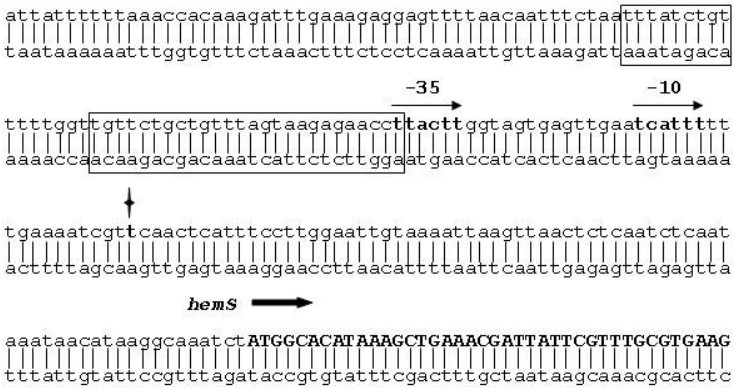


Figure 3-6. TSS mapping and promoter regulatory regions of the *hut* locus showing the H-box.

A. TSS's mapped by 5'RACE are indicated by a diamond (*hutA*) and arrowhead (*tonB*). Putative -10 and -35 promoter elements are shown with directionality, and the horizontal arrow and bold-face type indicate the *hutA* and *tonB* genes. The consensus sequence that interacts with *B. quintana* Irr (6) is boxed. Note the consensus is on the inverse complement (lower) strand.

B. TSS of *hems* is indicated in bold by the star. Potential -10 and -35 sites are indicated and the region containing the consensus sequence that interacts with *B. quintana* Irr (6) is boxed. The *hemS* gene is indicated by the horizontal arrow and bold-face type.

B)



G. REFERENCES

1. **Allen, D. M., J. S. Lehman, T. A. Green, M. L. Lindegren, I. M. Onorato, and W. Forrester.** 1994**.** HIV infection among homeless adults and runaway youth, United States, 1989-1992. Field Services Branch. Aids **8**:1593-1598.
2. **Alsmark, C. M., A. C. Frank, E. O. Karlberg, B. A. Legault, D. H. Ardell, B. Canback, A. S. Eriksson, A. K. Naslund, S. A. Handley, M. Huvet, B. La Scola, M. Holmberg, and S. G. Andersson.** 2004. The louse-borne human pathogen *Bartonella quintana* is a genomic derivative of the zoonotic agent *Bartonella henselae*. Proc. Natl. Acad. Sci. U S A **101**:9716-9721.
3. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215**:403-410.
4. **Ausubel, F.M. R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl.** 1995. Current Protocols in Molecular Biology. John Wiley & Sons Inc, New York, N.Y.
5. **Battisti, J. M., K. N. Sappington, L. S. Smitherman, N. L. Parrow, and M. F. Minnick.** 2006. Environmental signals generate a differential and coordinated expression of the heme receptor gene family of *Bartonella quintana*. Infect. Immun. **74**:3251-3261.
6. **Battisti, J. M., L. S. Smitherman, K. N. Sappington, N. L. Parrow, R. Raghavan, and M. F. Minnick.** 2007. Transcriptional regulation of the heme binding protein gene family of *Bartonella quintana* is accomplished by a novel promoter element and iron response regulator. Infect. Immun. **75**:4373-4385.

7. **Beale, S. I.** 1996. Biosynthesis of Hemes, p. 731-748. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, vol. I. ASM Press, Washington, D.C.

8. **Beck, C. F., and R. A. Warren.** 1988. Divergent promoters, a common form of gene organization. Microbiol. Rev. **52**:318-326.

9. **Bennett-Lovsey, R. M., A. D. Herbert, M. J. Sternberg, and L. A. Kelley.** 2008. Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins **70**:611-625.

10. **Bracken, C. S., M. T. Baer, A. Abdur-Rashid, W. Helms, and I. Stojiljkovic.** 1999. Use of heme-protein complexes by the *Yersinia enterocolitica* HemR receptor: histidine residues are essential for receptor function. J. Bacteriol. **181**:6063-6072.

11. **Chaudhuri, R. R., and M. J. Pallen.** 2006. xBASE, a collection of online databases for bacterial comparative genomics. Nucleic Acids Res. **34**:D335-D337.

12. **Elkins, C., P. A. Totten, B. Olsen, and C. E. Thomas.** 1998. Role of the *Haemophilus ducreyi* Ton system in internalization of heme from hemoglobin. Infect. Immun. **66**:151-160.

13. **Ferguson, A. D., and J. Deisenhofer.** 2002. TonB-dependent receptors-structural perspectives. Biochim. Biophys. Acta. **1565**:318-332.

14. **Ferguson, A. D., R. Chakraborty, B. S. Smith, L. Esser, D. van der Helm, and J. Deisenhofer.** 2002. Structural basis of gating by the outer membrane transporter FecA. Science **295**:1715-1719.

15. **Friedman, Y. E., and M. R. O'Brian.** 2003. A novel DNA-binding site for the ferric uptake regulator (Fur) protein from *Bradyrhizobium japonicum*. J. Biol. Chem. **278**:38395-401.

16. **Genco, C. A., and D. W. Dixon.** 2001. Emerging strategies in microbial haem capture. Mol. Microbiol. **39**:1-11.

17. **Hamza, I., S. Chauhan, R. Hassett, and M. R. O'Brian.** 1998. The bacterial irr protein is required for coordination of heme biosynthesis with iron availability. J. Biol. Chem. **273**:21669-21674.

18. **Hantke, K.** 2001. Iron and metal regulation in bacteria. Curr. Opin. Microbiol. **4**:172-177.

19. **Hotez, P. J.** 2008. Neglected infections of poverty in the United States of America. PLoS Negl. Trop. Dis. **2**:e256-e265.

20. **Johnston, A. W., J. D. Todd, A. R. Curson, S. Lei, N. Nikolaidou-Katsaridou, M. S. Gelfand, and D. A. Rodionov. 2007.** Living without Fur: the subtlety and complexity of iron-responsive gene regulation in the symbiotic bacterium *Rhizobium* and other alpha-proteobacteria. Biometals **20**:501-511.

21. **Kovach, M. E., R. W. Philips, P. H. Elzer, R. M. Roop II, and K. M. Peterson.** 1994. pBBR1MCS: a broad-host-range cloning vector. Biotechniques **16**:800-802.

22. **Kumar, S., and U. Bandyopadhyay.** 2005. Free heme toxicity and its detoxification systems in human. Toxicol. Lett. **157**:175-188.

23. **Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D.**

Thompson, T. J. Gibson, and D. G. Higgins. 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23**:2947-2948.

24. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**:402-408.

25. **Martinez, M., R. A. Ugalde, and M. Almiron.** 2005. Dimeric *Brucella abortus* Irr protein controls its own expression and binds haem. Microbiology **151**:3427-3433.

26. **McQuillan, G. M. and D. Kruszon-Moran.** 2008. HIV infection in the United States household population aged 18-49 years: Results from 1999-2006. NCHS data brief no. 4. National Center for Health Statistics, Hyattsville, MD.

27. **Minnick, M. F. and B. E. Anderson.** 2000. *Bartonella* Interactions with Host Cells, p. 97-118. *In* T. A. Oelschlaeger and J. Hacker (ed.), Bacterial Invasion into Eukaryotic Cells, vol. 33. Kluwer Academic / Plenum Publishers, New York, N.Y.

28. **Minnick, M. F., K. N. Sappington, L. S. Smitherman, S. G. Andersson, O. Karlberg, and J. A. Carroll.** 2003. Five-member gene family of *Bartonella quintana*. Infect. Immun. **71**:814-821.

29. **Mourino, S., C. R. Osorio, M. L. Lemos, and J. H. Crosa.** 2006. Transcriptional organization and regulation of the *Vibrio anguillarum* heme uptake gene cluster. Gene **374**:68-76.

30. **Murphy, E. R., R. E. Sacco, A. Dickenson, D. J. Metzger, Y. Hu, P. E. Orndorff, and T. D. Connell.** 2002. BhuR, a virulence-associated outer

membrane protein of *Bordetella avium*, is required for the acquisition of iron from heme and hemoproteins. Infect. Immun. **70**:5390-5403.

31. **Myers, W. F., J. V. Osterman, and C. L. Wisseman, Jr.** 1972. Nutritional studies of *Rickettsia quintana*: nature of the hematin requirement. J. Bacteriol. **109**:89-95.

32. **Ochsner, U. A., and M. L. Vasil.** 1996. Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa*: cycle selection of iron-regulated genes. Proc. Natl. Acad. Sci. U S A **93**:4409-4414.

33. **Ohl, M. E., and D. H. Spach.** 2000. *Bartonella quintana* and urban trench fever. Clin. Infect. Dis. **31**:131-135.

34. **Paiva-Silva, G. O., C. Cruz-Oliveira, E. S. Nakayasu, C. M. Maya-Monteiro, B. C. Dunkov, H. Masuda, I. C. Almeida, and P. L. Oliveira.** 2006. A heme-degradation pathway in a blood-sucking insect. Proc. Natl. Acad. Sci. U S A **103**:8030-8035.

35. **Pawelek, P. D., N. Croteau, C. Ng-Thow-Hing, C. M. Khursigara, N. Moiseeva, M. Allaire, and J. W. Coulton.** 2006. Structure of TonB in complex with FhuA, *E. coli* outer membrane receptor. Science **312**:1399-1402.

36. **Qi, Z., and M. R. O'Brian.** 2002. Interaction between the bacterial iron response regulator and ferrochelatase mediates genetic control of heme biosynthesis. Mol. Cell **9**:155-162.

37. **Rolain, J. M., C. Foucault, R. Guieu, B. La Scola, P. Brouqui, and D. Raoult.** 2002. *Bartonella quintana* in human erythrocytes. Lancet **360**:226-228.

38. **Rossi, P. H., J. D. Wright, G. A. Fisher, and G. Willis.** 1987. The urban homeless: estimating composition and size. Science **235**:1336-1341.

39. **Rudolph, G., G. Semini, F. Hauser, A. Lindemann, M. Friberg, H. Hennecke, and H. M. Fischer.** 2006. The Iron control element, acting in positive and negative control of iron-regulated *Bradyrhizobium japonicum* genes, is a target for the Irr protein. J. Bacteriol. **188**:733-744.

40. **Sangwan, I., S. K. Small, and M. R. O'Brian.** 2008. The *Bradyrhizobium japonicum* Irr protein is a transcriptional repressor with high-affinity DNA-binding activity. J. Bacteriol. **190**:5172-5177.

41. **Sasarman, A., M. Surdeanu, G. Szegli, T. Horodniceanu, V. Greceanu, and A. Dumitrescu.** 1968. Hemin-deficient mutants of *Escherichia coli* K-12. J. Bacteriol. **96**:570-572.

42. **Scherer, D. C., I. DeBuron-Connors, and M. F. Minnick.** 1993. Characterization of *Bartonella bacilliformis* flagella and effect of antiflagellin antibodies on invasion of human erythrocytes. Infect. Immun. **61**:4962-4971.

43. **Spach, D. H., and J. E. Koehler.** 1998. Bartonella-associated infections. Infect. Dis. Clin. North Am. **12**:137-155.

44. **Stojiljkovic, I., and K. Hantke.** 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. Embo J. **11**:4359-4367.

45. **Stojiljkovic, I., V. Hwa, L. de Saint Martin, P. O'Gaora, X. Nassif, F. Heffron, and M. So.** 1995. The *Neisseria meningitides* haemoglobin receptor: its role in iron utilization and virulence. Mol. Microbiol. **15**:531-541.

46. **Tappero, J. W., J. E. Koehler, T. G. Berger, C. J. Cockerell, T. H. Lee, M. P. Busch, D. P. Stites, J. Mohle-Boetani, A. L. Reingold, and P. E. LeBoit.** 1993. Bacillary angiomatosis and bacillary splenitis in immunocompetent adults. Ann. Intern. Med. **118**:363-365.

47. **Thomas, C. E., B. Olsen, and C. Elkins.** 1998. Cloning and characterization of tdhA, a locus encoding a TonB-dependent heme receptor from *Haemophilus ducreyi*. Infect. Immun. **66**:4254-4262.

48. **Todd, J. D., M. Wexler, G. Sawers, K. H. Yeoman, P. S. Poole, and A. W. Johnston.** 2002. RirA, an iron-responsive regulator in the symbiotic bacterium *Rhizobium leguminosarum*. Microbiology **148**:4059-4071.

49. **Towbin, H., T. Staehelin, and J. Gordon.** 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. Biotechnology **24**:145-149.

50. **Wang, R. F. and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100**: 195-199.

51. **Yang, J., K. Ishimori, and M. R. O'Brian.** 2005. Two heme binding sites are involved in the regulated degradation of the bacterial iron response regulator (Irr) protein. J. Biol. Chem. **280**:7671-7676.

52. **Zimmermann, R., V. A. Kempf, E. Schiltz, K. Oberle, and A. Sander.** 2003. Hemin binding, functional expression, and complementation analysis of Pap 31 from *Bartonella henselae*. J. Bacteriol. **185**:1739-1744.

