Functional identification of genes involved in heme uptake and utilization in B. henselae

Ma Feng Liu

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Université Pierre et Marie CURIE

THESIS

MA FENG LIU

Functional Identification of Genes Involved in Heme Uptake and Utilization in B. henselae

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Examinator
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Thesis director
Functional Identification of Genes Involved in Heme Uptake and Utilization in *B. henselae*

Abstract

*Bartonellae* are hemotropic bacteria, agents responsible for emerging zoonoses. These *Alphaproteobacteria* are heme auxotroph which must import heme for supporting their growth, as they cannot synthesize it. Therefore, *Bartonella* genome encodes for a complete heme uptake system allowing the transportation of this compound in the cytoplasm. Heme has also been shown to be used as an iron source for *Bartonella*. Similarly to other bacteria which use heme as an iron source, *Bartonellae* must degrade it to allow the release of iron from the tetrapyrrole ring in the cytoplasm. For *Bartonella*, the genes cluster devoted to the complete heme uptake system contains a gene encoding for a polypeptide that shares homologies with heme trafficking or degrading enzymes. Using complementation of an *E. coli* mutant strain impaired in heme degradation, we demonstrated that HemS from *Bartonella henselae* expressed in *E. coli* allows the release of iron from heme. Recombinant HemS of *B. henselae* binds heme and can degrade it in the presence of a suitable electron donor. Knocking down the expression of HemS in *B. henselae* reduces *B. henselae* ability to face H₂O₂ exposure. Except classical heme uptake system, these bacteria encode for four or five outer membrane heme binding proteins (Hbps). The structural genes of these highly homologous proteins are expressed differently according to oxygen, temperature, and heme concentration. These proteins were hypothesized to be involved in various cellular processes according to their ability to bind heme and their regulation profile. In this report, we investigated the roles of the four heme binding proteins of *Bartonella henselae*. We show that these heme binding proteins can bind Congo red *in vivo* and heme *in vitro*. They can enhance the efficiency of heme uptake when co-expressed with a heme transporter in *E. coli*. Using *B. henselae* Hbps knockdown mutants, we show that these proteins are involved in the defense against oxidative stress response, colonization of human endothelial cell and survival inside the flea.

Keywords: *Bartonella* / heme utilization / oxidative stress / HemS / heme binding proteins / endothelial cells invasion / flea transmission
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<tr>
<td>AP</td>
<td>Alkaline Phosphatase</td>
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<td>Bacillary Angiomatosis</td>
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<td>Bartonella repetitive protein A</td>
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<td>Cluster of Differentiation</td>
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<td>CFU</td>
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<td>human umbilical vein endothelial cells</td>
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<td>Ial</td>
<td>Invasion-associated locus</td>
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<td>infective endocarditis</td>
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<td>Omp</td>
<td>Outer membrane protein</td>
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<td>Opa</td>
<td>Opacity associated protein</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
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<td>peliosis hepatic</td>
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<td>TAAa</td>
<td>Trimeric autotransporter adhesins</td>
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<td>Variably expressed outer membrane proteins</td>
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INTRODUCTION
CHAPTER ONE

*Bartonella* and its survival in different microenvironments

The α-proteobacterium *Bartonellae* employs erythrocyte parasitism and arthropod-mediated transmission as common parasitism strategies (Dehio and Sander, 1999; Schulein et al., 2001). Currently, 26 distinct *Bartonella* species have been described (Table 1) (Kaiser et al., 2011). Each *Bartonella* species appears to be transmitted by specific blood-sucking arthropod vectors, and is highly adapted to one or several mammalian reservoir hosts, in which it causes long-lasting intra-erythrocytic bacteremia as a hallmark of infection (Schroder and Dehio, 2005). *Bartonellae* are also able to infect the non-reservoir host and cause various clinical manifestations without conspicuous erythrocyte parasitism (Schulein et al., 2001).

 Typically, cats are the natural reservoir for *Bartonella henselae* (*B. henselae*); infected cats usually develop intra-erythrocytic bacteremia (Kordick and Breitschwerdt, 1995). However, humans infected by *B. henselae* show no evidence of erythrocyte parasitism, although one group claimed that *B. henselae* can infect human erythrocytes *in vitro* (Pitassi et al., 2007). *Bartonella bacilliformis*, *Bartonella quintana* and *B. henselae* are the three most important human pathogens (Dehio, 2005; Florin et al., 2008; Guptill, 2010). Man is the reservoir host for *B. bacilliformis* and *B. quintana*, in whom they cause various clinical manifestations associated with both intra-erythrocytic bacteremia and endothelial cell infection (Hill et al., 1992; Maurin and Raoult, 1996). *B. bacilliformis* causes Oroya fever and verruga peruana (Herrer, 1953a, b). *B. quintana* causes trench fever (Vinson et al., 1969). *B. henselae* causes
cat scratch disease (CSD) and bacillary peliosis (Jones, 1993). Both *B. quintana* and *B. henselae* can cause bacillary angiomatosis in immunodeficient patients (Sander et al., 1996; Spach et al., 1995).

**Table 1:** *Bartonella* species, their natural reservoirs and vectors, and resulting human diseases. (Kaiser et al., 2011).

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<th>Bartonella species</th>
<th>Reservoir</th>
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<th>Human disease(s)</th>
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<tr>
<td><strong>Human-specific species</strong></td>
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<td><em>B. bacilliformis</em></td>
<td>Human</td>
<td>Sandfly</td>
<td>Carrión’s disease: Oroya fever and verruga peruana</td>
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<tr>
<td><em>B. quintana</em></td>
<td>Human</td>
<td>Body louse, cat flea, tick</td>
<td>Trench fever, endocarditis, bacillary angiomatosis</td>
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<td><strong>Zoonotic species</strong></td>
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<td><em>B. alsatica</em></td>
<td>Rabbit</td>
<td>Unknown</td>
<td>Endocarditis, lymphadenitis</td>
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<tr>
<td><em>B. clarridgeiae</em></td>
<td>Cat</td>
<td>Cat flea</td>
<td>Cat-scratch disease</td>
</tr>
<tr>
<td><em>B. elizabethae</em></td>
<td>Rat</td>
<td>Unknown</td>
<td>Endocarditis, neuroretinitis</td>
</tr>
<tr>
<td><em>B. grahamii</em></td>
<td>Mouse, vole</td>
<td>Rodent flea</td>
<td>Neuroretinitis</td>
</tr>
<tr>
<td><em>B. henselae</em></td>
<td>Cat, dog</td>
<td>Cat flea</td>
<td>Cat-scratch disease, endocarditis, bacillary angiomatosis, bacillary peliosis, neuroretinitis, bacteremia with fever</td>
</tr>
<tr>
<td><em>B. koehlerae</em></td>
<td>Cat</td>
<td>Unknown</td>
<td>Endocarditis</td>
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<td><em>B. rochalimae</em></td>
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<tr>
<td><em>B. tamiae</em></td>
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<td>Mite (?)</td>
<td>Bacteremia with fever</td>
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<tr>
<td><em>B. vinsonii subsp. arupensis</em></td>
<td>Mouse</td>
<td>Tick (?)</td>
<td>Bacteremia with fever</td>
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<tr>
<td><em>B. vinsonii subsp. berkoffii</em></td>
<td>Dog</td>
<td>Tick (?)</td>
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<td><em>B. washoensis</em></td>
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<td>Myocarditis, endocarditis (?)</td>
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<td>Unknown</td>
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<td><em>B. talpae</em></td>
<td>Vole</td>
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</tr>
<tr>
<td><em>B. taylorii</em></td>
<td>Mouse, vole</td>
<td>Rodent flea</td>
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</tr>
<tr>
<td><em>B. tribocorum</em></td>
<td>Rat</td>
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<td>Unknown</td>
</tr>
<tr>
<td><em>B. vinsonii subsp. Vinsonii</em></td>
<td>Vole</td>
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</table>
1. Analysis of *Bartonellae* genomes

The complete sequences of 10 *Bartonella* species genomes are now available.

Comparison of five *Bartonella* genomes (*B. bacilliformis*, *B. grahamii*, *B. henselae*, *B. quintana*, *B. tribocorum*) contained in the MicroScope database (https://www.genoscope.cns.fr/agc/microscope/home/index.php) revealed a low G+C content (38.2-38.8%), a low-coding density of 72.3 to 81.6% and a core genome containing 1016 genes.

**Table 2; Fig. 1** Comparison of five *Bartonella* genomes.

<table>
<thead>
<tr>
<th></th>
<th><em>B. bacilliformis</em></th>
<th><em>B. grahamii</em></th>
<th><em>B. henselae</em></th>
<th><em>B. quintana</em></th>
<th><em>B. tribocorum</em></th>
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<tbody>
<tr>
<td>Genome size (bp)</td>
<td>1,445,021</td>
<td>2,341,328</td>
<td>1,931,047</td>
<td>1,581,384</td>
<td>2,619,061</td>
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<tr>
<td>Total genes (bp)</td>
<td>1,401</td>
<td>2,330</td>
<td>1,906</td>
<td>1,502</td>
<td>2,650</td>
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<tr>
<td>Core genome (bp)</td>
<td>1,016</td>
<td>1,121</td>
<td>1,018</td>
<td>1,034</td>
<td>1,034</td>
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<tr>
<td>Specific genes (bp)</td>
<td>334</td>
<td>647</td>
<td>482</td>
<td>399</td>
<td>758</td>
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</tbody>
</table>

Comparison of *B. henselae* and *B. quintana* genomes indicates that the *B. quintana* is a reduced version of the *B. henselae* genome. *B. henselae* has a 1.93 megabase pair (Mb) genome containing 1,906 protein coding genes. Among them, 482 are unique to *B. henselae*. *B. quintana* has a 1.58 Mb genome encoding 1,502
proteins. Among them, 399 are unique to *B. quintana* (Alsmark et al., 2004). Like *B. quintana*, *B. bacilliformis* has a small genome consisting of a 1.44 Mb genome encoding 1,401 proteins, 334 of which are unique to *B. bacilliformis* (Fig 1; Table 2). Genomic analysis also revealed the presence of type IV secretion systems in “modern” species such as *B. henselae* and *B. quintana*, but not in “ancient” species, i.e. *B. bacilliformis*. Flagella of *B. bacilliformis* were replaced by the Trw T4SS in “modern” species (Harms and Dehio, 2012).

This relatively small core genome reflects specific adaptations to the host-associated lifestyle. A striking example of specific adaptation is that of heme. This important source of iron and porphyrin is particularly abundant in some of the niches colonized by *Bartonellae*, i.e., the intracellular spaces of erythrocytes and the mid-gut lumen of bloodsucking arthropods. The strict heme requirement for growth of *Bartonellae in vitro* correlates with the absence of a heme biosynthesis pathway in these organisms (Alsmark et al., 2004). Moreover, these bacteria contain genes encoding for four to five heme binding proteins and a heme uptake system.