CHAPTER FOUR

Bartonella quintana HemS is a Hemin Binding Protein with Potential Heme Oxygenase Activity

A. INTRODUCTION

An extraordinarily high hemin requirement for *in vitro* growth is a hallmark of *Bartonella* species, and meeting this requirement is undoubtedly pivotal to its ability to cause disease in the context of the human host. Recent work with *Bartonella quintana* has focused on the mechanisms of hemin acquisition resulting in the characterization of the hemin utilization locus (hut) and the hemin binding protein family members (HbpA-E). The ability to obtain iron, either free or from the breakdown of heme, is largely undefined in *Bartonella.* It has been established that iron and protoporphyrin IX are not sufficient for *in vitro* growth in the absence of hemin itself, but this may result from the lack of ferrochelatase rather than the inability to acquire iron (4, 152). Analysis of the *B. quintana* genome indicates two potential systems for iron uptake, namely the *yfe* (A-D) locus for manganese, zinc, and possibly ferric iron acquisition and the *ceuD/fat* (B-D) locus for siderophore-mediated iron acquisition (4). However, the *B. quintana yfe* locus lacks a homolog to *yfeE*, which was shown to be an essential component of the *Yersinia* system in complementation assays of an *E. coli* enterobactin mutant (15). Furthermore, there are no obvious candidates for siderophore synthesis genes in the *B. quintana* genome (Battisti and Minnick, unpublished observations). In addition to the ambiguity surrounding iron uptake, the mechanism of protection from heme-mediated cytotoxicity is unclear. One potential solution that provides iron and neutralizes heme-mediated oxidative damage is the utilization of a heme oxygenase (HO).

Originally characterized in mammalian cells, membrane associated HOs act in conjunction with NADPH-cytochrome P450 reductase to catalyze the breakdown of heme into biliverdin, carbon monoxide (CO) and free iron (Fe^{2+}). Biliverdin is then converted by biliverdin reductase to bilirubin (94). Heme breakdown can also occur via coupled oxidation which, like HO-mediated catabolism, generates *meso*-hydroxyheme and verdoheme intermediates. However, the two reactions can be distinguished by

inhibitors and end products. In particular, coupled oxidation does not occur in the presence of catalase whereas HO-mediated catabolism does. Also, coupled oxidation nonspecifically degrades heme generating a mix of biliverdin isomers, whereas HO-mediated catabolism proceeds to the specific generation of α-biliverdin in almost all instances (10, 94). Although the detoxification of free heme is thought to be the primary function of mammalian HOs, secondary functions include protection from cellular injury through generation of the antioxidants biliverdin and bilirubin and maintenance of homeostasis through CO signaling (19, 94) .

Unlike the situation described in eukaryotes, bacterial HOs are soluble, their *in vivo* reductase partners are largely undefined, the fate of biliverdin is unclear, and their primary function is presumably to release iron from heme. Due in part to considerable homology with human HO-1, one class of bacterial HO has been characterized in the Gram-positive pathogen *Corynebacterium diphtheriae* (192). Another example is the Gram-negative *Neisserial* HemO protein which also bears some resemblance to eukaryotic HOs. Studies suggest that the *Neisserial* HemO degrades heme into ferric-biliverdin IXα and CO rather than releasing free iron and biliverdin, but this may be the result of a weak interaction with a surrogate reductase partner (198). In *Pseudomonas aeruginosa*, PigA also has homology to the HemO/HmuO class of heme oxygenases and has been shown to function as such (140). Of note, this HO, like coupled oxidation, generates a mixture of biliverdin IX-β and biliverdin IX-δ rather than the biliverdin IX-α product characteristic of HO-catalyzed heme degradation. It is also unable to efficiently interact with the NADPH-cytochrome P450 reductase (140). The widespread use of heme by pathogenic bacteria implies that utilization of a HO to liberate iron from heme might also be widespread. Interestingly, one report indicates that database searches of bacterial genomes have identified homologs to HmuO and HemO in only *Deinococcus* and *Legionella*, suggesting that either the strategy of iron release from heme via HO-mediated degradation is rare or that many bacterial species encode a novel HO enzyme (140).

Not surprisingly, analysis of the *B. quintana* genome also indicates the absence of a mammalian HO homolog, but it does encode a potential hemin-degrading protein, HemS. The precise function of HemS homologs is somewhat controversial. Initial experiments

suggested that the *Shigella dysenteriae* homolog, ShuS, formed an oligomeric ferritin-like structure that functions as a heme-storage protein (191). This result was modified to suggest that ShuS complexed with heme exists as a dimer, while the oligomeric structure arises from heme-free ShuS bound to DNA (91). This occurrence led Kaur and Wilks to hypothesize a Dps-like role in protection of DNA from oxidative stress, but ShuS-bound DNA was not fully protected from oxidative stress and *shuS* was not able to complement an *E. coli dps* mutant (91). Examination of the *P. aeruginosa* homolog, PhuS, indicates that heme can be degraded by this protein in the absence of catalase but not in its presence. The authors attributed degradation to the coupled oxidation mechanism and proposed that PhuS functions as a heme-trafficking protein that facilitates the transfer of heme to PigA (106). The closely related *Yersinia enterocolitica* HemS was required for efficient utilization of heme as an iron source, but not as a porphyrin source, in complementation studies involving *E. coli* EB53 (165). *Y. enterocolitica* HemS was also required to prevent cytotoxicity, presumably resulting from the accumulation of excess heme, in *E. coli* strains expressing the recombinant hemin receptor, HemR, from a high-copy plasmid (166). Based on these observations, heme oxygenase activity was proposed for HemS despite the absence of sequence similarity to known HOs or experimental evidence of this catabolic capability (166, 192). Evidence of enzymatic activity was obtained for the ChuS homolog from *E. coli* O157:H7, which was shown to function as a HO by specific degradation of heme accompanied by generation of carbon monoxide in the presence of an electron source, *in vitro*. The generation of free iron and biliverdin was assumed based on spectral data and culture pigmentation (169). The suggestion that ChuS-mediated heme degradation occurs via coupled oxidation was addressed by the addition of catalase or superoxide dismutase to the reaction. Neither of these agents inhibited the decomposition of heme, but mutagenesis of a highly conserved histidine residue completely abolished enzymatic activity (168). These data provide strong evidence for the existence of a novel HO that has homologs in many bacterial species.

Due to the fact that heme is assumed to be the primary source of iron and porphyrin in the human host, especially in the intracellular erythrocyte environment, we hypothesized that HemS functions as a heme oxygenase. However, it should be noted that heme-trafficking/chaperone functions are not necessarily exclusive of heme oxygenase

functions. These studies were undertaken to elucidate the function of HemS in an effort to better understand heme/iron acquisition in *Bartonella*.

B. MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *E. coli* strains were routinely grown overnight at 37°C in Luria-Bertani (LB). Gene expression was induced by the addition of 0.1 mM to 2.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to mid-log phase cultures prior to an additional 4-6 h incubation. *Corynebacterium ulcerans* strains CU712 *hmuO*Δ and CU712 *hmuO*Δ/pCUhmuO (generously provided by Michael Schmitt) were routinely grown overnight at 37°C in heart infusion broth (HIB) containing 0.2% Tween 80 (HIBTW) unless otherwise stated (104, 135). Standard antibiotic concentrations were supplied as needed. Strains used in this study are listed in Table 4.1 (p. 94).

Analysis of sequence and structural similarity. The National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) and the *Rhizo*DB website (http://xbase.bham.ac.uk/rhizodb/) were used for genome searches and sequence retrievals. Multiple amino acid sequence alignments were performed with ClustalW version 2.0 and comparison with sequence databases were performed with BLAST (5, 108). Structural predictions were made with the Protein Homology/Analogy Recognition Engine (Phyre) version 0.2 (http://www.sbg.bio.ic.ac.uk/~phyre/) (92).

DNA preparations and plasmid construction. A DNeasy blood and tissue kit (Qiagen, Valencia, CA) was used for the preparation of genomic DNA. Small-scale plasmid purification was performed with a QIAprep miniprep kit (Qiagen) and large-scale preparations were obtained with a Wizard Plus midiprep kit (Promega, Madison, WI). Standard protocols were employed for PCR amplification, restriction digestion, ligation, and cloning (8). Primers used in this study are listed in Table 4.2 (p. 95).

HemS expression and purification. Overnight cultures of JM109/pQE30HemS were expanded and grown to mid-log phase then induced with 2.5 mM IPTG for 5-7 h. Cells were centrifuged at 10,000 x *g* for 10 min at 4°C, and resulting pellets were placed in -20°C until needed. Because attempts to purify soluble His_6-tagged HemS resulted in very low yields, purification was performed under denaturing conditions per QIAexpressionist (Qiagen) protocol with minor modifications. Briefly, denatured HemS was purified with Ni^{++}NTA (nickel nitrilotriacetate) resin per protocol except the column was washed twice with 4 mls Buffer C each time. Elution was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12.5% (wt/vol) acrylamide gels, which indicated partial purification of HemS in the third and fourth fractions of Buffer E. These fractions were combined and refolded against dialysis buffer (100 mM NaH_2PO_4, 600 mM NaCl, 20% glycerol, pH 8.0) in a 3500 MWCO (molecular weight cut-off) Slide-a-Lyzer cassette (Pierce, Rockford, IL) (135). Re-folded HemS was concentrated with PEG [poly(ethylene glycol)] 20,000 (Alfa Aesar, Ward Hill, MA). Pooled samples were further concentrated by ultrafiltration using an amicon stir cell with a 30,000 MWCO membrane. Samples were stored at -20°C until needed.

Spectral analysis of hemin binding. Hemin binding to HemS was assessed spectrophotometrically with an HP8453 A diode array UV/VIS spectrophotometer. Briefly, 81.25 nM of hemin was added incrementally to the sample cuvette containing 3 ml of HemS (27μM/L) or the reference cuvette (containing buffer only) in 6.25 nM aliquots. Hemin was incubated with HemS for 5 min at room temperature after each addition, and the absorbance spectrum from 300 nanometers (nm) to 800 nm was recorded for each. Data obtained for the reference cuvette were subtracted from data obtained from the sample cuvette to generate difference spectra. Data from two independent experiments were used for nonlinear regression analysis with Prism software from GraphPad (http://graphpad.com).

Hemin blots. A bicinchoninic acid protein kit (Sigma Chemicals, St. Louis, MO) was used for protein quantification. SDS-PAGE followed the general methods of Laemmli

(105). Gels were stained with Coomassie brilliant blue for visualization or transferred to nitrocellulose according to the methods of Towbin (8, 173). Hemin blots were basically performed as described (31). In brief, blots were blocked for 1 h with Tris-buffered saline (TBS-Tween 20) (10 mM Tris, 150 mM NaCl, 0.1% Tween 20) containing 2% nonfat dry milk then washed twice with TBS. Blots were then incubated for 1.5 h in TBS-Tween 20 containing 10 µg/ml hemin, washed three times (30 min each) in TBS-Tween 20, developed using enhanced chemiluminescence (ECL) reagents (Pierce, Rockford IL) and visualized with autoradiographic film (Eastman Kodak Co., Rochester, NY).

Electroporation of *Corynebacterium*. *Corynebacterium ulcerans* strains were transformed essentially as described (74). Ten ml of overnight CU712*hmuO*Δ culture was used to inoculate 100 ml of HIBTW supplemented with 20 ml of 20% glycerol. Cultures were incubated 1 h at 37°C then diluted to OD_{600} ~0.4. Cells were centrifuged at 10,000 x *g* for 10 min at 4°C and the resulting pellet was washed with 200 ml cold 15% glycerol. Cells were centrifuged again and resuspended in a final volume of 400 µl cold 15% glycerol. A 40 µl volume of this suspension was mixed with plasmid DNA (1.4 µg to 34.3 µg) in a 2mm gap electroporation cuvette (BTX, Holliston, MA). Cells were electroporated with a GenePulser (Bio-Rad, Hercules, CA) set at 2.5kV, 25µF and 200Ω. HIBTW (900 µl per cuvette) was added immediately after the pulse and cells were incubated at 30°C for 1.5 h prior to plating.

Complementation of *C. ulcerans hmuO*Δ. Complementation of *C. ulcerans* was performed as described previously (135). In brief, the *C. ulcerans* CU712*hmuO*Δ strain was transformed with the *E. coli-Corynebacterium* shuttle vector pKN2.6Z (kindly provided by M. Schmitt) containing *B. quintana hemS* (46). CU712*hmuO*Δ containing vector only served as a negative control and CU712*hmuO*Δ/pCUhmuO served as a positive control. Strains were grown overnight at 37°C in HIBTW then diluted 1:1000. 25 µl aliquots of these dilutions were used to inoculate HIBTW supplemented with 200 µg/ml of the iron chelator ethylenediamine-N,N'-bis(2-hydroxyphenyl acetic acid)

(EDDHA), alone, or in conjunction with 1 mM $FeSO_4$ or 7 μM hemin. Plates were scored for visible growth following overnight incubation at 37°C.

DNA Sequencing. An automated DNA sequencer (AB3130x1 genetic analyzer) and a BigDye Terminator cycle sequencing ready reaction kit 3.1 (ABI, Foster City, CA) were used to obtain sequence data. Data were analyzed with ChromasPro 1.13 software (http://www.technelysium.com.au/ChromasPro.html).

C. RESULTS

Sequence conservation and predicted structural similarities between *B. quintana* HemS and *E. coli* ChuS. Initial examination of *B. quintana* HemS focused on identifying similarities to other HemS homologs, specifically concentrating on the *E. coli* ChuS protein. BLAST analyses of ChuS from 0157:H7 against the *B. quintana* genome indicate 33% amino acid identity and 50% similarity across 327 of 347 HemS residues. The crystal structure of ChuS has been solved and indicates a novel HO structure consisting of 9 anti-parallel β-strands contributing to the formation of two large β sheets (168, 169). Several amino acids required for stabilization of the heme-binding interaction or enzymatic activity were identified. Clustal alignment of HemS with ChuS indicates complete conservation of all amino acid residues involved in stabilization of heme binding except for the replacement of one tyrosine (Tyr 315) with a phenylalanine in *B. quintana* HemS (See Fig. 4-1A, p. 96). Most importantly, the key histidine residue (His 193) for coordinating heme and required for oxygenase activity is conserved, as is the second coordinating residue, Arg 100 (168). The predicted three-dimensional structure of *B. quintana* HemS aligned with the *E. coli* ChuS structure determined by crystallography. This alignment shows positional conservation of the heme coordinating residues within the heme-binding cleft identified by crystallization of the ChuS-heme complex (Figure 4-1B, p. 97). Although HemS and ChuS share relatively low amino acid identity, these data suggest that the majority of critical residues for heme binding and oxygenation are conserved and the two proteins share a high degree of structural homology.