*Bartonella* genome analysis revealed that homologues of 38 genes shown to be essential for *Escherichia coli* are not present (Table 3). In *E. coli*, these essential genes are involved in biosynthesis of heme, tetrahydrofolate, thiamine, isoprenenoid, ubiquinone, undecaprenyl-PP, lipid A, phospholipid and murein. Two of these genes are involved in the biosynthesis and export of lipopolysaccharides. In addition, homologues of genes involved in cell division, chromosome partitioning, secretion, replication and oligoribonucleotide degradation are also not present in *Bartonella*
genomes. Likewise, many homologues of genes involved in the oxidative stress response are not present in *Bartonella* genomes. The absence of some of the above mentioned genes can be easily explained by the lifestyle of *Bartonella*. For example, *Bartonella* can replicate inside the erythrocyte and arthropod gut where heme is available (see above). The function of other genes involved in processes like cell division, oligoribonucleotide degradation and the oxidative stress response are undoubtedly assumed by functional analogs.
<table>
<thead>
<tr>
<th>Gene</th>
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<td>csrA</td>
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<td>hemB</td>
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<td>rlpB</td>
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<td>Uncharacterized protein</td>
</tr>
<tr>
<td>yihA</td>
<td>GTP binding protein</td>
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2. Diseases caused in humans; diagnosis and treatment of *Bartonellosis*

2.1. Clinical features

**Oroya fever and verruga peruana caused by *B. bacilliformis***

*Bartonellosis* caused by *B. bacilliformis* infection is referred to as Carrion’s disease. The pathogen is endemic in some areas of Peru and is transmitted by the sandfly *Lutzomyia verrucarum* (Caceres, 1993). Carrion’s disease is comprised of two different phases: an acute stage, that of Oroya fever, and an eruptive rash phase referred to as verruga peruana (Garcia-Caceres and Garcia, 1991; Schultz, 1968). During the acute stage, 80% of red blood cells are parasitized and lysed (Reynafarje and Ramos, 1961), leading to severe hemolytic anemia (Garcia-Caceres and Garcia, 1991). The mortality rate in untreated patients during this phase may attain 40-80% (Gray et al., 1990; Ihler, 1996; Schultz, 1968). The verruga peruana stage usually occurs four to eight weeks after symptoms of Oroya fever have resolved, and is characterized by hemangiomas (benign vascular tumors) (Garcia et al., 1990). Vasculature infection provokes endothelial cell hyperplasia and neovascularization, resulting in formation of new blood capillaries, a process defined as angiogenesis (Garcia et al., 1990). In this phase, proliferating endothelial cells, along with inflammatory and growth factors induced by *B. bacilliformis*, culminate in pyogenic granulomatous hemangiomas, referred to as verruga peruana (Garcia-Caceres and Garcia, 1991).

**Trench fever caused by *B. quintana***

*B. quintana* is the agent of trench fever, the common name for the acute febrile
syndrome. The louse *Pediculus humanus corporis* is the vector of *B. quintana* (Byam and Lloyd, 1920). Trench fever was epidemic during World Wars 1 and 2 (Hunt and Rankin, 1915; Maurin and Raoult, 1996). The most common characterization included acute onset of fever (39–40°C) lasting 1-3 days, followed by relapse, with decreasing episodes of fever every 5 days. Chronic bacteremia can persist up to 8 year after initial infection (Kostrzewski, 1950). Chronic infection causes endocarditis, bacillary angiomatosis and bacillary peliosis (Spach and Koehler, 1998). It has mainly been reported in the homeless, alcoholics and immunodeficient patients (Brouqui et al., 1999; Spach et al., 1995).

**Cat scratch disease caused by *B. henselae***

The most common disease caused by *B. henselae* in humans is cat scratch disease (CSD), a self-limiting lymphadenopathy transmitted by a cat scratch or bite (Anderson and Neuman, 1997). Regional lymphadenopathy (swollen enlarged lymph nodes) and suppurative papules at the site of inoculation are characteristic clinical manifestations of CSD. Symptoms can occur within several weeks or months and resolve spontaneously. Chronic *B. henselae* infection in immunocompromised individuals causes tumor proliferation of endothelial cells in the skin and internal organs, referred to as bacillary angiomatosis (BA) and peliosis hepatis (PH), respectively (Adal et al., 1994).

**2.2. Diagnosis and treatment**

In cases of suspected Bartonellosis, diagnosis is usually performed by blood culture and serological testing. Blood culture techniques have been successful, but
*Bartonella* can take up to two weeks to grow and is thus not useful for treatment. Numerous serological tests are available, such as indirect immunofluorescence assay (IFA) (Regnery et al., 1992). However, cross-reactivity exists with intracellular pathogens such as *Chlamydia pneumoniae* (Maurin et al., 1997) and *Coxiella burnetti* (La Scola and Raoult, 1996). Therefore, an additional diagnostic test is necessary, such as PCR performed on blood and various tissues including lymph nodes, skin and liver (Fenollar and Raoult, 2004). In an early work, amplification of 16S rRNA genes was used to detect *Bartonella* species (Kerkhoff et al., 1999). However, detection of the 16S rRNA gene is not convenient for distinguishing which *Bartonella* species is responsible for infection, since 16S rRNA genes in *Bartonella* species share > 97.8% similarity (Fenollar and Raoult, 2004). Recently, new target genes have been used to detect different *Bartonella* species such as the citrate synthase gene (*gltA*) (Patel et al., 1999), the riboflavin synthase α-chain gene (*ribC*) (Bereswill et al., 1999), the heat shock protein (*groEL*) (Zeaiter et al., 2002) and the 16S-23S rRNA intergenic spacer region (ITS) (Jensen et al., 2000).

Currently, *Bartonellosis* is treated with penicillin, chloramphenicol, doxycycline, erythromycin and tetracycline (Kordick et al., 1997; Regnery et al., 1996). Despite the fact that trench fever and CSD are self-limiting, antibiotics are recommended. Trench fever treated with chloramphenicol or tetracycline has given good results. CSD patients have been advised to use erythromycin and doxycycline (Maguina et al., 2009).

3. The cycle of *Bartonella* in the mammalian reservoir host
Intra-erythrocytic bacteremia caused by *Bartonella* in the host has been studied in different rodent models (*B. birtlesii*/mouse, *B. tribocorum*/rat) (Boulouis et al., 2001; Marignac et al., 2010; Schulein et al., 2001). The most detailed information came from the rat model of *B. tribocorum* infection (Schulein et al., 2001). After intravenous inoculation with *in-vitro*-grown *B. tribocorum*, the bacteria rapidly disappeared in the circulating blood system within a few hours, and blood remained sterile for 3-4 days (Schulein et al., 2001). Niches that supported bacterial division and rendered bacteria competent toward erythrocytes during that time have not been clearly identified. Although it was shown that endothelial cells are an important constituent of this primary niche (Dehio, 2005), it was also speculated that CD34⁺ progenitor cells could comprise the primary niche (Mandle et al., 2005).

About 4 to 5 days post-infection, bacteria seeded from the primary niche to the bloodstream are able to adhere and to invade mature erythrocytes (Schulein et al., 2001). Inside erythrocytes, bacteria multiply until reaching a steady number (an average of eight intracellular bacteria per infected erythrocyte), which is maintained for the remaining life span of the infected cell (Fig. 2) (Schulein et al., 2001). Bacterial persistence within erythrocytes is an original strategy enabling its persistence in its host, and the resulting long-lasting intra-erythrocytic bacteremia is considered a unique adaptation to the mode of transmission by bloodsucking arthropod vectors (Schulein et al., 2001). The number of infected erythrocytes decreases after day 6 (Schulein et al., 2001). However, the recurrent erythrocyte infection wave is detected after 3 to 6 days, suggesting that it was seeded from the
same primary niche (Fig. 2) (Dehio, 2001) and could be related to fever relapse in human trench fever (Schulein et al., 2001). Intra-erythrocytic bacteremia in the *B. tribocorum* rat model is persistent for eight to ten weeks (Schulein et al., 2001). This infectious procedure was also observed in a mouse model of *B. grahamii* and *B. birtlesii* (Koesling et al., 2001; Marignac et al., 2010).

Since the intravenous route is not the natural route of infection, Marignac et al. (2010) experimentally infected mice using *B. birtlesii* via the intravenous, subcutaneous, intradermal, oral or ocular route. The experiment showed that all routes of infection can cause bacteremia in infected mice (Marignac et al., 2010). This infection strategy is presumably shared by most *Bartonella* species, except for *B. bacilliformis*, which causes massive hemolysis of colonized human erythrocytes (Hendrix, 2000).

![Fig. 2 Model of the infection cycle of Bartonella in their reservoir host. Adapted from (Pulliainen and Dehio, 2012).](image-url)
4. Bartonella and erythrocyte parasitism

Mammalian erythrocytes are generally characterized as disks that are bi-concave in shape and red in color due to the spectral properties of the hemoglobins, the most abundant proteins in these cells (Morera and Mackenzie, 2011). Hemoglobin is responsible for the transport of more than 98% of oxygen. Mammalian erythrocytes are non-nucleated in their mature form. Moreover, they lose all other cell organelles, such as their mitochondria, Golgi apparatus and endoplasmic reticulum. Adult humans have roughly 4-6 million erythrocytes per microliter. Human erythrocytes live in blood circulation for about 100-120 days. At the end of their life span, they are removed from the circulation. There are currently more than 50 known membrane proteins in erythrocytes. These membrane proteins perform a wide diversity of functions, including transport of ions and molecules across the red cell membrane, adhesion and interaction with cells such as endothelial cells, and other currently unknown functions.