His_6-tagged HemS expression and hemin binding in *E. coli*. The successful construction of a pQE30 plasmid encoding a his_6-HemS fusion, termed pQE30HemS, was initially confirmed by DNA sequencing. Sequence analyses indicate the presence of a point mutation resulting in the substitution of an arginine residue at position 10 (Arg 10) with a leucine (data not shown). Despite the presence of this mutation, all experiments were performed using this clone. This decision was justified in part by difficulties in obtaining the clone and in part by the fact that Arg10 is not conserved in ChuS (See Fig. 4-1A, p. 96). Protein synthesis was confirmed by SDS-PAGE and those analyses indicate production of a unique inducible protein of ~40 KDa (See Fig. 4-2A, p. 98). This is in accordance with the predicted molecular mass of 39.4 KDa for native HemS. Expression of recombinant HOs in *E. coli* can confer a blue-green pigment to cells that others have attributed to the accumulation of biliverdin (169, 198). However, this phenomenon was not observed during expression of the *B. quintana* His_6-HemS protein regardless of expression level. Examination of the hemin binding ability of HemS by hemin blotting indicates that His_6-HemS binds hemin at levels well above background (See Fig. 4-2B, p. 98). Based on these data, the His_6-HemS protein is expressed and inducible in *E. coli.* Although HemS synthesis does not generate visible indications of biliverdin accumulation, it is able to bind hemin in a strong and specific manner independently of Arg 10.

Spectral analyses of hemin binding. For further exploration of the hemin binding capacity of His_6-HemS, the protein was purified and renatured for spectrophotometric analysis of heme binding. Difference spectra were obtained by subtracting the absorbance of hemin in buffer alone from the spectra obtained from hemin added to His_6-HemS in buffer. These difference spectra, similar to those obtained from other HOs, show a Soret maximum at 411 nm which is indicative of hemin binding (See Fig. 4-3A, p. 99). Additionally, the absorbance of bound hemin in 350 nm range is lower than that obtained from free hemin resulting in a trough (B. Lei, personal communication). Although β and α bands (at ~545 and 580nm, respectively) have been reported for other bacterial HOs, they are not evident in the difference spectra obtained for the hemin-His_6-

HemS complex. Presumably, the absence of these bands is due to the relatively low concentration of His_6-HemS compared to the concentrations of protein used in other studies (140, 169). Nonlinear regression analyses of the change in absorbance at 411 nm (ΔOD_{411}) versus hemin concentration best fit a one-binding site model (See Fig. 4-3B, p. 100). The estimated Kd is 5.9 $\pm$ 1.7 μM and the increase in absorbance appears to even out at ~25 μM suggesting a 1:1 ratio of hemin to protein. The estimated Kd reported here is higher (lower affinity) than that reported for several HOs, but fairly close to the estimated Kd of the IsdG hemin-degrading enzymes of *Staphylococcus aureus* and *Bacillus anthracis* (159, 160).

***B. quintana* HemS does not complement a *C. ulcerans* CU712*hmuO*Δ strain.** Attempts to reconstitute HemS heme oxygenase activity *in vitro* have been unsuccessful to date. An alternative strategy for examining the function of HemS is the complementation of a HO mutant strain. Unfortunately, few such strains exist. Despite the distant relationship between *B. quintana* and *C. ulcerans* and the fact that HmuO represents a different class of bacterial HO, the existence of CU712*hmuO*Δ provided a potential strain for complementation assays in an attempt to define the function of HemS *in vivo* (104). In order to explore the hypothesis that *hemS* could functionally repair the *hmuO* defect, the *B. quintana hemS* gene was cloned into the *Corynebacterium-E. coli* shuttle vector pKN2.6Z. Of note, attempts to identify or induce HemS from pKN2.6Z in *E. coli* were unsuccessful but the presence of the insert was confirmed by PCR and DNA sequencing (data not shown). CU712*hmuO*Δ strains containing pKNHemS or pKN2.6Z alone were tested for the ability to utilize hemin as the only source of iron. Control strains included the mutant, CU712*hmuO*Δ, and the mutant strain complemented with its own *hmuO* gene, CU712*hmuO*Δ/pCUhmuO. As expected, none of the strains grew on iron-chelated media and all of the strains grew on media supplemented with $FeSO_4$. Like CU712*hmuO*Δ, CU721*hmuO*Δ/pKNHemS was unable to grow on plates supplemented with hemin. In contrast, the strain complemented with *C. ulcerans hmuO* grew well with hemin as a sole iron source (See Fig. 4-4, p. 101). These results show that *B. quintana hemS* is not sufficient to restore growth of the *C. ulcerans hmuO*Δ strain on iron-depleted

medium supplemented with hemin. Not surprisingly, in light of the inability to induce HemS expression in *E. coli*, supplementation of media with IPTG did not alter these results.

D. DISCUSSION

Although one hemin acquisition system, the Hut locus, has been characterized in *B. quintana*, neither the fate of hemin in the cytoplasm nor the mechanisms used for iron acquisition are currently understood. We hypothesized that one member of the Hut locus, HemS, was able to bind and degrade hemin. The data presented herein clearly indicate that HemS has amino acid and structural similarities to the *E. coli* HO, ChuS, and that HemS is able to bind hemin. Experiments performed to date have not provided *in vitro* or *in vivo* evidence of heme oxygenase activity. Nevertheless, these results may be ascribed to the generation of a spurious mutation while cloning *hemS* and the complementation system chosen rather than an authentic absence of enzymatic activity.

With respect to the mutation of Arg10 in HemS, the replacement of the positively charged, basic side chain of arginine with the nonpolar side chain of leucine may have altered proper folding of HemS. The inability to purify large quantities of soluble His_6-HemS may also be attributable to this mutation, as several studies report the generation of soluble recombinant His-tagged heme oxygenases (140, 169, 198). Alternatively, the inability to reconstitute *in vitro* HO activity may simply have resulted from relatively low concentrations of His_6-HemS compounded by potentially incomplete renaturation. The use of a C-terminal histidine tag or a glutathione-S-transferase (GST) tag will be considered for future cloning and purification attempts. Until these potential issues have been resolved, the ability of HemS to function as a heme oxygenase *in vitro* cannot be ascertained with any confidence.

With respect to the *C. ulcerans* complementation system, it was initially chosen due to the absence of an alternative HO mutant strain. However, the fact that HmuO represents a different class of HO presents at least one potential pitfall that could result in the inability of one class to substitute for the other. Namely, the two classes of bacterial HOs may utilize different reductase partners *in vivo*. In support of this explanation, the *P.*

aeruginosa HO, PigA, which also belongs to the HmuO/HemO class of bacterial HOs was unable to efficiently use the NADPH-cytochrome P450 reductase system for *in vitro* heme degradation (140). As with the *in vitro* experiments, there are too many potential problems with the complementation assays for these negative data to definitively rule out the hypothesis that HemS possesses heme oxygenase activity.

An *E. coli chuS* mutant in an 0157:H7 background was recently generated, and may provide an alternative complementation system (176). The phenotype of this strain is currently unknown, but it may be unable to use hemin as a sole iron source if grown on iron-depleted media. In an effort to overcome the difficulties associated with attempting to complement the CU712*hmuO*Δ strain, collaborations are currently planned to examine the phenotype of the *chuS* mutant and, if an appropriate phenotype exists, attempt to complement it with *B. quintana hemS* (C. Martin, personal communication). Provided the mutant has a testable phenotype, the utilization of an *E. coli chuS* strain for complementation assays has a much higher chance of succeeding because of the homology between ChuS and HemS.

A final point of interest, albeit speculative, regarding potential heme oxygenase activity in *B. quintana* is the existence of a hypothetical gene (BQ09510) that shares 22% identity and 44% similarity with 165 of 296 amino acids of human biliverdin reductase A. Moreover, BQ09510 also shares 23% identity and 41% similarity across 254 amino acids to the cyanobacterial biliverdin reductase, BvdR (155). In addition to implying HO activity in *B. quintana*, a biliverdin reductase would also supply a cycling mechanism for the powerful antioxidant bilirubin. Evidence from mammalian cells suggests that minute amounts of bilirubin can protect lipids from massive hydrogen peroxide challenge. The ability of low concentrations of bilirubin to be highly cytoprotective has been attributed to the fact that oxidization of bilirubin generates biliverdin which is in turn reduced back to bilirubin by biliverdin reductase (157). Thus, examination of the function of BQ09510 would be an interesting avenue for future studies in terms of elucidating both heme metabolism and the closely related oxidative stress response.

Table 4.1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strain		
B. quintana		
JK31	low passage human isolate	J. Koehler
E. coli		
JM109	Host strain for cloning	Promega
TOP10F'	Host strain for cloning	Invitrogen
C. ulcerans		
CU712*humO*Δ	CU712 with a 305bp internal deletion of *hmuO*	M. Schmitt
CU712*hmuO*Δ/pCUhmuO	CU712*hmuO*Δ harboring pCUhmoO	M. Schmitt
CU712*hmu*OΔ/pKN2.6Z	CU712*hmuO*Δ harboring pKN2.6Z	This study
CU712*hmuO*Δ/pKNHemS	CU712*hmuO*Δ harboring pKNHemS	This study
Plasmid		
pKN2.6Z	*Corynebacterium-E. coli* shuttle vector	M. Schmitt
pKNHemS	pKN2.6Z with *B. quintana hemS*	This study
pQE30	Expression vector	Qiagen
pQE30HemS	pQE30 with *B. quintana hemS*	This study

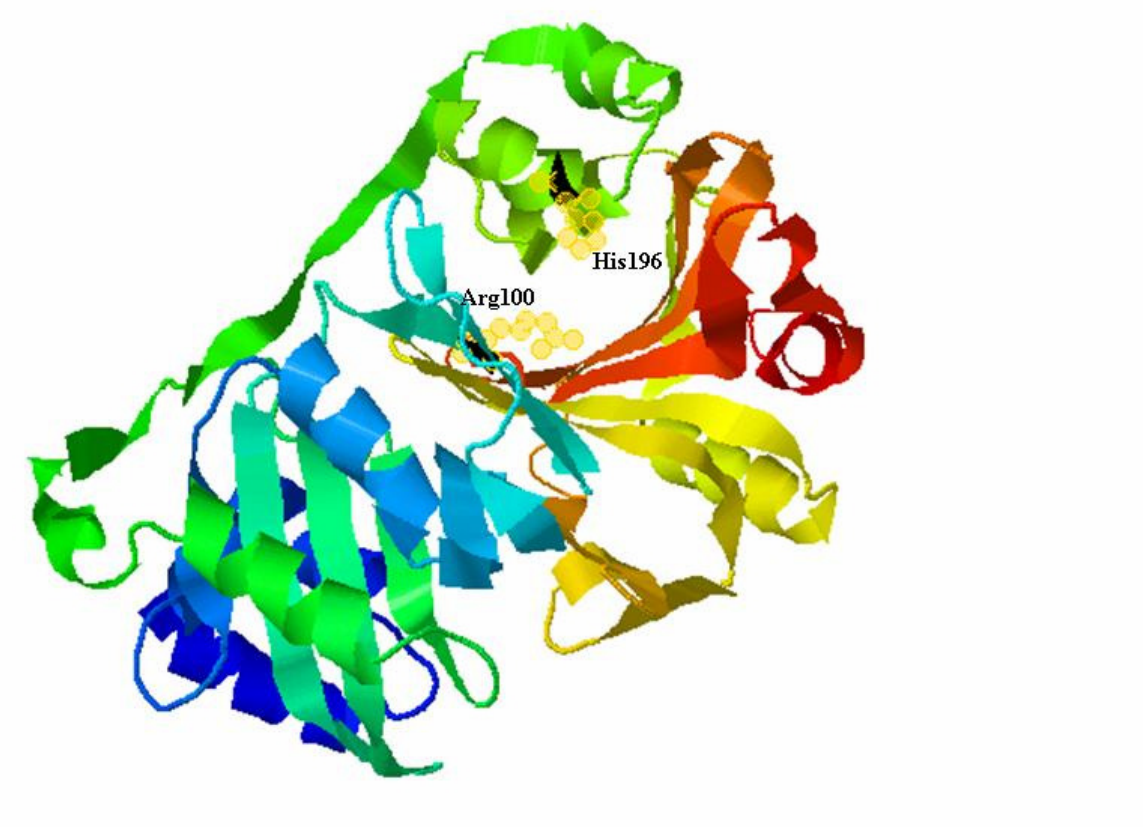
Table 4.2 Primers used in this study.

Primers	
SacIhemSfor	TGAGCTCGCTGAAACGATTATTCGTTTGC
HindIIIhemSrev	GGGAAGCTTTCGATATTTTTCAGCTTTGG
SalIhemSfor	CATGTCGACCTATGGCACATAAAGCTGAAACG
EcoRVhemSrev	GGATATCCGATATTTTTCAGCTTTGGGGC

A)

Figure 4-1. Similarity of *B. quintana* HemS to the *E. coli* heme oxygenase ChuS. A) Clustal alignment of *B. quintana* HemS with ChuS from *E. coli* 0157:H7 strain EDL933. Conserved residues involved in heme binding are boxed and shaded and the histidine (His193 of *E. coli*) required for enzymatic activity is indicated by the large star. A dot indicates weakly conserved residues, a colon indicates strongly conserved residues, and a star indicates fully conserved residues. B) Three-dimensional model of HemS shows structural similarity to *E. coli* ChuS. The predicted positions of conserved residues required for coordinating and degrading heme are highlighted in yellow.