During the primary phase (hematic) of bartonellosis caused by B. bacilliformis, about 80% of circulating erythrocytes are lysed (Reynafarje and Ramos, 1961). Other infections caused by B. henselae and B. quintana have not been associated with evidence of hemolysis (Iwaki-Egawa and Ihler, 1997; Rolain et al., 2003a), although the B. henselae and B. quintana genomes contain genes coding for hemolysin activity (http://www.ncbi.nlm.nih.gov/nuccore/NC_005956.1;http://www.ncbi.nlm.nih.gov/nuccore/NC_005955.1). Bartonella entry into erythrocytes is an active process, since mature erythrocytes do not contain an active cytoskeleton that can be subverted by
bacteria to assist in its uptake (Dramsi and Cossart, 1998). Moreover, Bartonella adhesion is unaffected when erythrocytes are pretreated with inhibitors of glycolysis (NaF) or a thiol reagent (N-ethyl maleimide), indicating that the erythrocyte does not contribute to energy-dependent adhesion (Walker and Winkler, 1981). Treatment of B. bacilliformis with a respiratory inhibitor (KCN), a protonmotive force uncoupler (dinitrophenol) or thiol reagent (N-ethyl maleimide), significantly reduces its ability to adhere to erythrocytes, an indication that this process is energy-dependent (Walker and Winkler, 1981). The multiple-surface-exposed proteins of human erythrocytes, including α- and β-subunits of spectrin, band 3 protein and glycophorin A/B, were shown to interact with B. bacilliformis (Buckles and McGinnis Hill, 2000). Bartonella factors that contribute to erythrocyte parasitism have been identified, mainly flagella-mediated motility and surface proteins associated with the Trw type IV secretion system (T4SS), deformin and IalA/IalB.

4.1. Flagella

Bacteria adhesion is the first step in the erythrocyte invasion process. Interestingly, B. bacilliformis adherence is related to its motility, and this motility is related to expression of multiple polar flagella. Each B. bacilliformis possesses a polar tuft composed of one to ten flagella (Scherer et al., 1993). Early works showed that non-motile variants of B. bacilliformis exhibit weaker binding to erythrocytes, suggesting that this process requires bacterial motility (Benson et al., 1986; Walker and Winkler, 1981). Later, Scherer et al. showed that invasion of human erythrocytes significantly decreases when bacteria are pre-incubated with antiflagellin antibodies (Scherer et al., 1993). Recently, it was shown that a non-flagellated mutant displayed
75% reduced binding to human erythrocytes; moreover, this phenotype could be rescued by complementation with the wild type locus (Battisti and Minnick, 1999). However, many other *Bartonella* species (e.g. *B. henselae, B. quintana*) that do not encode flagella are able to invade erythrocytes (Kordick and Breitschwerdt, 1995; Rolain et al., 2002). The apparent exchange of flagella for Trw T4SS (see below) in many other *Bartonella* species (e.g. *B. henselae, B. quintana*) and the common function of both virulence factors in erythrocyte invasion indicate that Trw T4SS may somehow have functionally replaced the flagella, either directly or indirectly (Dehio, 2008).

### 4.2. Deformin

One feature of the *B. bacilliformis* interaction with human erythrocytes is production of deeply invaginated pits and trenches in the erythrocyte membrane, considered an invasion passage for bacteria (Benson et al., 1986). This phenomenon is triggered by deformin, a bacterial factor secreted by *B. bacilliformis* and *B. henselae* (Iwaki-Egawa and Ihler, 1997), but that is not responsible for the hemolytic activity caused by *B. bacilliformis* (Hendrix, 2000). Supernatants of culture medium from both *B. bacilliformis* and *B. henselae* can cause invagination of human red cell membranes and formation of intracellular vacuoles, although *B. henselae* cannot invade human erythrocytes (Iwaki-Egawa and Ihler, 1997). Earlier reports showed that deformin was a heat-sensitive protein of 65 or 130 kDa (Mernaugh and Ihler, 1992; Xu et al., 1995). Subsequently, the same group showed that deformin activity was associated with a small hydrophobic molecule having high affinity for albumin
(Derrick and Ihler, 2001). At present, no further data concerning this putative deformin activity have been published, and the nature of the deforming factor remains mysterious. How deformin causes invagination pits and trenches at the erythrocyte surface remains unknown.

4.3. IalA and IalB

Another factor involved in erythrocyte invasion in *B. bacilliformis* is that of the invasion-associated loci (*ial, ialA* and *ialB*). The *ial* locus is highly conserved within the genus *Bartonella* (Mitchell and Minnick, 1997). Earlier work showed that expression of IalA and IalB in *E. coli* confers an invasion phenotype upon human erythrocytes (Mitchell and Minnick, 1995). Consistent with these phenotypes, the *B. bacilliformis ialA/ialB* mutant strain was strongly impaired in its erythrocyte invasion activity (10-fold less) (Mitchell and Minnick, 1995). Recently, it was shown that the *ialA/B* mutant of *B. birtlesii* adheres normally to erythrocytes but is impaired in invasion (Vayssier-Tausat et al., 2010). The gene *ialA* encodes a 21 kDa protein, a nucleoside polyphosphate hydrolase of the MutT motif family (Cartwright et al., 1999; Conyers and Bessman, 1999). The function of IalA was presumed to be that of eliminating toxic nucleotide derivatives from the cell and regulating the levels of important signaling nucleotides and their metabolites (Cartwright et al., 1999). Homologs of IalA in other pathogens were shown to be crucial for infectivity (Edelstein et al., 2005; Ismail et al., 2003). A homolog in *Rickettsia prowazekii* was suggested to buffer bacterial homeostasis against stressful conditions during growth in a hostile intracellular environment (Gaywee et al., 2002). Thus, it is reasonable to
assume that IalA may indirectly contribute to invasion.

Recently, it was shown that *B. bacilliformis* ialB mutant strains are severely impaired in their ability to invade *in vitro* human erythrocytes. The capacity to invade erythrocytes was restored by complementation with the wild type locus (Coleman and Minnick, 2001). An ialB mutant of *B. birtlesii* was shown to be unable to generate bacteremia in mice (Mavris et al., 2005). The ialB gene encodes for an 18 kDa protein that shares 63.6% sequence conservation with Ail of *Yersinia enterocolitica*, a 17 kDa protein involved in attachment and invasion of epithelial cells (Miller and Falkow, 1988). IalB was shown to be an outer membrane fraction in *B. henselae* (Chenoweth et al., 2004a; Coleman and Minnick, 2001). The crystal structure of IalB of *B. henselae* also confirmed its localization in the outer membrane (Koebnik et al., 2000). It is thus easily conceivable that IalB may be directly involved in erythrocyte invasion, possibly by interacting with one or more of the red blood cell surface proteins bound by *Bartonella*. However, the erythrocyte ligands that are bound to IalB remain unidentified.

### 4.4. Type IV secretion systems (T4SSs)

Type IV secretion systems (T4SSs) act as transporters of Gram-negative bacteria, injecting bacterial virulence factors and DNA substrates into eukaryotic target cells (Dehio, 2008). An increasing number of bacteria are shown to encode type IV secretion systems. The prototype of T4SSs is the VirB-D4 system of *Agrobacterium tumefaciens*, which delivers oncogenic nucleoprotein particles to plant cells (Christie, 2004). Eleven proteins encoded by the *virB* operon and one protein encoded by *virD4*
build up a pilus and pore complex, which spans both the Gram-negative bacterial membrane and the host cell membrane, allowing bacteria to transport a nucleoprotein complex from the bacterial cytoplasm directly into the host cytoplasm (Dehio, 2004)(Fig. 3).

**Fig 3.** Model of VirB/VirD4 T4SS machinery. T4SS machinery spans the inner and outer membranes of Gram-negative bacteria. This transporter is able to secrete substrates from the bacterial cytoplasm directly into the cytoplasm of infected host cells and into the extracellular milieu. Adapted from (Schroder and Dehio, 2005).

The VirB-D4 type IV secretion system has been recently identified in *B. henselae*, which shares extensive homology with the prototypic *virB* locus of *Agrobacterium tumefaciens* and the *virB* locus of *Brucella* spp. (O’Callaghan et al., 1999; Schmiederer and Anderson, 2000) (Fig 4). The difference is that *B. henselae* has no
VirB1, while VirB5 and VirB7 were replaced by two unrelated proteins of 17 kDa and 15 kDa, which do not have extensive amino acid sequence similarities to other known proteins (Dehio, 2001) (Fig 4). The 17 kDa protein was identified as an immunodominant protein in *B. henselae* and is well conserved in the genus *Bartonella* (except from *B. bacilliformis*) (Sweger et al., 2000). *Bartonella* species (except for *B. bacilliformis*) encode two distinct T4SSs, VirB-D4 and Trw, both of which are essential for infection in an animal model (Schulein and Dehio, 2002; Seubert et al., 2003).

![Diagram of T4SS proteins](image)

**Fig 4.** The VirB-like type IV secretion systems of (a) *Agrobacterium tumefaciens*, (b) *Brucella suis* and (c) *Bartonella henselae* and *Bartonella tribocorum*. Homologous genes are represented in the same number. Adapted from (Seubert et al., 2002).

*B. tribocorum* rat models and *B. birtlesii* mouse models showed that mutants impaired in VirB/VirD4 and Trw T4SS lost their ability to cause intra-erythrocytic parasitism in infected rats/mice (Mavris et al., 2005; Saenz et al., 2007). The loss of intra-erythrocytic parasitism in mammals can be explained by the inability to infect the primary niche, or they may be the consequence of a defect in invasion of erythrocytes. Schulein and Dehio (2002) showed that the VirB/VirD4 system is
required at an early infection phase, prior to the onset of bacteremia occurring on day 4 or 5 (Schulein and Dehio, 2002), suggesting that the VirB/VirD4 system plays an important role in establishing a primary niche infection.

The second T4SS of *Bartonella*, Trw, was first identified in *B. henselae*, where it is upexpressed and activated during endothelial cell infection (Seubert et al., 2003). The Trw system of *B. tribocorum* shares high sequence similarity with the Trw conjugation machinery of broad-host-range antibiotic resistance plasmid R388 of enterobacterial origin (Dehio, 2004) (Fig 5). Except for the presence of multiple tandem gene duplications of *trwL* and *trwJ-trwH*, the *trw* genes of *Bartonella* are co-linear with the respective genes of plasmid R388 (Dehio, 2008) (Fig 5).

![Fig 5](image-url)  
*Fig 5.* Organization of *B. tribocorum* Trw (Seubert et al., 2003) and R388-Trw (de Paz et al., 2005; Seubert et al., 2003). Homologous genes have the same color. *B. tribocorum* presents seven TrwL repetitions and five TrwJ-TrwI-TrwH repetitions. Adapted from (de Paz et al., 2005; Seubert et al., 2003).

Complementation assays showed that functions are also conserved between Trw
of *Bartonella* and plasmid R388 (de Paz et al., 2005; Seubert et al., 2003). The *trwE* mutant in *B. tribocorum* cannot establish a bacteremic infection course in a rat model. However, this mutant is still able to infect a primary niche (Dehio, 2004). Likewise, it was hypothesized that Trw is required for colonization of erythrocytes and intra-erythrocytic replication. Recently, it was shown that the mutant of *trw* (*trwD, trwE, trwF, trwJ2, trwL1, trwL2*) in *B. birtlesii* was impaired in adhesion and invasion into mouse erythrocytes both *in vitro* and *in vivo* (Vayssier-Taussat et al., 2010).