B)



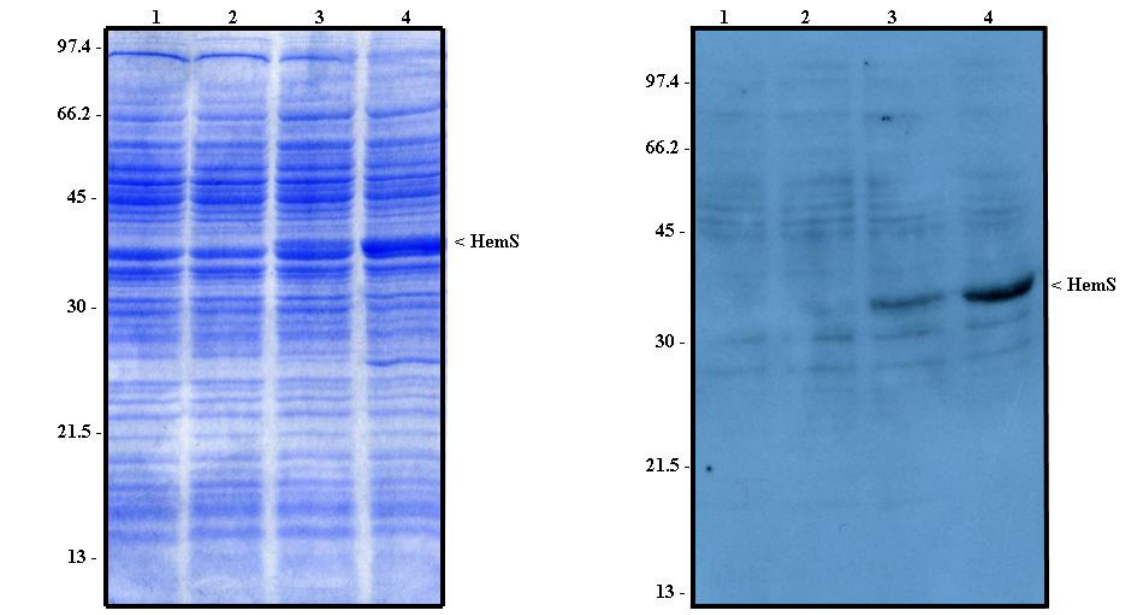


Figure 4-2. Recombinant his_6-tagged HemS is able to bind hemin. Lanes: 1, JM109/pQE30 (uninduced); 2, JM109/pQE30 (induced); 3, JM109/pQE30HemS (uninduced); 4, JM109/pQE30HemS (induced). A) Coomassie blue stained SDS-PAGE gel. B) Corresponding hemin blot showing hemin bound to HemS. Molecular mass standards are indicated on the left in kilodaltons.

A)

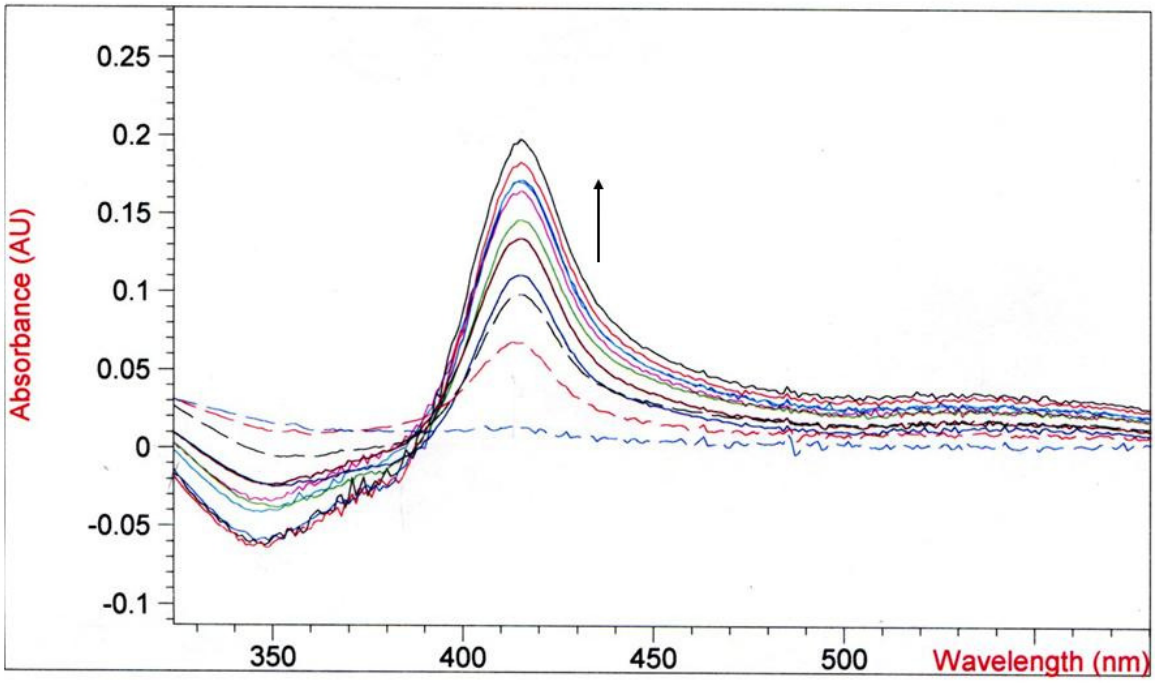
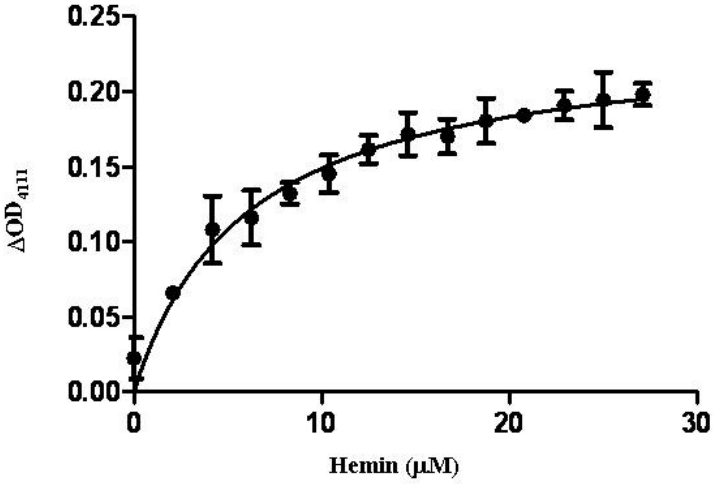


Figure 4-3. Spectral analysis of hemin binding. A) Difference spectra of His_6-HemS with increasing amounts of hemin added shows a Soret maximum at 411 nm and a trough peak at ~350 nm. The increased absorption at 411 nm following addition of increasing amounts of hemin is indicated by the arrow. B) Nonlinear regression of the change in absorbance at 411 nm versus the concentration of hemin. Data shown are the mean and standard deviation of two independent experiments.

B)



Strain	Supplement		
	200μg/ml EDDHA	1mM FeSO4	7μM Hemin
CU712*hmuO*Δ	No growth	+++	No growth
CU712*hmuO*Δ/pCUhmuO	No growth	+++	+++
CU712*hmuO*Δ/pKN2.6Z	No growth	+++	No growth
CU712*hmuO*Δ/pKNHemS	No growth	+++	No growth

Figure 4-4. Complementation of *C. ulcerans hmuO*Δ strains. Strains were plated to HIB containing 200 μg/ml EDDHA alone, or supplemented with 1 mM $FeSO_4$, or 7 μM hemin and visually scored for growth after overnight incubation. "+++" indicates a lawn. Results are representative of three separate experiments.

CHAPTER FIVE

DISCUSSION

Neither free iron nor free hemin is abundant in the human host and the scarcity of these nutrients is exacerbated by the innate iron/hemin sequestration response to infection (186). Pathogens must overcome these limitations if they are to be successful. Elucidation of the mechanisms and control of bacterial hemin/iron acquisition will enhance understanding of this aspect of bacterial adaptation to its host environment, may indicate novel regulation of virulence determinants in response to environmental cues, and has the potential to identify novel targets for the generation of new antimicrobial agents by rational drug design. The need for novel antibiotics is predicted to become increasingly urgent due to the continued emergence of multidrug resistance in several pathogens (128) Previous studies indicated that *Bartonella* required an extraordinary amount of hemin for *in vitro* growth, possessed a novel family of hemin binding proteins (HbpA-E), and was able to rapidly shift between the extreme differences in free hemin availability found in the human host and the body louse (123, 152). These reports laid the foundation for the current studies, which were undertaken with the aim of characterizing the hemin/iron acquisition and regulation mechanisms employed by *B. quintana*.

The initial exploration of potential hemin receptors focused on the Hbps. An HbpA homolog, Pap31, from *B. henselae* reportedly functioned as a hemin receptor when recombinantly expressed in *E. coli* and the *hbp* genes were shown to be transcriptionally responsive to the alteration of external cues associated with colonization of either the body louse or the human blood stream, including oxygen and hemin concentration (13, 199). Contrary to the prevailing hypothesis of the time, *B. quintana* HbpA was unable to function as a hemin receptor in *E. coli*. The exact function of the Hbps is still unclear, but their importance in mediating human infection is underscored by the fact that one of them (HbpE) is a predominant outer membrane protein recognized by sera obtained from infected individuals (23). Furthermore, their specific interaction with both hemin and hemoglobin suggests this binding has a biologically relevant purpose. The potential

relevance of the hemin/hemoglobin interaction is accentuated by the fact that the closely related *Neisserial opa* genes are upregulated when cells are grown on media containing hemoglobin as the only iron source relative to that found when transferrin is the sole iron source (87).

Although purely speculative, the possibility of heme bound to the Hbps acting as a CO sensor is intriguing. Carbon monoxide is a potent second messenger molecule in the human body that increases expression of the anti-inflammatory cytokine, IL-10, in macrophages (19). Interestingly, cytokine profiles obtained from homeless people with chronic *B. quintana* bacteremia show a specific increase in IL-10 secretion that is believed to contribute to bacterial persistence (29). Thus while CO may signal an anti-inflammatory state in the human host it seems plausible that it may also serve as a signal to the bacterium indicating an environment conducive to the establishment of bacteremia. Alternatively, it has been hypothesized that the Hbps may induce a microaerobic environment by binding O_2, or serve as a surface hemin storage site. Regardless of their actual function(s), the Hbp-hemin interaction represents a fascinating aspect of *Bartonella* biology that clearly warrants future study.

The transcriptional response of the *hbps* served as the impetus for study of hemin and/or iron-responsive gene regulation. The identification of a Fur homolog in *B. quintana* led to the investigation of this protein as the potential mediator of hemin-responsive transcriptional control of the *hbp*genes (131). *B. quintana fur* is in a unique arrangement relative to the *fur* gene of other bacterial species in that it is 17 bp downstream from the *secA* gene encoding the ATPase component of the general secretory pathway (182). Although *B. quintana fur* encodes a functional repressor that recognizes the *E. coli* "Fur box" consensus sequence when recombinantly expressed under control of an exogenous promoter, no support for endogenous promoter activity or *in vivo* expression was obtained in these studies. These data add to a growing body of evidence that challenges the γ-proteobacterial model of Fur as the primary iron-responsive gene regulator by showing that its role is lessened or even absent in α-proteobacteria (148). It is within the realm of possibility that *B. quintana fur* is expressed under some undetermined condition, perhaps requiring a novel *Bartonella*-specific sigma factor. In support of this possibility, forced overexpression of Fur in *B. quintana* resulted in

decreased transcription of *hbpC* and *tonB* relative to control strains (14). As the gut of the body louse is expected to have the highest concentration of free hemin, it would be interesting to examine *fur* expression, perhaps using the *fur* promoter-*gfp* fusion constructs described by Parks *et al*, in *B. quintana* during a louse infection (131).

The inability to identify a native promoter for *fur* led to investigation of the Fur superfamily transcriptional regulator, Irr. Although attempts to generate an *irr* knockout were not successful, Irr overexpression in *B. quintana* resulted in a "bloodstream-like" profile of *hbp* transcription. Specifically, *hbpA, D,* and *E* transcript levels were increased relative to that observed in controls. Additionally, purified recombinant Irr was shown to bind to a conserved motif found in the promoter region of the *hbps*, termed the H-box (14). These data, combined with the absence of a role for Fur, suggest that Irr has assumed the role of primary hemin-responsive transcriptional regulator in *Bartonella*. It would be interesting to examine the effects of Irr overexpression on the *B. quintana* genome to see if it also regulates potential virulence determinants.

Upon successfully identifying a major transcriptional regulator of the *hbp* genes, efforts were refocused on identification of the principal hemin uptake system of *B. quintana*. By genomic analyses, the *hut* locus was chosen as the most likely candidate. The ability of HutA to function as a hemin receptor was confirmed by complementation assays using an *E. coli* strain unable to either synthesize or import porphyrin. Complementation assays were also used to establish the TonB-dependence of HutA-mediated hemin transport. Both the structure of HutA and the genetic organization of the *hut* locus are remarkably similar to hemin acquisition systems described in other Gram-negative bacteria (165). The extensive conservation found in bacterial hemin uptake systems makes them attractive candidates for the generation of new antibiotics. At least one study has examined the possibility of exploiting bacterial hemin/hemoglobin uptake systems for the delivery of non-iron metalloporphyrins. Gallium protoporphyrin IX was toxic to a range of bacterial pathogens, but relatively well tolerated by human fibroblasts and a mouse model (167). This report provides preliminary evidence confirming the viability of exploiting hemin acquisition systems as an antimicrobial approach.