Duplicated genes *trwL* and *trwJ* of *Bartonella* encode for variant forms of surface-exposed pilus components that may bind erythrocyte surface antigens (Seubert et al., 2003). Proteins encoded by other duplicated genes of *trwl* and *trwH* are required for pilus elongation and its anchorage to the outer membrane, respectively (Dehio, 2008). However, the *trw* system of *Bartonella* lacks *trwB*, which is required for substrate transport in other T4SSs (Cascales and Christie, 2003) and plasmid R388 (Seubert et al., 2003). It was suggested that the function of Trw T4SS is to establish binding to erythrocytes rather than translocation of effector molecules to the host cell (Dehio, 2004). Interestingly, ectopic expression of Trw of *B. tribocorum* in *B. henselae* and *B. quintana* conferred upon them the ability to infect rat erythrocytes, suggesting that the Trw system plays a role in determining the host specificity of erythrocyte infection (Vayssier-Taussat et al., 2010). Recently, it was showed that only TrwJ1 and TrwJ2 were expressed and localized at the cell surface of *B. birtlesii* and were bound to band 3, one of the major outer membrane glycoproteins of mouse erythrocyte (Deng et al., 2012).
5. *Bartonella* interaction with endothelial cells

Another remarkable pathologic feature of *Bartonella* infection is that it causes vasoproliferative lesions in a process of pathological angiogenesis (Dehio, 2004). Typical manifestations of *Bartonella*-triggered tumor formation are verruga peruana (*B. bacilliformis*) and bacillary angiomatosis (BA) (*B. quintana* and *B. henselae*). The lesions consist of proliferating endothelial cells, bacteria and a mixture of macrophages/monocytes and polymorphonuclear neutrophils (Kostianovsky and Greco, 1994; Manders, 1996). *Bartonella* were found in aggregates both surrounding and within endothelial cells. Clearance of the infection by antibiotics caused complete regression of vascular lesions, indicating that vascular endothelial cells are a target for *Bartonella* colonization *in vivo* (Manders, 1996). Primary human umbilical vein endothelial cells (HUVEC) have been used as a model to study the interaction between the human vascular endothelium and *Bartonella*. Infection of vascular endothelial cells with *Bartonella in vitro* results in cytoskeletal rearrangements that mediate bacterial internalization caused by activation of the small GTPase Rho (Dehio, 2001); activation of transcription factor nuclear factor NF-kappaB, which mediates recruitment and adhesion of leukocytes (Fuhrmann et al., 2001); inhibition of apoptosis (Kirby and Nekorchuk, 2002); and direct mitogenic stimulation (Conley et al., 1994), resulting in endothelial proliferation and formation of vasoproliferative tumors (Pulliainen and Dehio, 2009). These cellular changes might be linked to *Bartonella* trimeric autotransporter adhesins (TAAs) (BadA) (see below) (Riess et al., 2004) and the VirB/VirD4 type IV system (Schulein et al., 2005).
5.1. BadA

*Bartonella* adhesion A (BadA) of *B. henselae* (Riess et al., 2004), together with variably expressed omps (Vomps) of *B. quintana* (Zhang et al., 2004) and *Bartonella* repetitive protein A (BrpA) of *B. vinsonii* (Zhang et al., 2004), are defined as *Bartonella* trimeric autotransporter adhesins (TAAs). TAAs are widespread in α-, β- and γ-proteobacteria and are related to the pathogenicity of many Gram-negative bacteria (Girard and Mourez, 2006). All share a similar trimeric ‘lollipop-like’ structure. They have been designated as membrane anchor, stalk, neck and head, respectively (Linke et al., 2006) (Fig 6).

Fig 6. Domain organization of representative trimeric autotransporter adhesins (A) and full model structure of YadA (B). Head domains are shown in red, stalk in green,
membrane anchor in orange and neck in khaki. Sequences are: BadA of *B. henselae*, NadA of *Neisseria meningitidis*, YadA of *Yersinia enterocolitica*, Hia of *Haemophilus influenzae*, UspA1 of *Moraxella catarrhalis* and XadA of *Xanthomonas oryzae*. Adapted from (Linke et al., 2006).

The C-terminal membrane anchor domain is highly conserved throughout the TAA family (Girard and Mourez, 2006). In *Y. enterocolitica*, it is known to form a pore by building a trimeric 12-stranded β-barrel in the outer membrane, and head and stalk domains are transported to the cell surface through the pore (Zhang et al., 2004). BadA of *B. henselae* is a large TAA with a monomeric molecular weight of 340 kDa (Riess et al., 2004); Expression of BadA forms a dense layer on the bacterial surface (length ~240 nm) (O'Rourke et al., 2011). The N-terminal signal sequence of BadA is followed by the head, which is composed of three domains: an N-terminal YadA-like head repeat, a Trp ring and a GIN domain (Kaiser et al., 2012). The BadA head is linked to the anchor by a long, highly repetitive neck-stalk module rich in coiled coils. These modules differ significantly in length between *Bartonella* species (Kaiser et al., 2012) and even among different *B. henselae* isolates (Riess et al., 2007).

It was demonstrated that BadA of *B. henselae* is essential for adhesion to host endothelial cells and extracellular matrix proteins. Expression of BadA is crucial for activation of hypoxia-inducible factor 1 and secretion of pro-angiogenic cytokines (VEGF, interleukin-8) (Riess et al., 2004), related to the capacity of *B. henselae* to trigger a pro-angiogenic host cell response. Sera (~70-80%) of *B. henselae*-infected patients contain BadA-specific antibodies, suggesting that BadA is an
immunodominant protein and a promising vaccine candidate (Wagner et al., 2008). The head of BadA was shown to play a role in auto-aggregation, binding to collagen and triggering pro-angiogenic host cell responses (Kaiser et al., 2008). The mutant strain of \textit{B. henselae} lacking the ‘neck and stalk’ region is impaired in fibronectin binding, there is no effect on host cell adhesion or induction of VEGF secretion (Kaiser et al., 2008). Genes encoding BadA-homologous TAAs have been found in all other \textit{Bartonella} species investigated thus far (O'Rourke et al., 2011), suggesting that there exists a conserved role for them in \textit{Bartonella} pathogenicity (Gilmore et al., 2005; Zhang et al., 2004). In the case of \textit{B. quintana}, TAAs (Vomps) are also involved in auto-aggregation of the bacteria and collagen binding (Zhang et al., 2004). It was later shown that the strain of \textit{B. quintana} lacking expression of \textit{vomp} did not induce VEGF secretion (Schulte et al., 2006) and the \textit{vomp} mutant strain of \textit{B. quintana} did not cause bacteremia in the animal model (MacKichan et al., 2008).

5.2. T4SS: the VirB/VirD4 type IV system

As described above, the VirB/VirD4 type IV system was shown to be related to endothelial cell parasitism. In fact, the VirB/VirD4 type IV system was revealed to be a 17 kDa immunodominant antigen, a VirB5 homolog encoded in the \textit{virB} operon in \textit{B. henselae} (Padmalayam et al., 2000). Subsequently, the VirB/VirD4 type IV system was widely studied. The VirB/VirD4 type IV system of \textit{B. henselae} is able to transport \textit{Bartonella} effector proteins (Beps) BepA to BepG, which are encoded downstream from the \textit{virB/virD4} locus of \textit{B. henselae} (Schulein et al., 2005), into the host cell cytosol (Schmid et al., 2006). These Beps contain a conserved Bep intracellular
delivery (BID) domain close to the C-terminus, which, together with a C-terminal positively charged tail sequence, constitutes a bipartite signal for T4SS-mediated protein translocation (Schroder et al., 2011). The main functions of the Beps are as follows: modification of the endothelial cell, including formation of the cellular structure invasome that mediates cell invasion (Rhomberg et al., 2009); activation of proinflammatory reaction factors (nuclear factor (NF)-κB, IL-8, ICAM-1); inhibition of endothelial cell apoptosis; and capillary-like sprout formation of endothelial cell aggregates (O'Rourke et al., 2011). The functions of BepA to BepG have recently been studied. BepA inhibits endothelial cell apoptosis and is responsible for capillary sprout formation in a more complex infection model (Schmid et al., 2006). BepF has been implicated in triggering the formation of invasomes together with BepC (Truttmann et al., 2011). BepG, that triggers cytoskeletal rearrangements, potently inhibits sprout formation (Scheidegger et al., 2009). Recently, it has been demonstrated that the virB/virD4 system of B. henselae is able to transfer DNA into endothelial cells, and BepD increases the transfer rate, suggesting that T4SS-dependent DNA transfer into host cells may occur naturally during human infection with Bartonella, and its capacity for DNA transfer increases its potential use in gene therapy and vaccination (Schroder et al., 2011).

6. Bartonellae and their vectors

Bartonella species are transmitted to mammals by various arthropod vectors, mainly including sandflies (Townsend, 1913), human lice (Varela et al., 1954), cat fleas (Chomel et al., 1996), some rodent fleas (Morick et al., 2010), various hard tick
species (Eskow et al., 2001; Kruszewska and Tylewska-Wierzbanowska, 1996) and various biting fly species (Dehio et al., 2004; Halos et al., 2004). A single mammal host may be infected by many *Bartonella* species (Table 1) and one vector can also be infected by various *Bartonella* species (Bown et al., 2004; Rolain et al., 2003b). However, the diversity of *Bartonella* species DNA identified in ectoparasites is much broader than that of species detected in their mammalian hosts (Tsai et al., 2011a; Tsai et al., 2011b), suggesting that *Bartonella* species are better adapted to their vectors than to their mammal hosts (Tsai et al., 2011a). Mechanisms leading to fitness in vectors and their mammal hosts warrant further experimental investigation.

### 6.1. *B. bacilliformis* and sandflies

*Lutzomyia verrucarum*, a native sandfly species in Peru, is the natural vector of *B. bacilliformis* (Townsend, 1913). Townsend was the first to propose *L. verrucarum* as an etiological agent of human bartonellosis (Oroya fever) based on epidemiological data (Townsend, 1913). Subsequently, in order to experimentally demonstrate that *B. bacilliformis* is the etiological agent of human bartonellosis, several experimental infection models were created in monkeys, which are able to reproduce *B. bacilliformis* bacteremia (Noguchi et al., 1929). Battistini et al., (1931) established that sandfly bites can induce verruga peruana in monkeys under experimental conditions; they demonstrated that *B. bacilliformis* could be isolated from blood of infected monkeys, and they revealed the presence of *B. bacilliformis* in midgut and feces of sandflies (Battistini, 1931). In addition to *L. verrucarum*, another unknown vector might exist as a potential transmission vector because of inconsistency between...
the distribution of Carrion’s disease and *L. verrucarum* (Scheidegger et al., 2009).

6.2. *B. quintana* and human body lice

The ability of body lice (*Pediculus humanus corporis*) to transmit *B. quintana* was first reported in 1920 (Byam and Lloyd, 1920). Recently, *B. quintana* was detected by PCR in body lice of homeless persons in France, Russia, Japan and the USA (Bonilla et al., 2009; Foucault et al., 2002; Rydkina et al., 1999; Sasaki et al., 2002). *B. quintana* replicates in the louse intestinal tract without affecting the life span of the louse (Seki et al., 2007). Transmission to humans is thought to be due to inoculation during scratching of *B. quintana* contained in louse feces (Raoult and Roux, 1999). *B. quintana* is able to survive and remain infectious up to one year in louse feces (Kostrzewski, 1950). Up until now, there has been no evidence demonstrating that *B. quintana* can be transmitted to offspring (eggs and larvae) of infected lice. In addition to humans, *B. quintana* is also able to infect other mammals (monkeys, cats and dogs) (Breitschwerdt et al., 2007; Kelly et al., 2006; O'Rourke et al., 2005). *B. quintana* can also be detected in cat fleas (Rolain et al., 2003b) and ticks (Chang et al., 2001).

6.3. *B. henselae* and cat fleas

The role of cat fleas (*Ctenocephalides felis*) in transmission of *B. henselae* was reported in northern California in the early 1990s (Koehler et al., 1994). Subsequently, it was experimentally demonstrated that infected fleas can transmit *B. henselae* to cats (Chomel et al., 1996). *B. henselae* replicates in the gut of the cat flea and is able to survive several days in flea feces (Chomel et al., 2009). Bacteria were shown to be
present in flea gut 3 h post-feeding, and persisted for more than 9 days after inoculation (Higgins et al., 1996). Similarly to louse feces, flea feces appear to be the principal infectious source of *B. henselae*. Transmission from cats to humans occurs through cat scratch. Apart from cat scratch, the role of cat bites in transmission has also been suggested (Demers et al., 1995; Jendro et al., 1998).

6.4. Ticks

The first demonstration of the role of ticks in *Bartonella* infection was provided by Noguchi, who showed that *B. bacilliformis* was transmitted from infected to normal rhesus monkeys by a bite from the tick *Dermacentor andersoni* (Noguchi, 1926). The first case report of human *B. henselae* infection associated with tick bite transmission was described by Lucey et al. (Lucey et al., 1992). Later, it was reported that *Bartonella* DNA was detected in patients exposed to tick bites (Billeter et al., 2008). Hard ticks, including *Ixodes* spp., *Dermacentor* spp., *Rhipicephalus* spp. and *Haemaphysalis* from many parts of the world have been shown to harbor *Bartonella* DNA (Billeter et al., 2008). However, the presence of *Bartonella* DNA in ticks does not prove vector competence. Recently, it was claimed that transmission of any *Bartonella* spp. from ticks to animals or humans has not been established (Telford and Wormser, 2010). The specific role of ticks in *Bartonella* transmission requires further studies.
CHAPTER TWO
Iron/heme uptake in *Bartonella*

Iron is one of the most important elements necessary for growth of most living organisms, including bacteria. As an enzyme catalytic center, it is believed to be involved in various biological processes, including photosynthesis, respiration, the tricarboxylic acid cycle, oxygen transport, gene regulation and DNA biosynthesis (Krewulak and Vogel, 2008).

Despite the fact that iron is abundant on the earth’s surface, very little free iron is biologically available. In aerobic inorganic environments, iron is present essentially in an oxidized ferric form Fe(III), which aggregates into highly insoluble oxyhydroxide polymers. The poor solubility of iron ($10^{-17}$M at pH 7.0) cannot satisfy growth requirements of bacteria for aerobic growth ($10^{-7}$ to $10^{-5}$M). In mammals, 99.9% of iron is intracellular. Extracellular iron (0.1%) is bound to iron carrier proteins such as transferrin and lactoferrin. Transferrin is found in serum and lactoferrin is found in lymph and mucosal secretions (Wandersman and Delepelaire, 2004). Moreover, approximately 95% of iron in mammals is sequestered in protoporphyrin IX to form heme, which is primarily bound to hemoproteins such as hemopexin and hemoglobin (Otto et al., 1992).

Bacteria growing aerobically in cell-free aquatic or soil-based media generally encounter insoluble Fe(III). Following colonization of multicellular organisms, pathogenic bacteria must also confront iron limitation, since no free iron is available. Bacteria have developed several iron supply strategies to acquire iron for overcoming
the iron shortage encountered in their biotopes. They use two general mechanisms to obtain iron/heme. The first involves direct contact between the bacteria and the exogenous iron/heme source. The second consists of bacterial synthesis and release of siderophores or hemophores into the extracellular medium to trap iron or heme.

1. Free Fe(II) and its transport

Under acidic and anaerobic conditions, ferrous iron Fe(II) predominates. This soluble iron can diffuse freely through the outer membrane porins and is transported through the cytoplasmic membrane via a high-affinity ferrous ion uptake (feo) system in many Gram-negative bacteria (Kammler et al., 1993). In Escherichia coli, feoA and B encode for the Fe(II) transporter (Kammler et al., 1993) (Fig.1). These feo genes are conserved in many bacteria, but feoB is often present in the absence of feoA (Andrews et al., 2003). The feoB gene is present in ~50% of the bacterial genomes sequenced to date (Hantke, 2003). FeoB was identified as an ATP-dependent transporter (Kammler et al., 1993) and has been shown to function as a G protein (Hantke, 2003). Feo mutants of Salmonella enterica and E. coli colonize mouse intestine less efficiently than the wild type (Stojiljkovic et al., 1993; Tsolis et al., 1996). In Legionella pneumophila, disruption of feoB decreases Fe(II) uptake, leading to reduced intracellular growth (Robey and Cianciotto, 2002). FeoB is the most important iron uptake system for Helicobacter pylori, which colonizes the stomach, an acidic environment (Velayudhan et al., 2000).
2. Transferrin and lactoferrin iron transport

Many bacterial species such as *Neisseria* and *Haemophilus influenzae* have transferrin and/or lactoferrin receptors (Cornelissen and Sparling, 1994; Fuller et al., 1998). Iron is stripped by these receptors from transferrin and lactoferrin and iron-free proteins are released. The functional transferrin receptor in *Neisseria* consists of TbpA and TbpB subunits. TbpA is analogous to TonB-dependent outer membrane receptors and TbpB is a lipoprotein anchored in the outer leaflet of the outer membrane (Legrain et al., 1993). TbpA is strictly required, whereas the requirement for TbpB is not as stringent. Uptake of iron by TbpA is TonB-ExbB-ExbD and pmf-dependent (Cornelissen et al., 1997). The bipartite lactoferrin LbpAB receptors of *Neisseria* (Schryvers et al., 1998) and *H. influenzae* (Ekins et al., 2004) function similarly, with only Fe(III) crossing the outer membrane. In the periplasm, Fe(III) binds FbpA in association with the bipartite receptor TbpAB (Gomez et al., 1998). FbpA shuttles
Fe(III) across the periplasm to an ABC permease in the cytoplasmic membrane (Ekins et al., 2004). This ABC permease translocates ferric iron across the cytoplasmic membrane into the cytosol.

3. Siderophores and hemophores

3.1. Synthesis and excretion of siderophores

To overcome the problem of the low solubility of Fe (III), many bacteria synthesize and secrete strong iron chelators: siderophores. Siderophores chelate iron from the extracellular medium; the iron-loaded siderophore is then taken up by the cell through a specific uptake system (Fig.2). Siderophores are low-molecular-weight compounds (400 to 1,000 Da) produced by microorganisms. They are synthesized by non-ribosomal cytoplasmic peptide synthetases resembling machinery used for the biosynthesis of peptide antibiotics. Following their synthesis, siderophores are excreted into the extracellular medium. These molecules are thought to be too large to diffuse through the envelope. In *E. coli*, *entS*, which is located in the enterobactin biosynthesis and transport gene cluster, encodes a membrane protein belonging to the major facilitator superfamily. The proteins of this family are involved in proton motive-force-dependent membrane efflux pumps. The protein EntS was shown to be directly involved in enterobactin export (Furrer et al., 2002).
3.2. Hemophores

Hemophores have been found in Gram-negative and Gram-positive bacteria. Hemophores are either anchored to the cell surface or released into the extracellular medium to capture free heme or to extract heme from hemoproteins such as hemoglobin in the external medium and present it to specific outer membrane receptors (Wandersman and Delepelaire, 2004, 2012).

The HasA hemophore

HasA from *Serratia marcescens* was the first hemophore to be discovered (Letoffé et al., 1994a). Later, hemophores were identified in *P. aeruginosa* (Letoffé et al., 1998), *P. fluorescens* (Idei et al., 1999), *Y. pestis* (Rossi et al., 2001), *Bacillus*...
anthracis (Fabian et al., 2009) and Porphyromonas gingivalis (Gao et al., 2010). HasA hemophores are conserved in several Gram-negative species (Wandersman and Delepelaire, 2012).

In S. marcescens, HasA, a 19 kDa monomer, exhibits very high affinity for heme \((K_d \approx 10^{-11} \text{M})\) at 1:1 stoichiometry (Izadi-Pruneyre et al., 2006). The crystal structure of holo HasA shows a globular protein with a two-faced fold (4 \(\alpha\)-helices on one face and 7 \(\beta\)-strands on the other) and a heme pocket with potential iron ligands (Arnoux et al., 1999). The heme iron atom is ligated by tyrosine 75 and histidine 32 (Letoffe et al., 2001). Histidine 83 also plays an important role in heme binding, since it stabilizes Tyr-75 and strengthens the Tyr-75 iron coordination bond (Wolff et al., 2002) (Fig. 3).

![Crystal structure of holo-HasA from S. marcescens](image)

**Fig 3.** Crystal structure of holo-HasA from S. marcescens. Heme is ligated by tyrosine 75 and histidine 32. Histidine 83 stabilizes Tyr-75 and strengthens the Tyr-75 iron coordination bond. Adapted from (Arnoux et al., 1999).

HasA is secreted by ABC transporters made up of three envelope proteins associated in a protein complex (Letoffe et al., 1994b) (Fig 4). This complex includes an inner membrane ATPase, HasD, a membrane fusion protein, HasE, located in the
inner membrane and HasF, an outer membrane protein of the TolC family (Wandersman and Delepelaire, 1990) (Fig 4).