Based on its function as a hemin transport system, the *hut* locus was expected to be transcriptionally responsive to alterations in available hemin and this was confirmed by

qRT-PCR. Surprisingly, the relative difference in transcription levels was modest despite the fact that samples were obtained from bacteria grown on media supplemented with enormously different hemin concentrations. These relatively small changes in *hut* locus transcription could hypothetically result from the existence of a hemin-storage reservoir or another buffering system.

B. quintana strains overexpressing Irr, Fur, and RirA, were used to investigate the transcriptional regulation of the *hut* locus. Irr was identified as the primary mediator of transcriptional changes in the *hut* locus, even though Fur overexpression did result in decreased transcription of *tonB* when strains were grown in the presence of excess hemin. Unlike the *hbp* genes, some of which were induced by overexpression of Irr, the *hut* locus genes were repressed by Irr overexpression at all hemin concentrations tested. These observations suggest that Irr is able to function as both an inducer and a repressor in *B. quintana*. A similar situation has been reported in *Bradyrhizobium japonicum*, where the effect of Irr is a function of the location of the binding motif, termed an iron control element (ICE), relative to the start of the gene (149). In accordance with these observations, consensus sequences similar to the H-box were identified in the *hutA/tonB* promoter region and in the *hemS* promoter region. These sites overlap the predicted -10/-35 promoter site of *hutA* and the predicted -35 promoter site of *hemS*. In contrast, the H-box identified in the promoter region of the *hbp* family members is at least 50 bp upstream of the start of the gene (14). Thus, these data appear to fit the *B. japonicum* model where Irr functions as a repressor when bound near the transcriptional start site of a gene and an inducer when bound farther upstream. As the consensus sequence encompassing the H-box is fairly large, an interesting area of future research would be to refine the motif to essential nucleotides by DNase footprinting and/or site-directed mutagenesis using a reporter gene fused to a promoter that contains the H-box.

While the majority of the Hut locus proteins have obvious roles, HemS is the subject of some controversy. Numerous functions have been attributed to HemS homologs in other bacterial species including DNA binding, heme trafficking and heme sequestration (90, 91, 191). The most interesting function for these proteins is a heme oxygenase activity attributed to the *E. coli* homolog, ChuS (169). The possibility that *B. quintana* HemS functions as heme oxygenase is interesting for several reasons. First, no heme

oxygenase with similarity to human HO-1, and therefore the major class of bacterial HO, is evident in the *B. quintana* genome. Second, although systems with homology to ferric iron uptake (*yfeA-D*) and siderophore transport (*fatB-D*) are present in the genome, iron supplied with protoporphyrin IX is not adequate to support *in vitro* growth of *Bartonella* (152), nor are genes encoding a siderophore biosynthesis system readily apparent (Battisti and Minnick, unpublished data). Third, even if *Bartonella* is able to acquire free iron, its availability in human erythrocytes is expected to be negligible. Fourth, *B. quintana* has a marked absence of genes encoding iron-storage molecules (13). Collectively these observations suggest an undefined mechanism in the acquisition of iron by *B. quintana*. Clearly, the presence of a HO would provide an iron source as long as heme was available. Finally, ChuS, and by default HemS, has no structural similarity to any known HO but has homologs in a number of bacterial pathogens. Given the scarcity of iron in the human host, inhibition of a unique HO presents an ideal target for therapeutic intervention.

Experiments to date have shown that HemS binds hemin with a 1:1 stoichiometry. The absence of enzymatic activity in *in vivo* experiments may result from the strain used for complementation analyses and in *in vitro* experiments may result from either the mutation in the His_6-HemS construct or the relatively low concentration of protein used in the experiments. Alternatively, Suits *et al*. reported that fresh ChuS was required to prevent aggregation (168). However, generating sufficient amounts of HemS for *in vitro* examination of enzymatic activity without stockpiling several preparations would be problematic with the current construct. An alternative and possibly more sensitive *in vitro* approach consists of measuring the release of ^{55}Fe from [^{55}Fe] protoporphyrin IXα (136). However, this method may be a last resort due to the expense associated with obtaining radiolabeled hemin. The identification of a *chuS* mutant in *E. coli* 0157:H7 is a promising avenue for examining the ability of HemS to function as a HO *in vivo* and the generation of a suitable construct for initiation of these studies is currently underway.

In summary, the studies described herein were undertaken with the aim of identifying the mechanism and regulation of hemin acquisition in *B. quintana*. Data obtained from these studies have paved the way for further characterization of the Hbp family of proteins, which do not appear to function as hemin receptors but may serve an accessory

role in hemin acquisition. These studies have also contributed to the generation of a new model of iron and hemin-responsive transcriptional regulation whereby the transcriptional control provided by the Fur protein in γ-proteobacteria is provided by the related Irr protein in α-proteobacteria. The primary hemin acquisition system of *B. quintana* was identified and shown to be under transcriptional control of Irr. Initial examination of the function of HemS has been undertaken and several difficulties that need to be addressed in order to further investigate the function of this enzyme have been identified. Numerous questions regarding hemin acquisition and utilization in *Bartonella* remain. Arguably, the most interesting of these include the function of the Hbps and the fate of hemin upon delivery to the cytosol. The answers to these questions, combined with the results presented here, will enhance understanding of the ability of these unique pathogens to cause disease in the human host.

REFERENCES FOR CHAPTERS ONE, TWO, FOUR AND FIVE

1. **Agyei, A. D., N. W. Runham, and N. Blackstock.** 1992. Histochemical changes in the midgut of two ixodid tick species *Boophilus microplus* and *Rhipicephalus appendiculatus* during digestion of the blood meal. Exp Appl Acarol **13:**187-212.
2. **Al-Qutub, M. N., P. H. Braham, L. M. Karimi-Naser, X. Liu, C. A. Genco, and R. P. Darveau.** 2006. Hemin-dependent modulation of the lipid A structure of *Porphyromonas gingivalis* lipopolysaccharide. Infect Immun **74:**4474-4485.
3. **Alcantara, V., J. M. Rolain, A. G. Eduardo, M. J. Raul, and D. Raoult.** 2009. Molecular detection of *Bartonella quintana* in human body lice from Mexico City. Clin Microbiol Infect.
4. **Alsmark, C. M., A. C. Frank, E. O. Karlberg, B. A. Legault, D. H. Ardell, B. Canback, A. S. Eriksson, A. K. Naslund, S. A. Handley, M. Huvet, B. La Scola, M. Holmberg, and S. G. Andersson.** 2004. The louse-borne human pathogen *Bartonella quintana* is a genomic derivative of the zoonotic agent *Bartonella henselae*. Proc Natl Acad Sci U S A **101:**9716-9721.
5. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J Mol Biol **215:**403-410.
6. **Anderson, B., K. Sims, R. Regnery, L. Robinson, M. J. Schmidt, S. Goral, C. Hager, and K. Edwards.** 1994. Detection of *Rochalimaea henselae* DNA in specimens from cat scratch disease patients by PCR. J Clin Microbiol **32:**942-948.
7. **Ashrafian, H.** 2003. Hepcidin: the missing link between hemochromatosis and infections. Infect Immun **71:**6693-6700.
8. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1995. Current protocols in molecular biology. John Wiley & Sons, New York.
9. **Avidor, B., M. Graidy, G. Efrat, C. Leibowitz, G. Shapira, A. Schattner, O. Zimhony, and M. Giladi.** 2004. *Bartonella koehlerae*, a new cat-associated agent of culture-negative human endocarditis. J Clin Microbiol **42:**3462-3468.
10. **Avila, L., H. W. Huang, C. O. Damaso, S. Lu, P. Moenne-Loccoz, and M. Rivera.** 2003. Coupled oxidation vs heme oxygenation: insights from axial ligand mutants of mitochondrial cytochrome b5. J Am Chem Soc **125:**4103-4110.
11. **Balla, J., H. S. Jacob, G. Balla, K. Nath, J. W. Eaton, and G. M. Vercellotti.** 1993. Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage. Proc Natl Acad Sci U S A **90:**9285-9289.
12. **Battisti, J. M., and M. F. Minnick.** 2008. Laboratory maintenance of *Bartonella quintana*. Curr Protoc Microbiol **Chapter 3:**Unit 3C 1 1-3C 1 13.
13. **Battisti, J. M., K. N. Sappington, L. S. Smitherman, N. L. Parrow, and M. F. Minnick.** 2006. Environmental signals generate a differential and coordinated expression of the heme receptor gene family of *Bartonella quintana*. Infect Immun **74:**3251-3261.
14. **Battisti, J. M., L. S. Smitherman, K. N. Sappington, N. L. Parrow, R. Raghavan, and M. F. Minnick.** 2007. Transcriptional regulation of the heme

binding protein gene family of *Bartonella quintana* is accomplished by a novel promoter element and iron response regulator. Infect Immun **75:**4373-4385.

15. **Bearden, S. W., T. M. Staggs, and R. D. Perry.** 1998. An ABC transporter system of *Yersinia pestis* allows utilization of chelated iron by *Escherichia coli* SAB11. J Bacteriol **180:**1135-1147.
16. **Benslimani, A., F. Fenollar, H. Lepidi, and D. Raoult.** 2005. Bacterial zoonoses and infective endocarditis, Algeria. Emerg Infect Dis **11:**216-224.
17. **Bermond, D., H. J. Boulouis, R. Heller, G. Van Laere, H. Monteil, B. B. Chomel, A. Sander, C. Dehio, and Y. Piemont.** 2002. *Bartonella bovis* Bermond *et al.* sp. nov. and *Bartonella capreoli* sp. nov., isolated from European ruminants. Int J Syst Evol Microbiol **52:**383-390.
18. **Bermond, D., R. Heller, F. Barrat, G. Delacour, C. Dehio, A. Alliot, H. Monteil, B. Chomel, H. J. Boulouis, and Y. Piemont.** 2000. *Bartonella birtlesii* sp. nov., isolated from small mammals (*Apodemus* spp.). Int J Syst Evol Microbiol **50 Pt 6:**1973-1979.
19. **Bilban, M., A. Haschemi, B. Wegiel, B. Y. Chin, O. Wagner, and L. E. Otterbein.** 2008. Heme oxygenase and carbon monoxide initiate homeostatic signaling. J Mol Med **86:**267-279.
20. **Birtles, R. J., T. G. Harrison, N. A. Saunders, and D. H. Molyneux.** 1995. Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpa*e comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. Int J Syst Bacteriol **45:**1-8.
21. **Bogumila, S., and M. Adamska.** 2005. [*Capreolus capreolus* and *Ixodes ricinus* as a reservoir of *Bartonella* in north-western Poland]. Wiad Parazytol **51:**139-143.
22. **Bonilla, D. L., H. Kabeya, J. Henn, V. L. Kramer, and M. Y. Kosoy.** 2009. *Bartonella quintana* in body lice and head lice from homeless persons, San Francisco, California, USA. Emerg Infect Dis **15:**912-915.
23. **Boonjakuakul, J. K., H. L. Gerns, Y. T. Chen, L. D. Hicks, M. F. Minnick, S. E. Dixon, S. C. Hall, and J. E. Koehler.** 2007. Proteomic and immunoblot analyses of *Bartonella quintana* total membrane proteins identify antigens recognized by sera from infected patients. Infect Immun **75:**2548-2561.
24. **Breitschwerdt, E. B., R. G. Maggi, M. Varanat, K. E. Linder, and G. Weinberg.** 2009. Isolation of *Bartonella vinsonii* subsp. *berkhoffii* genotype II from a boy with epithelioid hemangioendothelioma and a dog with hemangiopericytoma. J Clin Microbiol **47:**1957-1960.
25. **Brouqui, P., B. Lascola, V. Roux, and D. Raoult.** 1999. Chronic *Bartonella quintana* bacteremia in homeless patients. N Engl J Med **340:**184-189.
26. **Brouqui, P., and D. Raoult.** 1996. *Bartonella quintana* invades and multiplies within endothelial cells in vitro and in vivo and forms intracellular blebs. Res Microbiol **147:**719-731.
27. **Brouqui, P., A. Stein, H. T. Dupont, P. Gallian, S. Badiaga, J. M. Rolain, J. L. Mege, B. La Scola, P. Berbis, and D. Raoult.** 2005. Ectoparasitism and vector-borne diseases in 930 homeless people from Marseilles. Medicine (Baltimore) **84:**61-68.
28. **Buchanan, S. K.** 2005. Bacterial metal detectors. Mol Microbiol **58:**1205-1209.