**Fig 4.** Organization of the *has* operon of *S. marcescens*. HasDEF and SecB allow secretion of the HasA hemophore into the extracellular medium, where it binds heme and returns it to the HasR receptor. Adapted from (Wandersman and Delepelaire, 2004).

HasA is able to capture heme from hemoglobin, holo-hemopexin and myoglobin (Benevides-Matos and Biville, 2010; Wandersman and Delepelaire, 2004). In *S. marcescens*, heme is directly taken up at concentrations higher than $10^{-6}$M in the presence of iron depletion. Hemophore HasA is required to mediate heme uptake only under strong iron depletion conditions and low heme concentrations (lower than $10^{-7}$M) (Benevides-Matos and Biville, 2010).

**The HxuCBA system of *H. influenzae***

Another hemophore system has been described in *H. influenzae* that lacks the
heme biosynthetic pathway and requires exogenous heme for aerobic growth (White and Granick, 1963). In *H. influenzae*, the gene cluster *hxuC, hxuB*, and *hxuA* is required for heme-hemopexin utilization (Cope et al., 1995). HuxA is a 100 kDa protein that can be found on the *H. influenzae* cell surface and in culture supernatants (Cope et al., 1994). In the reconstituted *E. coli* system, most HuxA remains associated with the cell and only a small fraction of HxuA is released into the extracellular medium (Fournier et al., 2011). HxuA is secreted by a signal-peptide-dependent pathway requiring one helper protein, HxuB (Cope et al., 1995). HxuB was hypothesized as being involved in release of soluble HxuA from the cell surface (Cope et al., 1995). Unlike HasA, there is no heme capture by HxuA, but heme is released into the medium when HxuA interacts with heme-hemopexin (Wandersman and Delepelaire, 2012). In reconstituted *E. coli* strain C600ΔhemA, expression of HxuC alone enabled use of free heme or hemoglobin as a heme source (Fournier et al., 2011). However, co-expression of HxuA, HxuB and HxuC in C600ΔhemA is required for heme acquisition from heme-loaded hemopexin (Fournier et al., 2011). It was shown that when HxuA interacts with holo-hemopexin, heme is released from hemopexin and immediately binds its cognate receptor HxuC, which transports heme through the outer membrane (Fournier et al., 2011).

**4. Receptors for siderophores and heme/hemophores**

**4.1 Receptors for siderophores**

Loaded siderophore complexes are too large to freely pass through the porins. The first step in ferri-siderophore internalization requires a specific outer membrane
receptor having high affinity ($K_d$ 0.1-100nM) for the ferri-siderophore complex (Stintzi and Raymond, 2000). Outer membrane receptors involved in recognition and transport of iron-loaded siderophores are specific, but have structural and functional characteristics in common (Koster, 2001). These receptors all display a plug and barrel organization (Biville, 2008; Krewulak and Vogel, 2008). The N-terminus part of the protein, which constitutes the plug, is folded inside a β-barrel anchored in the outer membrane. The plug delimits two interacting surfaces, one on the outside of the membrane and the other on the periplasmic side. The substrate sits on the top of the plug, facing the extracellular medium. This brings it into contact with residues of the extracellular loops and the plug apices. There is no channel large enough to accommodate the substrate. Determination of the crystal structure of FecA complexed with ferric citrate and of FhuA complexed with iron-containing ferrichrome has provided information on the transport process (Locher et al., 1998; Yue et al., 2003). Substrate binding triggers small changes in the conformation of the plug apices, with larger movements observed on the periplasmic side where the TonB box is found ( Ferguson and Deisenhofer, 2004). The TonB box consists of five conserved residues towards the N-terminus of outer membrane siderophore receptors. These residues have been implicated in the functioning of the TonB complex (Postle, 1993). It is thought that substrate binding leads to closure of the outside loops around the substrate and movement on the periplasmic side, allowing the TonB complex to function. Closing of the outside loops prevents the substrate from being released into the medium (Eisenhauer et al., 2005).
4.2 Receptors for heme

Heme receptors were subdivided into three categories: the first group recognizes free heme, the second recognizes host hemoproteins (Fig 5) and the third interacts with hemophores (Fig 4) (Tong and Guo, 2009). The receptors that recognize hemoproteins and hemophores can also take up free heme. All these receptors share overall common structural attributes within the family and also share significant homology with siderophore receptors (Wilks and Burkhard, 2007).

The outer membrane receptor binds heme free or bound to hemoproteins, and transports it into the periplasm via a TonB-dependent process (Fig 5). Inside the periplasm, heme is bound by the heme periplasmic binding protein, which addresses it to an inner membrane ATP binding cassette (ABC) transporter (Fig 5). Inside the cytoplasm, heme is rapidly transferred to a heme oxygenase (HO)/heme-degrading protein which are able to release iron from heme (Fig. 5).
5. The *TonB-ExbB-ExbD* complex

Transportation of ferri-siderophores or heme through the outer membrane is energy-dependent. This transport against a concentration gradient depends on a complex of three proteins in the cytoplasmic membrane: *TonB*, *ExbB* and *ExbD* (Moeck and Coulton, 1998). This *TonB-ExbB-ExbD* complex transduces the energy generated by the electrochemical gradient across the cytoplasmic membrane (Bradbeer, 1993). *TonB-ExbB-ExbD* proteins are found in many Gram-negative bacteria. The structure and function of this system has been extensively studied in *E. coli*.

The *TonB* protein is associated with the inner membrane, with a large part of the
protein occupying the periplasmic space (Fig. 6). TonB spans the periplasm and directly contacts outer membrane active transport proteins. ExbB and ExbD are integral cytoplasmic membrane proteins. ExbB has three transmembrane segments and its N-terminus projects into the periplasm (Fig. 6). ExbD has only one transmembrane domain and most of the protein is in the periplasm (Braun and Braun, 2002) (Fig. 6). The three proteins, ExbB, ExbD and TonB, seem to act as a complex, since ExbB and ExbD interact with each other in vitro (Braun et al., 1996).

![Fig. 6 The structure of the complex TonB-ExbB-ExbD. OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane. Adapted from Tong and Guo, 2009.](image)

### 6. Transport across the periplasm and cytoplasmic membrane

Transport of ferri-siderophore complexes and heme across the periplasmic space and the cytoplasmic membrane is mediated by periplasmic binding proteins associated with inner membrane transporters. The periplasmic binding proteins shuttle
ferri-siderophore or heme from the outer membrane receptor or the periplasm and deliver it to a cognate permease in the inner membrane (Koster, 2001). The periplasmic binding proteins are less specific than the outer membrane receptors. ABC permeases are responsible for transport across the inner membrane. ABC permeases consist of a periplasmic domain and an inner membrane complex energized by an ABC ATPase (Koster, 2001).

7. Fate of the ferri-siderophore and heme in the cytoplasm

After internalization, iron must be released from the siderophore. Two mechanisms have been proposed for release of iron from the ferri-siderophore into the cytoplasm. Spontaneous dissociation of the ferri-siderophore may be related to the relatively low affinity of siderophores for Fe (II). A specific ferric reductase is probably required for this pathway (Matzanke et al., 2004). The second mechanism involves intracellular breakdown of the ferri-siderophore, implying that siderophores are used only once. In *E. coli*, the use of ferri-enterobactin requires esterase activity encoded by the *fes* gene, located in a cluster of genes involved in enterobactin biosynthesis and uptake. Fes is a cytoplasmic esterase that hydrolyzes ferri-enterobactin, producing 2,3-dihydroxybenzoyl serine (Brickman and McIntosh, 1992). Iron is released from heme as soon as it is transported to the cytoplasm (see paper one, Introduction).

8. Regulation of iron and heme uptake in Gram-negative bacteria

Iron is an essential element for most bacteria. However, overload iron may be harmful because of its toxic properties. Via the Fenton reaction (Fe(II)+H₂O₂→
Fe(III)+OH⁻+OH⁻), oxidized iron and toxic hydroxyl radicals are created, the latter of which can damage biological macromolecules (Halliwell and Gutteridge, 1984). Many bacteria incorporate intracellular iron into iron storage proteins. Three classes of iron storage proteins have been characterized in bacteria: ferritins (Abdul-Tehrani et al., 1999), heme-containing bacterioferritins (Andrews et al., 1989) and Dps, or small DNA binding polypeptides found only in prokaryotes (Almiron et al., 1992). Ferritin and bacterioferritin take up soluble Fe(II) and catalyze its oxidation to give Fe(III), using oxygen and peroxide, respectively, as electron acceptors. The insoluble ferric iron Fe(III) is then deposited in the central cavity to give a ferrihydrite core of about 5,000 iron ions (Carrondo, 2003). Dp DNA binding proteins catalyze peroxide-dependent Fe(II) oxidation and protect DNA against oxidative damage; they store 500 iron ions per 12-mer (Andrews et al., 2003). Thus, under iron deficiency conditions, genes involved in iron uptake are expressed. When the intracellular iron concentration is high, these genes are repressed.

In many bacteria such as *E. coli* (Hantke, 1981), *V. cholerae* (Litwin et al., 1992) and *Y. pestis* (Staggs and Perry, 1991), genes involved in iron and heme uptake are negatively regulated by the global determinant ferric uptake regulator protein (Fur). Fur is a homodimer composed of two identical 17 kDa subunits (Coy and Neilands, 1991). The N-terminal domain of Fur is involved in DNA binding and the C-terminal of Fur, which is rich in His residues, is involved in iron binding and dimerisation (Stojiljkovic and Hantke, 1995). Under iron-rich conditions, Fur binds to Fe²⁺ and forms a Fur-Fe²⁺ complex which binds the Fur box, a 19 bp consensus sequence
Generally, the Fur box is located in between the -35 and -10 sites of Fur-regulated promoters (Baichoo and Helmann, 2002). Binding of Fur protein to the Fur box represses transcription of iron/heme uptake genes. On the other hand, when iron is limiting, divalent iron will be released from Fur, which no longer binds the Fur box, thus allowing RNA polymerase to bind the promoter region and start transcription.

Another regulatory mechanism involves specific extracytoplasmic function (ECF) sigma factors. Most sigma factors are associated with membrane-bound anti-sigma factors. An extracytoplasmic signal provokes release of the sigma factor from its cognate anti-sigma factor, thus leading to transcription of target operons (Helmann, 2002; Raivio and Silhavy, 2001). The best documented example and the first to be fully characterized is the *E. coli* ferric citrate uptake system (Braun et al., 2003). FecI, the iron starvation ECF sigma, binds to the RNA core polymerase, initiating transcription of *fecABCDE* genes which encode the outer membrane receptor and binding-protein-dependent ABC permease. Under conditions of iron depletion, binding of ferric citrate to its specific outer membrane receptor FecA without transport is sufficient to initiate the signaling cascade leading to induction of the *fecABCDE* operon (Braun et al., 2003).