29. **Capo, C., N. Amirayan-Chevillard, P. Brouqui, D. Raoult, and J. L. Mege.** 2003. *Bartonella quintana* bacteremia and overproduction of interleukin-10: model of bacterial persistence in homeless people. J Infect Dis **187:**837-844.
30. **Carlson, P. E., Jr., K. A. Carr, B. K. Janes, E. C. Anderson, and P. C. Hanna.** 2009. Transcriptional profiling of *Bacillus anthracis* Sterne (34F2) during iron starvation. PLoS One **4:**e6988.
31. **Carroll, J. A., S. A. Coleman, L. S. Smitherman, and M. F. Minnick.** 2000. Hemin-binding surface protein from *Bartonella quintana*. Infect Immun **68:**6750-6757.
32. **Casadevall, A.** 2008. Evolution of intracellular pathogens. Annu Rev Microbiol **62:**19-33.
33. **Cescau, S., H. Cwerman, S. Letoffe, P. Delepelaire, C. Wandersman, and F. Biville.** 2007. Heme acquisition by hemophores. Biometals **20:**603-613.
34. **Chang, C. C., B. B. Chomel, R. W. Kasten, V. Romano, and N. Tietze.** 2001. Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in California. J Clin Microbiol **39:**1221-1226.
35. **Chenoweth, M. R., G. A. Somerville, D. C. Krause, K. L. O'Reilly, and F. C. Gherardini.** 2004. Growth characteristics of *Bartonella henselae* in a novel liquid medium: primary isolation, growth-phase-dependent phage induction, and metabolic studies. Appl Environ Microbiol **70:**656-663.
36. **Chomel, B. B., H. J. Boulouis, E. B. Breitschwerdt, R. W. Kasten, M. Vayssier-Taussat, R. J. Birtles, J. E. Koehler, and C. Dehio.** 2009. Ecological fitness and strategies of adaptation of *Bartonella* species to their hosts and vectors. Vet Res **40:**29.
37. **Chomel, B. B., A. C. Wey, and R. W. Kasten.** 2003. Isolation of *Bartonella washoensis* from a dog with mitral valve endocarditis. J Clin Microbiol **41:**5327-5332.
38. **Chung, C. Y., R. W. Kasten, S. M. Paff, B. A. Van Horn, M. Vayssier-Taussat, H. J. Boulouis, and B. B. Chomel.** 2004. *Bartonella* spp. DNA associated with biting flies from California. Emerg Infect Dis **10:**1311-1313.
39. **Comer, J. A., T. Diaz, D. Vlahov, E. Monterroso, and J. E. Childs.** 2001. Evidence of rodent-associated *Bartonella* and *Rickettsia* infections among intravenous drug users from Central and East Harlem, New York City. Am J Trop Med Hyg **65:**855-860.
40. **Comer, J. A., C. Flynn, R. L. Regnery, D. Vlahov, and J. E. Childs.** 1996. Antibodies to *Bartonella* species in inner-city intravenous drug users in Baltimore, Md. Arch Intern Med **156:**2491-2495.
41. **Cueto, M.** 1996. Tropical medicine and bacteriology in Boston and Peru: studies of Carrion's disease in the early twentieth century. Med Hist **40:**344-364.
42. **Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor.** 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J Clin Microbiol **31:**872-881.
43. **Delpino, M. V., J. Cassataro, C. A. Fossati, F. A. Goldbaum, and P. C. Baldi.** 2006. *Brucella* outer membrane protein Omp31 is a haemin-binding protein. Microbes Infect **8:**1203-1208.

44. **Drancourt, M., J. L. Mainardi, P. Brouqui, F. Vandenesch, A. Carta, F. Lehnert, J. Etienne, F. Goldstein, J. Acar, and D. Raoult.** 1995. *Bartonella* (*Rochalimaea*) *quintana* endocarditis in three homeless men. N Engl J Med **332:**419-423.
45. **Drancourt, M., L. Tran-Hung, J. Courtin, H. Lumley, and D. Raoult.** 2005. *Bartonella quintana* in a 4000-year-old human tooth. J Infect Dis **191:**607-611.
46. **Drazek, E. S., C. A. Hammack, and M. P. Schmitt.** 2000. *Corynebacterium diphtheriae* genes required for acquisition of iron from haemin and haemoglobin are homologous to ABC haemin transporters. Mol Microbiol **36:**68-84.
47. **Droz, S., B. Chi, E. Horn, A. G. Steigerwalt, A. M. Whitney, and D. J. Brenner.** 1999. *Bartonella koehlerae* sp. nov., isolated from cats. J Clin Microbiol **37:**1117-1122.
48. **Drummelsmith, J., and C. Whitfield.** 2000. Translocation of group 1 capsular polysaccharide to the surface of *Escherichia coli* requires a multimeric complex in the outer membrane. EMBO J **19:**57-66.
49. **Duncan, A. W., H. S. Marr, A. J. Birkenheuer, R. G. Maggi, L. E. Williams, M. T. Correa, and E. B. Breitschwerdt.** 2008. *Bartonella* DNA in the blood and lymph nodes of Golden Retrievers with lymphoma and in healthy controls. J Vet Intern Med **22:**89-95.
50. **Easterbrook, J. D., J. B. Kaplan, N. B. Vanasco, W. K. Reeves, R. H. Purcell, M. Y. Kosoy, G. E. Glass, J. Watson, and S. L. Klein.** 2007. A survey of zoonotic pathogens carried by Norway rats in Baltimore, Maryland, USA. Epidemiol Infect **135:**1192-1199.
51. **Elkins, C., P. A. Totten, B. Olsen, and C. E. Thomas.** 1998. Role of the *Haemophilus ducreyi* Ton system in internalization of heme from hemoglobin. Infect Immun **66:**151-160.
52. **Engel, P., and C. Dehio.** 2009. Genomics of host-restricted pathogens of the Genus *Bartonella*. Genome Dyn **6:**158-169.
53. **Eremeeva, M. E., H. L. Gerns, S. L. Lydy, J. S. Goo, E. T. Ryan, S. S. Mathew, M. J. Ferraro, J. M. Holden, W. L. Nicholson, G. A. Dasch, and J. E. Koehler.** 2007. Bacteremia, fever, and splenomegaly caused by a newly recognized bartonella species. N Engl J Med **356:**2381-2387.
54. **Fenollar, F., S. Sire, and D. Raoult.** 2005. *Bartonella vinsonii* subsp. *arupensis* as an agent of blood culture-negative endocarditis in a human. J Clin Microbiol **43:**945-947.
55. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J Bacteriol **115:**717-722.
56. **Florin, T. A., T. E. Zaoutis, and L. B. Zaoutis.** 2008. Beyond cat scratch disease: widening spectrum of *Bartonella henselae* infection. Pediatrics **121:**e1413-1425.
57. **Foucault, C., P. Brouqui, and D. Raoult.** 2006. *Bartonella quintana* characteristics and clinical management. Emerg Infect Dis **12:**217-223.
58. **Foucault, C., S. Ranque, S. Badiaga, C. Rovery, D. Raoult, and P. Brouqui.** 2006. Oral ivermectin in the treatment of body lice. J Infect Dis **193:**474-476.

59. **Fournier, P. E., H. Lelievre, S. J. Eykyn, J. L. Mainardi, T. J. Marrie, F. Bruneel, C. Roure, J. Nash, D. Clave, E. James, C. Benoit-Lemercier, L. Deforges, H. Tissot-Dupont, and D. Raoult.** 2001. Epidemiologic and clinical characteristics of *Bartonella quintana* and *Bartonella henselae* endocarditis: a study of 48 patients. Medicine (Baltimore) **80:**245-251.
60. **Fournier, P. E., J. B. Ndihokubwayo, J. Guidran, P. J. Kelly, and D. Raoult.** 2002. Human pathogens in body and head lice. Emerg Infect Dis **8:**1515-1518.
61. **Fournier, P. E., C. Taylor, J. M. Rolain, L. Barrassi, G. Smith, and D. Raoult.** 2007. *Bartonella australis* sp. nov. from kangaroos, Australia. Emerg Infect Dis **13:**1961-1962.
62. **Furci, L. M., P. Lopes, S. Eakanunkul, S. Zhong, A. D. MacKerell, Jr., and A. Wilks.** 2007. Inhibition of the bacterial heme oxygenases from *Pseudomonas aeruginosa* and *Neisseria meningitidis*: novel antimicrobial targets. J Med Chem **50:**3804-3813.
63. **Gallardo, A., G. Mougabure Cueto, and M. I. Picollo.** 2009. *Pediculus humanus capitis* (head lice) and *Pediculus humanus humanus* (body lice): response to laboratory temperature and humidity and susceptibility to monoterpenoids. Parasitol Res **105:**163-167.
64. **Gangopadhyay, A. K., and P. K. Sharma.** 2001. Bacillary angiomatosis in an immune-competent patient. Indian J Dermatol Venereol Leprol **67:**37-38.
65. **Gasquet, S., M. Maurin, P. Brouqui, H. Lepidi, and D. Raoult.** 1998. Bacillary angiomatosis in immunocompromised patients. AIDS **12:**1793-1803.
66. **Genco, C. A., and D. W. Dixon.** 2001. Emerging strategies in microbial haem capture. Mol Microbiol **39:**1-11.
67. **Gottlieb, M. S., H. M. Schanker, P. T. Fan, A. Saxon, and J. D. Weisman.** 1981. *Pneumocystis* Pneumonia---Los Angeles. Morbidity and Mortality Weekly Report **30:**1-3.
68. **Graca-Souza, A. V., C. Maya-Monteiro, G. O. Paiva-Silva, G. R. Braz, M. C. Paes, M. H. Sorgine, M. F. Oliveira, and P. L. Oliveira.** 2006. Adaptations against heme toxicity in blood-feeding arthropods. Insect Biochem Mol Biol **36:**322-335.
69. **Gundi, V. A., B. Davoust, A. Khamis, M. Boni, D. Raoult, and B. La Scola.** 2004. Isolation of *Bartonella rattimassiliensis* sp. nov. and *Bartonella phoceensis* sp. nov. from European *Rattus norvegicus*. J Clin Microbiol **42:**3816-3818.
70. **Gundi, V. A., C. Taylor, D. Raoult, and B. La Scola.** 2009. *Bartonella rattaustraliani* sp. nov., *Bartonella queenslandensis* sp. nov. and *Bartonella coopersplainsensis* sp. nov., from Australian rats. Int J Syst Evol Microbiol.
71. **Hamza, I., Z. Qi, N. D. King, and M. R. O'Brian.** 2000. Fur-independent regulation of iron metabolism by Irr in *Bradyrhizobium japonicum*. Microbiology **146 (Pt 3):**669-676.
72. **Hantke, K.** 2001. Iron and metal regulation in bacteria. Curr Opin Microbiol **4:**172-177.
73. **Hantke, K.** 1987. Selection procedure for deregulated iron transport mutants (fur) in *Escherichia coli* K 12: fur not only affects iron metabolism. Mol Gen Genet **210:**135-139.

74. **Haynes, J. A., and M. L. Britz.** 1989. Electrotransformation of *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*: growth in tween 80 increases transformation frequencies. FEMS Microbiol Lett **61:**329-334.
75. **Heller, R., M. Kubina, P. Mariet, P. Riegel, G. Delacour, C. Dehio, F. Lamarque, R. Kasten, H. J. Boulouis, H. Monteil, B. Chomel, and Y. Piemont.** 1999. *Bartonella alsatica* sp. nov., a new *Bartonella* species isolated from the blood of wild rabbits. Int J Syst Bacteriol **49 Pt 1:**283-288.
76. **Heller, R., P. Riegel, Y. Hansmann, G. Delacour, D. Bermond, C. Dehio, F. Lamarque, H. Monteil, B. Chomel, and Y. Piemont.** 1998. *Bartonella tribocorum* sp. nov., a new *Bartonella* species isolated from the blood of wild rats. Int J Syst Bacteriol **48 Pt 4:**1333-1339.
77. **Hoar, B. R., B. B. Chomel, D. L. Rolfe, C. C. Chang, C. L. Fritz, B. N. Sacks, and T. E. Carpenter.** 2003. Spatial analysis of *Yersinia pestis* and *Bartonella vinsonii* subsp. *berkhoffii* seroprevalence in California coyotes (*Canis latrans*). Prev Vet Med **56:**299-311.
78. **Horton, R. M., H. D. Hunt, S. N. Ho, J. K. Pullen, and L. R. Pease.** 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene **77:**61-68.
79. **Houpikian, P., and D. Raoult.** 2005. Blood culture-negative endocarditis in a reference center: etiologic diagnosis of 348 cases. Medicine (Baltimore) **84:**162-173.
80. **Hunt, T. A., W. T. Peng, I. Loubens, and D. G. Storey.** 2002. The *Pseudomonas aeruginosa* alternative sigma factor PvdS controls exotoxin A expression and is expressed in lung infections associated with cystic fibrosis. Microbiology **148:**3183-3193.
81. **Inoue, K., H. Kabeya, H. Shiratori, K. Ueda, M. Y. Kosoy, B. B. Chomel, H. J. Boulouis, and S. Maruyama.** 2009. *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., isolated from *Apodemus* mice in Japan. Int J Syst Evol Microbiol.
82. **Ito, S., and J. W. Vinson.** 1965. Fine structure of *Rickettsia quintana* cultivated in vitro and in the louse. J Bacteriol **89:**481-495.
83. **Jackson, L. A., and D. H. Spach.** 1996. Emergence of *Bartonella quintana* infection among homeless persons. Emerg Infect Dis **2:**141-144.
84. **Jaggar, J. H., A. Li, H. Parfenova, J. Liu, E. S. Umstot, A. M. Dopico, and C. W. Leffler.** 2005. Heme is a carbon monoxide receptor for large-conductance Ca2+-activated K+ channels. Circ Res **97:**805-812.
85. **Jeanclaude, D., P. Godmer, D. Leveiller, P. Pouedras, P. E. Fournier, D. Raoult, and J. M. Rolain.** 2009. *Bartonella alsatica* endocarditis in a French patient in close contact with rabbits. Clin Microbiol Infect.
86. **Johnston, A. W., J. D. Todd, A. R. Curson, S. Lei, N. Nikolaidou-Katsaridou, M. S. Gelfand, and D. A. Rodionov.** 2007. Living without Fur: the subtlety and complexity of iron-responsive gene regulation in the symbiotic bacterium *Rhizobium* and other alpha-proteobacteria. Biometals **20:**501-511.
87. **Jordan, P. W., and N. J. Saunders.** 2009. Host iron binding proteins acting as niche indicators for *Neisseria meningitidis*. PLoS One **4:**e5198.

88. **Kaplan, J. E., C. Benson, K. H. Holmes, J. T. Brooks, A. Pau, and H. Masur.** 2009. Guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America. MMWR Recomm Rep **58:**1-207; quiz CE201-204.
89. **Karem, K. L., C. D. Paddock, and R. L. Regnery.** 2000. *Bartonella henselae*, *B. quintana*, and *B. bacilliformis*: historical pathogens of emerging significance. Microbes Infect **2:**1193-1205.
90. **Kaur, A. P., I. B. Lansky, and A. Wilks.** 2009. The role of the cytoplasmic heme-binding protein (PhuS) of *Pseudomonas aeruginosa* in intracellular heme trafficking and iron homeostasis. J Biol Chem **284:**56-66.
91. **Kaur, A. P., and A. Wilks.** 2007. Heme inhibits the DNA binding properties of the cytoplasmic heme binding protein of *Shigella dysenteriae* (ShuS). Biochemistry **46:**2994-3000.
92. **Kelley, L. A., and M. J. Sternberg.** 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc **4:**363-371.
93. **Kerkhoff, F. T., A. M. Bergmans, A. van Der Zee, and A. Rothova.** 1999. Demonstration of *Bartonella grahamii* DNA in ocular fluids of a patient with neuroretinitis. J Clin Microbiol **37:**4034-4038.
94. **Kikuchi, G., T. Yoshida, and M. Noguchi.** 2005. Heme oxygenase and heme degradation. Biochem Biophys Res Commun **338:**558-567.
95. **Koehler, J. E., F. D. Quinn, T. G. Berger, P. E. LeBoit, and J. W. Tappero.** 1992. Isolation of *Rochalimaea* species from cutaneous and osseous lesions of bacillary angiomatosis. N Engl J Med **327:**1625-1631.
96. **Koehler, J. E., M. A. Sanchez, C. S. Garrido, M. J. Whitfeld, F. M. Chen, T. G. Berger, M. C. Rodriguez-Barradas, P. E. LeBoit, and J. W. Tappero.** 1997. Molecular epidemiology of bartonella infections in patients with bacillary angiomatosis-peliosis. N Engl J Med **337:**1876-1883.
97. **Koehler, J. E., M. A. Sanchez, S. Tye, C. S. Garrido-Rowland, F. M. Chen, T. Maurer, J. L. Cooper, J. G. Olson, A. L. Reingold, W. K. Hadley, R. R. Regnery, and J. W. Tappero.** 2003. Prevalence of *Bartonella* infection among human immunodeficiency virus-infected patients with fever. Clin Infect Dis **37:**559-566.
98. **Kordick, D. L., E. J. Hilyard, T. L. Hadfield, K. H. Wilson, A. G. Steigerwalt, D. J. Brenner, and E. B. Breitschwerdt.** 1997. *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). J Clin Microbiol **35:**1813-1818.
99. **Kordick, D. L., B. Swaminathan, C. E. Greene, K. H. Wilson, A. M. Whitney, S. O'Connor, D. G. Hollis, G. M. Matar, A. G. Steigerwalt, G. B. Malcolm, P. S. Hayes, T. L. Hadfield, E. B. Breitschwerdt, and D. J. Brenner.** 1996. *Bartonella vinsonii* subsp. *berkhoffii* subsp. nov., isolated from dogs; *Bartonella vinsonii* subsp. *vinsonii*; and emended description of *Bartonella vinsonii*. Int J Syst Bacteriol **46:**704-709.
100. **Kosoy, M., C. Morway, K. W. Sheff, Y. Bai, J. Colborn, L. Chalcraft, S. F. Dowell, L. F. Peruski, S. A. Maloney, H. Baggett, S. Sutthirattana, A. Sidhirat, S. Maruyama, H. Kabeya, B. B. Chomel, R. Kasten, V. Popov, J.**

Robinson, A. Kruglov, and L. R. Petersen. 2008. *Bartonella tamiae* sp. nov., a newly recognized pathogen isolated from three human patients from Thailand. J Clin Microbiol **46:**772-775.

101. **Kosoy, M., M. Murray, R. D. Gilmore, Jr., Y. Bai, and K. L. Gage.** 2003. *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated from a human patient. J Clin Microbiol **41:**645-650.
102. **Krieg, S., F. Huche, K. Diederichs, N. Izadi-Pruneyre, A. Lecroisey, C. Wandersman, P. Delepelaire, and W. Welte.** 2009. Heme uptake across the outer membrane as revealed by crystal structures of the receptor-hemophore complex. Proc Natl Acad Sci U S A **106:**1045-1050.
103. **Kumar, S., and U. Bandyopadhyay.** 2005. Free heme toxicity and its detoxification systems in human. Toxicol Lett **157:**175-188.
104. **Kunkle, C. A., and M. P. Schmitt.** 2007. Comparative analysis of hmuO function and expression in *Corynebacterium* species. J Bacteriol **189:**3650-3654.
105. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680-685.
106. **Lansky, I. B., G. S. Lukat-Rodgers, D. Block, K. R. Rodgers, M. Ratliff, and A. Wilks.** 2006. The cytoplasmic heme-binding protein (PhuS) from the heme uptake system of *Pseudomonas aeruginosa* is an intracellular heme-trafficking protein to the delta-regioselective heme oxygenase. J Biol Chem **281:**13652-13662.
107. **Lara, F. A., U. Lins, G. H. Bechara, and P. L. Oliveira.** 2005. Tracing heme in a living cell: hemoglobin degradation and heme traffic in digest cells of the cattle tick *Boophilus microplus*. J Exp Biol **208:**3093-3101.
108. **Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins.** 2007. Clustal W and Clustal X version 2.0. Bioinformatics **23:**2947-2948.
109. **LeBoit, P. E., T. G. Berger, B. M. Egbert, J. H. Beckstead, T. S. Yen, and M. H. Stoler.** 1989. Bacillary angiomatosis. The histopathology and differential diagnosis of a pseudoneoplastic infection in patients with human immunodeficiency virus disease. Am J Surg Pathol **13:**909-920.
110. **Li, D. M., Q. Y. Liu, D. Z. Yu, J. Z. Zhang, Z. D. Gong, and X. P. Song.** 2007. Phylogenetic analysis of *Bartonella* detected in rodent fleas in Yunnan, China. J Wildl Dis **43:**609-617.
111. **Li, J. S., D. J. Sexton, N. Mick, R. Nettles, V. G. Fowler, Jr., T. Ryan, T. Bashore, and G. R. Corey.** 2000. Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis **30:**633-638.
112. **Litwin, C. M., and S. B. Calderwood.** 1993. Role of iron in regulation of virulence genes. Clin Microbiol Rev **6:**137-149.
113. **Lopez, C. S., and J. H. Crosa.** 2007. Characterization of ferric-anguibactin transport in *Vibrio anguillarum*. Biometals **20:**393-403.
114. **Maillard, R., P. Riegel, F. Barrat, C. Bouillin, D. Thibault, C. Gandoin, L. Halos, C. Demanche, A. Alliot, J. Guillot, Y. Piemont, H. J. Boulouis, and M. Vayssier-Taussat.** 2004. *Bartonella chomelii* sp. nov., isolated from French domestic cattle (*Bos taurus*). Int J Syst Evol Microbiol **54:**215-220.

115. **Margileth, A. M., and D. F. Baehren.** 1998. Chest-wall abscess due to cat-scratch disease (CSD) in an adult with antibodies to *Bartonella clarridgeiae*: case report and review of the thoracopulmonary manifestations of CSD. Clin Infect Dis **27:**353-357.
116. **Marie, J. L., P. E. Fournier, J. M. Rolain, S. Briolant, B. Davoust, and D. Raoult.** 2006. Molecular detection of *Bartonella quintana*, *B. elizabethae*, *B. koehlerae*, *B. doshiae*, *B. taylorii*, and *Rickettsia felis* in rodent fleas collected in Kabul, Afghanistan. Am J Trop Med Hyg **74:**436-439.
117. **Martinez, M., R. A. Ugalde, and M. Almiron.** 2005. Dimeric *Brucella abortus* Irr protein controls its own expression and binds haem. Microbiology **151:**3427-3433.
118. **Matsumoto, K., Z. L. Berrada, E. Klinger, H. K. Goethert, and S. R. Telford, 3rd.** 2008. Molecular detection of *Bartonella schoenbuchensis* from ectoparasites of deer in Massachusetts. Vector Borne Zoonotic Dis **8:**549-554.
119. **Maurin, M., and D. Raoult.** 1996. *Bartonella* (*Rochalimaea*) *quintana* infections. Clin Microbiol Rev **9:**273-292.
120. **Minnick, M. F.** 1994. Identification of outer membrane proteins of *Bartonella bacilliformis*. Infect Immun **62:**2644-2648.
121. **Minnick, M. F., and B. E. Anderson.** 2006. The Genus *Bartonella*, p. 467-492. *In* M. Dworkin, S. Falkow, E. Rosenberg, K. Schleifer, and E. Stackebrandt (ed.), Prokaryotes: A handbook on the biology of bacteria, 3rd ed, vol. 5. Springer, New York.
122. **Minnick, M. F., and J. M. Battisti.** 2009. Pestilence, persistence and pathogenicity: infection strategies of *Bartonella*. Future Microbiol **4:**743-758.
123. **Minnick, M. F., K. N. Sappington, L. S. Smitherman, S. G. Andersson, O. Karlberg, and J. A. Carroll.** 2003. Five-member gene family of *Bartonella quintana*. Infect Immun **71:**814-821.
124. **Murphy, E. R., R. E. Sacco, A. Dickenson, D. J. Metzger, Y. Hu, P. E. Orndorff, and T. D. Connell.** 2002. BhuR, a virulence-associated outer membrane protein of *Bordetella avium*, is required for the acquisition of iron from heme and hemoproteins. Infect Immun **70:**5390-5403.
125. **Myers, W. F., L. D. Cutler, and C. L. Wisseman, Jr.** 1969. Role of erythrocytes and serum in the nutrition of *Rickettsia quintana*. J Bacteriol **97:**663-666.
126. **Mylonakis, E., and S. B. Calderwood.** 2001. Infective endocarditis in adults. N Engl J Med **345:**1318-1330.
127. **Nakatogawa, H., A. Murakami, and K. Ito.** 2004. Control of SecA and SecM translation by protein secretion. Curr Opin Microbiol **7:**145-150.
128. **Nordmann, P., T. Naas, N. Fortineau, and L. Poirel.** 2007. Superbugs in the coming new decade; multidrug resistance and prospects for treatment of *Staphylococcus aureus*, *Enterococcus* spp. and *Pseudomonas aeruginosa* in 2010. Curr Opin Microbiol **10:**436-440.
129. **O'Connell, M. D., H. Hunt, and A. C. Rankin.** 1915. Trench Fever. The Lancet **186:**1368-1369.
130. **O'Halloran, H. S., K. Draud, M. Minix, A. K. Rivard, and P. A. Pearson.** 1998. Leber's neuroretinitis in a patient with serologic evidence of *Bartonella elizabethae*. Retina **18:**276-278.

131. **Park, S. Y., K. L. Kelminson, A. K. Lee, P. Zhang, R. E. Warner, D. H. Rehkopf, S. B. Calderwood, and J. E. Koehler.** 2001. Identification, characterization, and functional analysis of a gene encoding the ferric uptake regulation protein in *Bartonella* species. J Bacteriol **183:**5751-5755.
132. **Perkins-Balding, D., M. T. Baer, and I. Stojiljkovic.** 2003. Identification of functionally important regions of a haemoglobin receptor from *Neisseria meningitidis*. Microbiology **149:**3423-3435.
133. **Plettenberg, A., T. Lorenzen, B. T. Burtsche, H. Rasokat, T. Kaliebe, H. Albrecht, T. Mertenskotter, J. R. Bogner, A. Stoehr, and H. Schofer.** 2000. Bacillary angiomatosis in HIV-infected patients--an epidemiological and clinical study. Dermatology **201:**326-331.
134. **Probert, W., J. K. Louie, J. R. Tucker, R. Longoria, R. Hogue, S. Moler, M. Graves, H. J. Palmer, J. Cassady, and C. L. Fritz.** 2009. Meningitis due to a "*Bartonella washoensis*"-like human pathogen. J Clin Microbiol **47:**2332-2335.
135. **Puri, S., and M. R. O'Brian.** 2006. The hmuQ and hmuD genes from *Bradyrhizobium japonicum* encode heme-degrading enzymes. J Bacteriol **188:**6476-6482.
136. **Raffin, S. B., C. H. Woo, K. T. Roost, D. C. Price, and R. Schmid.** 1974. Intestinal absorption of hemoglobin iron-heme cleavage by mucosal heme oxygenase. J Clin Invest **54:**1344-1352.
137. **Raoult, D., O. Dutour, L. Houhamdi, R. Jankauskas, P. E. Fournier, Y. Ardagna, M. Drancourt, M. Signoli, V. D. La, Y. Macia, and G. Aboudharam.** 2006. Evidence for louse-transmitted diseases in soldiers of Napoleon's Grand Army in Vilnius. J Infect Dis **193:**112-120.
138. **Raoult, D., C. Foucault, and P. Brouqui.** 2001. Infections in the homeless. Lancet Infect Dis **1:**77-84.
139. **Raoult, D., J. B. Ndihokubwayo, H. Tissot-Dupont, V. Roux, B. Faugere, R. Abegbinni, and R. J. Birtles.** 1998. Outbreak of epidemic typhus associated with trench fever in Burundi. Lancet **352:**353-358.
140. **Ratliff, M., W. Zhu, R. Deshmukh, A. Wilks, and I. Stojiljkovic.** 2001. Homologues of neisserial heme oxygenase in gram-negative bacteria: degradation of heme by the product of the *pigA* gene of *Pseudomonas aeruginosa*. J Bacteriol **183:**6394-6403.
141. **Reeves, W. K., T. E. Rogers, L. A. Durden, and G. A. Dasch.** 2007. Association of *Bartonella* with the fleas (*Siphonaptera*) of rodents and bats using molecular techniques. J Vector Ecol **32:**118-122.
142. **Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins.** 1990. The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N Engl J Med **323:**1573-1580.
143. **Rodriguez, G. M., and I. Smith.** 2003. Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. Mol Microbiol **47:**1485-1494.
144. **Rolain, J. M., P. Brouqui, J. E. Koehler, C. Maguina, M. J. Dolan, and D. Raoult.** 2004. Recommendations for treatment of human infections caused by *Bartonella* species. Antimicrob Agents Chemother **48:**1921-1933.
145. **Rolain, J. M., C. Foucault, R. Guieu, B. La Scola, P. Brouqui, and D. Raoult.** 2002. *Bartonella quintana* in human erythrocytes. Lancet **360:**226-228.