The *has* operon of *S. marcescens* encodes for a completely hemophore-dependent heme uptake system. Two genes upstream from the *has* operon encode for HasI (an ECF sigma factor) and HasS (an anti-sigma factor) (Fig.7).
When there is enough heme, HasS anti-sigma activity is turned off by binding of holo-HasA to HasR, and HasI induces transcription of hasS and the has operon (Fig. 7A). This leads to accumulation of HasS molecules which are inactive as long as HasR is loaded with holo-hasA. HasS molecules become active when HasR ceases to be occupied by holo-HasA. Thus, as soon as there is a heme shortage, HasS is released from HasR and can then bind HasI, which thus cannot activate has operon transcription (Fig. 7B) (Biville et al., 2004; Cwerman et al., 2006).

9. The heme uptake system in Bartonella

9.1. Heme requirements in Bartonella

In 1921, Sikora first reported propagation of Rickettsia pediculi (B. quintana) on human blood agar. Later, it was shown that erythrocytes contain an essential factor or factors for multiplication of B. quintana (Vinson, 1966). Hemoglobin was suspected to be the critical factor provided by them (Vinson, 1966). Hemoglobin alone could not replace erythrocytes in the medium, but a combination of hemoglobin and high serum concentrations enabled multiplication of B. quintana, suggesting that serum contains a
substance that is able to promote growth of *Bartonella* (Vinson, 1966). Myers et al. (1969) showed that hemoglobin and heme, but not protoporphyrin, were able to substitute for the red cell lysate in the presence of serum, and the requirement for heme was relatively high (20 to 40 µg/ml) in *B. quintana* (Myers et al., 1969). This high requirement had already suggested that it functions not as a nutrient precursor in formation of hemoproteins, but rather, as a catalyst for destruction of hydrogen peroxide (Lascelles, 1962). Further analysis indicated that starch or charcoal can replace serum or bovine serum albumin for growth and it was suggested that serum does not play an essential nutritive role, but rather serves as a detoxification function (Lascelles, 1962). Recently, studies on *B. henselae* showed that protoporphyrin IX alone or together with FeCl₂ or FeCl₃ as well as transferrin or lactoferrin did not support growth, indicating that *B. henselae* alone cannot synthesize heme, whereas heme is essential for sustaining growth of *Bartonella* with both an iron and heme source (Sander et al., 2000b). This observation was consistent with the hypothesis that *Bartonella* species lack most of the enzymes required to synthesize heme (Alsmark et al., 2004). Except for solid medium, several liquid media have been developed for culture of *Bartonella* (Chenoweth et al., 2004b; Maggi et al., 2005; Riess et al., 2008; Schwartzman et al., 1993; Wong et al., 1995). Whether in solid or liquid medium, supplementation with blood, erythrocytes or serum seems to be required for growth of *Bartonella*.

### 9.2. The heme uptake system in *Bartonella*

Genomic analysis of *Bartonella* indicated that these bacteria neither encode for
siderophore biosynthesis pathway, nor for a complete Fe$^{3+}$ transport system (Table 1). Only genes sharing strong homology with all compounds of the Fe$^{2+}$ uptake system already characterized in *Yersinia pestis* (Perry et al., 2007) and *Photorhabdus luminescens* (Watson et al., 2010) are present in *Bartonella* genomes.

**Table 1.** Genes involved in heme/iron uptake of *Bartonella*.

<table>
<thead>
<tr>
<th></th>
<th><strong>Bartonella</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Transport of iron (Fe$^{2+}$)</td>
<td>YfeABCD</td>
</tr>
<tr>
<td>Sidrophore biosynthesis</td>
<td>No</td>
</tr>
<tr>
<td>Synthesis of heme</td>
<td>No</td>
</tr>
<tr>
<td>Heme uptake system</td>
<td>HutA, HutB, HmuV, HutC, HemS</td>
</tr>
<tr>
<td>Heme binding proteins</td>
<td>HbpA, B, C, D</td>
</tr>
<tr>
<td>Energy suppliers</td>
<td>TonB, ExbB, ExbD</td>
</tr>
<tr>
<td>Iron storage</td>
<td>No</td>
</tr>
<tr>
<td>Regulation</td>
<td>Fur/Irr/RirA</td>
</tr>
</tbody>
</table>

In addition, *Bartonella* genomes encode for a complete heme uptake system (Table 1). The cluster of genes devoted to heme uptake includes heme receptor HutA, a TonB-like energy transducer, an ABC transporter comprised of three proteins, HutB, HutC and HmuY, and a putative cytoplasmic heme storage/degradation protein, HemS (Fig 8).
Fig. 8 Proposed organization of the heme acquisition system of *Bartonella*. The heme uptake system consists of HutA, a TonB-dependent heme receptor, HutB, HutC and HmuV (ABC transport system), and HemS (heme trafficking or degradation protein). Adapted from (Minnick and Battisti, 2009).

Sequence comparison showed that HutA of *B. quintana* has conserved FRAP and NPNL domains with HemR of *Yersinia enterocolitica* and HumR of *Yersinia pestis* (Parrow et al., 2009). It was shown that HutA of *B. quintana* exhibited TonB-dependent heme transport activity through complementation of the *E. coli* hemA mutant (Parrow et al., 2009). However, experiments were performed in the presence of high heme concentrations in liquid medium and indicated that weak growth restoration could be the consequence of mutation-enhancing outer membrane permeability (Olczak et al., 2008). Such complementation assays failed for an *E. coli* K-12 hemA mutant expressing HutA from *B. birtlesii* when grown on heme-supplemented solid medium (Nijssen et al., 2009). Finally, *Bartonella* encode for an outer membrane heme binding protein family--HbpA-E for *B. quintana* and HbpA-D for *B. henselae*, which do not have sequence similarity to classical heme receptors (see paper two) (Carroll et al., 2000).

9.3. Regulation of heme uptake in *Bartonella*

Like other bacteria, *Bartonella* need to create a balance between obtaining sufficient heme in a limited environment for survival and preventing accumulation of intracellular heme that is toxic. *Bartonellae* are able to survive in a mammal heme-limiting environment and in heme-repleted arthropod gut; thus, the heme
acquisition process is tightly regulated in Bartonella. It has been shown that the transcription level of hut locus genes was slightly increased (~1.5 fold) in response to low heme limitations (0.05 mM) and ~2.2-fold-decreased in response to heme excess (2.5 mM) (Parrow et al., 2009). In Bartonella, there are at least three iron/heme-responsive regulators, namely Fur, Irr (iron response regulator) and RirA (rhizobial iron regulator A).

The B. henselae Fur protein has 38% amino acid identity with E. coli and Vibrio cholerae Fur (Park et al., 2001). Corresponding fur genes were also identified in B. bacilliformis and B. quintana (Park et al., 2001). Functional analysis of B. henselae fur indicated that it is able to complement a V. cholerae fur mutant (Park et al., 2001). Recently, it was shown that B. quintana fur was able to complement an E. coli fur mutant. Overexpression of fur represses hbpC and tonB expression of B. quintana in the presence of heme excess. Other genes of the hut locus and other hbp genes are not repressed under the same conditions (Parrow et al., 2009). However, the Fur protein was not detected in B. quintana using immunoblotting, thus suggesting a weak fur expression level (Parrow et al., 2009).

In Bartonella genomes, two alternative iron-responsive regulators have been identified (Battisti et al., 2007). One is the Rhizobial iron regulator RirA, which was first identified in Rhizobium leguminosarum (Todd et al., 2005). In B. quintana, it was shown that rirA overexpression does not affect transcription of hut locus genes regardless of the ambient heme concentration (Parrow et al., 2009).

The second regulator is Irr (iron response regulator), a member of the Fur family
that responds directly to intracellular heme concentrations rather than to iron in \textit{Bradyrhizobium japonicum} (Yang et al., 2006). In contrast to Fur, which is widespread in all Gram-negative bacteria, Irr is restricted to members of the \(\alpha\)-proteobacteria (Rudolph et al., 2006). Homologues of Irr were found in all \textit{Rhizobia} (Rudolph et al., 2006). Irr was first identified in \textit{B. japonicum} as a transcriptional repressor of \textit{hemB} which encodes for heme biosynthetic enzyme \(\delta\)-aminolevulinic acid dehydratase (Hamza et al., 1998). Irr has been described as a regulator of iron- and heme-associated genes in \textit{B. japonicum} (Hamza et al., 2000), \textit{R. leguminosarum} (Singleton et al., 2010) and \textit{B. abortus} (Martinez et al., 2005). \textit{B. japonicum} Irr expression is under the transcriptional control of Fur. It was shown that \textit{B. japonicum} Fur binds to the \textit{irr} gene promoter with high affinity, although without a consensus Fur box (Friedman and O'Brian, 2003). However, \textit{R. leguminosarum} \textit{irr} expression is not mediated by Fur, and purified Fur cannot bind the promoter region of Irr (Wexler et al., 2003). Overexpression of Irr was shown to repress transcription of the \textit{hut} locus of \textit{B. quintana} (Parrow et al., 2009). These data suggested that Irr plays an important role as a heme-responsive transcriptional regulator in \textit{Bartonella} (Parrow et al., 2009).
CHAPTER THREE
Research significance and Purpose of the work

Research significance

Arthropod vector-borne *Bartonellae* species have strong heme requirements for growth *in vitro*. *In vivo*, *Bartonellae* cause long-standing bacteremia as a hallmark of infection in its reservoir host (Schulein et al., 2001). Invasion of erythrocytes was hypothesized as being a strategy for obtaining heme that cannot be synthesized by them. When confronting invading erythrocytes, *Bartonellae* must replicate and gain competence in a primary niche, characterized as the endothelial cell (Dehio, 2005).

Sequence analysis showed that *Bartonellae* genomes encode for a complete heme uptake system. In the gene cluster devoted to heme uptake, HutA was identified as a heme receptor in *B. quintana* (Parrow et al., 2009). However, the fate of heme in the cytoplasm was unknown. In addition to the classical heme uptake system, *Bartonellae* synthesize heme binding proteins located in the outer membrane. The regulation profile of heme binding protein expression has been widely investigated, but its function remains to be defined. It was suggested that heme binding proteins of *Bartonellae* play an important role during transition between a low heme concentration biotope (inside mammalian host) and a high heme concentration biotope (flea gut).
Purpose of the work

In this project, we focused on identification of bacterial genes involved in heme uptake and utilization, mainly the hemS and hbp families of *B. henselae*, using two strategies. The first approach was to characterize the function of genes via use of a heterologous *E. coli* model. The second strategy was to elucidate physiological effects related to knockdown of *hemS* and *hbpA-D* in *B. henselae*.