146. **Rolain, J. M., M. Franc, B. Davoust, and D. Raoult.** 2003. Molecular detection of *Bartonella quintana, B. koehlerae, B. henselae, B. clarridgeiae, Rickettsia felis*, and *Wolbachia pipientis* in cat fleas, France. Emerg Infect Dis **9:**338-342.
147. **Rotily, M., Y. Obadia, H. Tissot-Dupont, and D. Raoult.** 1997. Trench fever among homeless people in Marseille, France: a seroprevalence survey. J Epidemiol Community Health **51:**205.
148. **Rudolph, G., H. Hennecke, and H. M. Fischer.** 2006. Beyond the Fur paradigm: iron-controlled gene expression in rhizobia. FEMS Microbiol Rev **30:**631-648.
149. **Rudolph, G., G. Semini, F. Hauser, A. Lindemann, M. Friberg, H. Hennecke, and H. M. Fischer.** 2006. The Iron control element, acting in positive and negative control of iron-regulated *Bradyrhizobium japonicum* genes, is a target for the Irr protein. J Bacteriol **188:**733-744.
150. **Rydkina, E. B., V. Roux, E. M. Gagua, A. B. Predtechenski, I. V. Tarasevich, and D. Raoult.** 1999. *Bartonella quintana* in body lice collected from homeless persons in Russia. Emerg Infect Dis **5:**176-178.
151. **Saenz, H. L., P. Engel, M. C. Stoeckli, C. Lanz, G. Raddatz, M. Vayssier-Taussat, R. Birtles, S. C. Schuster, and C. Dehio.** 2007. Genomic analysis of *Bartonella* identifies type IV secretion systems as host adaptability factors. Nat Genet **39:**1469-1476.
152. **Sander, A., S. Kretzer, W. Bredt, K. Oberle, and S. Bereswill.** 2000. Hemin-dependent growth and hemin binding of *Bartonella henselae*. FEMS Microbiol Lett **189:**55-59.
153. **Sasarman, A., M. Surdeanu, G. Szegli, T. Horodniceanu, V. Greceanu, and A. Dumitrescu.** 1968. Hemin-deficient mutants of *Escherichia coli* K-12. J Bacteriol **96:**570-572.
154. **Scherer, D. C., I. DeBuron-Connors, and M. F. Minnick.** 1993. Characterization of *Bartonella bacilliformis* flagella and effect of antiflagellin antibodies on invasion of human erythrocytes. Infect Immun **61:**4962-4971.
155. **Schluchter, W. M., and A. N. Glazer.** 1997. Characterization of cyanobacterial biliverdin reductase. Conversion of biliverdin to bilirubin is important for normal phycobiliprotein biosynthesis. J Biol Chem **272:**13562-13569.
156. **Schmitt, M. P.** 1997. Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. J Bacteriol **179:**838-845.
157. **Sedlak, T. W., M. Saleh, D. S. Higginson, B. D. Paul, K. R. Juluri, and S. H. Snyder.** 2009. Bilirubin and glutathione have complementary antioxidant and cytoprotective roles. Proc Natl Acad Sci U S A **106:**5171-5176.
158. **Seki, N., T. Sasaki, K. Sawabe, M. Matsuoka, Y. Arakawa, E. Marui, and M. Kobayashi.** 2006. Epidemiological studies on *Bartonella quintana* infections among homeless people in Tokyo, Japan. Jpn J Infect Dis **59:**31-35.
159. **Skaar, E. P., A. H. Gaspar, and O. Schneewind.** 2006. *Bacillus anthracis* IsdG, a heme-degrading monooxygenase. J Bacteriol **188:**1071-1080.

160. **Skaar, E. P., A. H. Gaspar, and O. Schneewind.** 2004. IsdG and IsdI, heme-degrading enzymes in the cytoplasm of *Staphylococcus aureus*. J Biol Chem **279:**436-443.
161. **Skaar, E. P., M. Humayun, T. Bae, K. L. DeBord, and O. Schneewind.** 2004. Iron-source preference of *Staphylococcus aureus* infections. Science **305:**1626-1628.
162. **Smith, H. M., R. Reporter, M. P. Rood, A. J. Linscott, L. M. Mascola, W. Hogrefe, and R. H. Purcell.** 2002. Prevalence study of antibody to ratborne pathogens and other agents among patients using a free clinic in downtown Los Angeles. J Infect Dis **186:**1673-1676.
163. **Spach, D. H., A. S. Kanter, M. J. Dougherty, A. M. Larson, M. B. Coyle, D. J. Brenner, B. Swaminathan, G. M. Matar, D. F. Welch, R. K. Root, and et al.** 1995. *Bartonella* (*Rochalimaea*) *quintana* bacteremia in inner-city patients with chronic alcoholism. N Engl J Med **332:**424-428.
164. **Stojiljkovic, I., A. J. Baumler, and K. Hantke.** 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a fur titration assay. J Mol Biol **236:**531-545.
165. **Stojiljkovic, I., and K. Hantke.** 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. EMBO J **11:**4359-4367.
166. **Stojiljkovic, I., and K. Hantke.** 1994. Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersinia enterocolitica*. Mol Microbiol **13:**719-732.
167. **Stojiljkovic, I., V. Kumar, and N. Srinivasan.** 1999. Non-iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. Mol Microbiol **31:**429-442.
168. **Suits, M. D., N. Jaffer, and Z. Jia.** 2006. Structure of the *Escherichia coli* O157:H7 heme oxygenase ChuS in complex with heme and enzymatic inactivation by mutation of the heme coordinating residue His-193. J Biol Chem **281:**36776-36782.
169. **Suits, M. D., G. P. Pal, K. Nakatsu, A. Matte, M. Cygler, and Z. Jia.** 2005. Identification of an *Escherichia coli* O157:H7 heme oxygenase with tandem functional repeats. Proc Natl Acad Sci U S A **102:**16955-16960.
170. **Telfer, S., M. Begon, M. Bennett, K. J. Bown, S. Burthe, X. Lambin, G. Telford, and R. Birtles.** 2007. Contrasting dynamics of *Bartonella* spp. in cyclic field vole populations: the impact of vector and host dynamics. Parasitology **134:**413-425.
171. **Todd, J. D., G. Sawers, and A. W. Johnston.** 2005. Proteomic analysis reveals the wide-ranging effects of the novel, iron-responsive regulator RirA in *Rhizobium leguminosarum* bv. *viciae*. Mol Genet Genomics **273:**197-206.
172. **Tong, Y., and M. Guo.** 2009. Bacterial heme-transport proteins and their heme-coordination modes. Arch Biochem Biophys **481:**1-15.
173. **Towbin, H., T. Staehelin, and J. Gordon.** 1992. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. Biotechnology **24:**145-149.

174. **Turgut, M., D. Alabaz, M. Karakas, M. Kavak, N. Aksaray, E. Alhan, F. Cevlik, and I. Tuncer.** 2004. Bacillary angiomatosis in an immunocompetent child with a grafted traumatic wound. J Dermatol **31:**844-847.
175. **Vanderpool, C. K., and S. K. Armstrong.** 2001. The *Bordetella bhu* locus is required for heme iron utilization. J Bacteriol **183:**4278-4287.
176. **Vareille, M., F. Rannou, N. Thelier, A. L. Glasser, T. de Sablet, C. Martin, and A. P. Gobert.** 2008. Heme oxygenase-1 is a critical regulator of nitric oxide production in enterohemorrhagic *Escherichia coli*-infected human enterocytes. J Immunol **180:**5720-5726.
177. **Varela, G., J. W. Vinson, and C. Molina-Pasquel.** 1969. Trench fever. II. Propagation of *Rickettsia quintana* on cell-free medium from the blood of two patients. Am J Trop Med Hyg **18:**708-712.
178. **Viguier, C., O. C. P, P. Clarke, and M. O'Connell.** 2005. RirA is the iron response regulator of the rhizobactin 1021 biosynthesis and transport genes in *Sinorhizobium meliloti* 2011. FEMS Microbiol Lett **246:**235-242.
179. **Vinson, J. W.** 1966. In vitro cultivation of the rickettsial agent of trench fever. Bull World Health Organ **35:**155-164.
180. **Vinson, J. W., and H. S. Fuller.** 1961. Studies on trench fever. I. Propagation of *Rickettsia*-like microorganisms from a patient's blood. Pathol Microbiol (Basel) **24(Suppl):**152-166.
181. **Vinson, J. W., G. Varela, and C. Molina-Pasquel.** 1969. Trench fever. 3. Induction of clinical disease in volunteers inoculated with *Rickettsia quintana* propagated on blood agar. Am J Trop Med Hyg **18:**713-722.
182. **Vrontou, E., and A. Economou.** 2004. Structure and function of SecA, the preprotein translocase nanomotor. Biochim Biophys Acta **1694:**67-80.
183. **Wandersman, C., and I. Stojiljkovic.** 2000. Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. Curr Opin Microbiol **3:**215-220.
184. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene **100:**195-199.
185. **Weaver, L. K., P. A. Pioli, K. Wardwell, S. N. Vogel, and P. M. Guyre.** 2007. Up-regulation of human monocyte CD163 upon activation of cell-surface Toll-like receptors. J Leukoc Biol **81:**663-671.
186. **Weinberg, E. D.** 1984. Iron withholding: a defense against infection and neoplasia. Physiol Rev **64:**65-102.
187. **Welch, D. F., K. C. Carroll, E. K. Hofmeister, D. H. Persing, D. A. Robison, A. G. Steigerwalt, and D. J. Brenner.** 1999. Isolation of a new subspecies, *Bartonella vinsonii* subsp. *arupensis*, from a cattle rancher: identity with isolates found in conjunction with *Borrelia burgdorferi* and *Babesia microti* among naturally infected mice. J Clin Microbiol **37:**2598-2601.
188. **Wennerhold, J., and M. Bott.** 2006. The DtxR regulon of *Corynebacterium glutamicum*. J Bacteriol **188:**2907-2918.
189. **Westling, K., A. Farra, C. Jorup, A. Nordenberg, B. Settergren, and E. Hjelm.** 2008. *Bartonella henselae* antibodies after cat bite. Emerg Infect Dis **14:**1943-1944.

190. **Wexler, M., J. D. Todd, O. Kolade, D. Bellini, A. M. Hemmings, G. Sawers, and A. W. Johnston.** 2003. Fur is not the global regulator of iron uptake genes in *Rhizobium leguminosarum*. Microbiology **149:**1357-1365.
191. **Wilks, A.** 2001. The ShuS protein of *Shigella dysenteriae* is a heme-sequestering protein that also binds DNA. Arch Biochem Biophys **387:**137-142.
192. **Wilks, A., and M. P. Schmitt.** 1998. Expression and characterization of a heme oxygenase (Hmu O) from *Corynebacterium diphtheriae*. Iron acquisition requires oxidative cleavage of the heme macrocycle. J Biol Chem **273:**837-841.
193. **Wyckoff, E. E., G. F. Lopreato, K. A. Tipton, and S. M. Payne.** 2005. *Shigella dysenteriae* ShuS promotes utilization of heme as an iron source and protects against heme toxicity. J Bacteriol **187:**5658-5664.
194. **Yang, J., K. Ishimori, and M. R. O'Brian.** 2005. Two heme binding sites are involved in the regulated degradation of the bacterial iron response regulator (Irr) protein. J Biol Chem **280:**7671-7676.
195. **Yang, J., I. Sangwan, A. Lindemann, F. Hauser, H. Hennecke, H. M. Fischer, and M. R. O'Brian.** 2006. *Bradyrhizobium japonicum* senses iron through the status of haem to regulate iron homeostasis and metabolism. Mol Microbiol **60:**427-437.
196. **Zeaiter, Z., P. E. Fournier, H. Ogata, and D. Raoult.** 2002. Phylogenetic classification of *Bartonella* species by comparing groEL sequences. Int J Syst Evol Microbiol **52:**165-171.
197. **Zheng, M., B. Doan, T. D. Schneider, and G. Storz.** 1999. OxyR and SoxRS regulation of *fur*. J Bacteriol **181:**4639-4643.
198. **Zhu, W., A. Wilks, and I. Stojiljkovic.** 2000. Degradation of heme in gram-negative bacteria: the product of the *hemO* gene of *Neisseriae* is a heme oxygenase. J Bacteriol **182:**6783-6790.
199. **Zimmermann, R., V. A. Kempf, E. Schiltz, K. Oberle, and A. Sander.** 2003. Hemin binding, functional expression, and complementation analysis of Pap 31 from *Bartonella henselae*. J Bacteriol **185:**1739-1744.