The first set of experiments focused on characterizing the function of HemS of *B. henselae* in iron release from heme. We initially examined the capacity of HemS to act as a heme-degrading protein by complementation of the *E. coli* mutant impaired in iron release from heme. Our second aim was to express and purify recombinant HemS and examine the capacity of purified recombinant HemS to bind heme and degrade it in the presence of a suitable electron donor. If HemS could degrade heme, then it was reasonable to believe that HemS could prevent deleterious effects of heme accumulation in the cytoplasm. Moreover, homologues of many genes involved in the oxidative stress response in *E. coli* are not present in *Bartonellae* genomes. Thus, our final goal was to determine the effect of HemS knockdown upon the oxidative stress response.

The second part of our project sought to characterize the function of heme binding proteins of *B. henselae*. Our primary goal was to examine the ability of heme binding proteins to bind Congo red/heme *in vivo* and *in vitro*. The functions of heme binding proteins in heme utilization remain to be defined. The second aim was to verify whether heme binding proteins of *B. henselae* can transport heme when expressed in the *E. coli* hemA mutant. We next determined whether heme binding
proteins modify the heme uptake process through co-expression of heme binding proteins and the heme transporter in *E. coli* mutant.

*Bartonellae* must overcome immune defenses such as oxidative stress produced by the mammal host in response to bacterial invasion and in the arthropod vector gut. However, many homologues of genes involved in the oxidative stress response in *E. coli* are not present in the *Bartonellae* genome. It was proposed that heme binding proteins might be a way of facing oxidative stress using the intrinsic peroxidase activity of heme (Battisti et al., 2006). The third goal was to check whether heme binding proteins are involved in the oxidative stress response through knockdown expression of heme binding proteins in *B. henselae*. Finally, the effect of heme binding protein knockdown expression upon colonization in the endothelial cell and the flea was investigated, since bacteria must face oxidative stress inside endothelial cells and in the flea gut.
RESULTS
Heme-degrading protein HemS is involved in the oxidative stress response of *Bartonella henselae*

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State of the art concerning the fate of heme in the cytoplasm

Iron release from heme requires heme degradation. Some species, including *Pseudomonas aeruginosa* (Ratliff et al., 2001), *Neisseria meningitidis* (Zhu et al., 2000b) and *Corynebacterium diphtheria* (Hirotsu et al., 2004), possess enzymes homologous to human oxygenase (HO) which can degrade heme to iron, biliverdin and carbon monoxide in the presence of a suitable electron donor such as ascorbate or NADPH-cytochrome P450 reductase (Fig. 1). Characterized HO family members are listed in Table 1.

![Heme degradation diagram](image)

**Fig. 1** Heme degradation, as catalyzed by the heme oxygenase reaction. Adapted from (Wilks and Burkhard, 2007).

However, many species that can use heme as an iron source do not have a heme oxygenase homologue. In *Staphylococcus aureus*, two enzymes, IsdG and IsdI, can degrade heme (Skaar et al., 2004). IsdG heme-degrading enzymes have been identified in *S. aureus* (Skaar et al., 2004), *Bacillus antracis* (Skaar et al., 2006), *Bradyrhizobium japonicum* (Puri and O'Brian, 2006), *Mycobacterium tuberculosis* (Chim et al., 2010) and *Brucella melitensis* (Table 1) (Puri and O'Brian, 2006). IsdG homologues were predicted to be encoded in the *Alphaproteobacteria, Streptomyces, Deinococcus-Thermus* and *Chloroflexi* (Anzaldi and Skaar, 2010). The IsdG family,
which differs from HO, degrades heme to release free iron and staphylobilin (5-oxo-δ-bilirubin and 15-oxo-β-bilirubin) (Fig. 2) in the presence of a reducing agent (Reniere et al., 2007; Reniere et al., 2010).

![5-oxo-δ-bilirubin and 15-oxo-β-bilirubin](image)

**Fig 2.** Linear representation of heme degradation products produced by the IsdG family. Adapted from (Reniere et al., 2010).

It was shown that YfeX and EfeB of *E. coli K12* remove iron from heme without destroying the tetrapyrrole ring (Letoffe et al., 2009). Recently, it was shown that YfeX exhibits peroxidase activity possibly responsible for iron release from heme (Dailey et al., 2011). Some cytoplasmic heme binding proteins of Gram-negative bacteria have been hypothesized to function as heme trafficking or storage proteins (Table 1).
Table 1. Characterized heme oxygenase, heme-degrading and heme-trafficking proteins in bacteria.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Organism</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ShuS</td>
<td><em>Shigella dysenteriae</em></td>
<td>Heme trafficking</td>
<td>(Wilks, 2001)</td>
</tr>
<tr>
<td>HemS</td>
<td><em>Yersinia enterocolitica</em></td>
<td>Heme trafficking</td>
<td>(Schneider et al., 2006)</td>
</tr>
<tr>
<td>HutZ</td>
<td><em>Vibrio cholerae</em></td>
<td>Heme trafficking</td>
<td>(Thompson et al., 1999)</td>
</tr>
<tr>
<td>PhuS</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Heme trafficking or storage</td>
<td>(Wyckoff et al., 2004)</td>
</tr>
<tr>
<td>PigA</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Heme oxygenase</td>
<td>(Ratliff et al., 2001)</td>
</tr>
<tr>
<td>BphO</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Heme oxygenase</td>
<td>(Wegele et al., 2004)</td>
</tr>
<tr>
<td>HmuO</td>
<td><em>Corynebacterium diphtheriae</em></td>
<td>Heme oxygenase</td>
<td>(Wilks and Schmitt, 1998)</td>
</tr>
<tr>
<td>HemO</td>
<td><em>Neisseria meningitidis</em></td>
<td>Heme oxygenase</td>
<td>(Zhu et al., 2000b)</td>
</tr>
<tr>
<td>ChuS</td>
<td><em>Escherichia coli O157:H7</em></td>
<td>Heme oxygenase</td>
<td>(Suits et al., 2005)</td>
</tr>
<tr>
<td>HutZ</td>
<td><em>Vibrio cholerae</em></td>
<td>Heme-trafficking or storage</td>
<td>(Wyckoff et al., 2004)</td>
</tr>
<tr>
<td>IsdG</td>
<td><em>Staphylococcus aureus</em></td>
<td>Heme-degrading enzyme</td>
<td>(Skaar et al., 2004)</td>
</tr>
<tr>
<td>IsdG</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>Heme-degrading enzyme</td>
<td>(Haley et al., 2011)</td>
</tr>
<tr>
<td>IsdI</td>
<td><em>Staphylococcus aureus</em></td>
<td>Heme-degrading enzyme</td>
<td>(Skaar et al., 2004)</td>
</tr>
<tr>
<td>IsdG</td>
<td><em>Bacillus anthracis</em></td>
<td>Heme-degrading monoxygenase</td>
<td>(Skaar et al., 2006)</td>
</tr>
<tr>
<td>HmuQ</td>
<td><em>Bradyrhizobium japonicum</em></td>
<td>Heme-degrading enzyme</td>
<td>(Puri and O'Brian, 2006)</td>
</tr>
<tr>
<td>HmuD</td>
<td><em>Bradyrhizobium japonicum</em></td>
<td>Heme-degrading enzyme</td>
<td>(Puri and O'Brian, 2006)</td>
</tr>
<tr>
<td>MhuD</td>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Heme-degrading enzyme</td>
<td>(Chim et al., 2010)</td>
</tr>
</tbody>
</table>

Heme oxygenases have been shown to be important in heme iron utilization. Inactivation of *N. gonorrhoeae hemO* caused a growth defect when the mutant was grown in liquid culture in which heme was the only iron source (Zhu et al., 2000a). Disruption of *B. anthracis isdG* causes growth inhibition when heme is the sole iron source (Skaar et al., 2006). The *Helicobacter pylori hugZ* mutant showed poor growth when hemoglobin was provided as an iron source under iron-restricted conditions (Guo et al., 2008). Disruption of *P. aeruginosa pigA* impaired heme utilization in iron-poor media supplemented with heme, while this defect was abolished by addition of FeSO₄ (Ratliff et al., 2001; Wyckoff et al., 2005). Deletion of *Y. enterocolitica*
HemS is lethal, but *hemS* expression in *E. coli* prevents heme toxicity (Stojiljkovic and Hantke, 1994).
Introduction

*Bartonellae* species have a strong heme requirement for growth *in vitro* (Myers et al., 1969). *In vivo*, erythrocyte persistence is a hallmark of *Bartonellae* infection, a possible strategy used by *Bartonella* species for obtaining heme that cannot be synthesized by them (Alsmark et al., 2004). Moreover, heme added to an iron-depleted medium supported growth of *B. henselae*, thus indicating the utility of heme as an iron source (Sander et al., 2000a). Analysis of the complete genomic sequences of *Bartonella* genomes showed that they encode for a complete heme transport system shown to be active in the presence of high heme concentrations in *B. quintana* (Parrow et al., 2009). In the cluster of genes devoted to heme uptake, one gene, *hemS*, shares homology with both heme-degrading and heme-trafficking enzymes. The function of HemS in heme uptake remains unknown.

Homologues of a number of genes involved in the oxidative stress response in *E. coli* are not present in *Bartonellae* genomes. In addition, many bacterial catalases contain heme as a prosthetic group (Frankenberg et al., 2002). Thus, it is reasonable to suggest that HemS may act to protect *Bartonellae* from the oxidative stress response.

In this report, we investigated the function of HemS of *B. henselae* using a functional complementation *Escherichia coli* mutant that cannot release iron from heme. The recombinant protein was expressed and purified from *E. coli* and tested for heme binding and degradation. Finally, the effect of *hemS* knockdown on the oxidative stress response was also investigated in *B. henselae*.
Heme Degrading Protein HemS Is Involved in Oxidative Stress Response of Bartonella henselae

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Abstract

Bartonella are hemotropic bacteria, agents of emerging zoonoses. These bacteria are heme auxotrophs, Alphaaproteobacteria which must import heme for supporting their growth, as they cannot synthesize it. Therefore, Bartonella genome encodes a complete heme uptake system allowing the transportation of this compound across the outer membrane, the periplasm and the inner membranes. Heme has been proposed to be used as an iron source for Bartonella since these bacteria do not synthesize a complete system required for iron Fe3+ uptake. Similarly to other bacteria which use heme as an iron source, Bartonella must transport this compound into the cytosol and degrade it to allow the release of iron from the tetrapyrole ring. For Bartonella, the gene cluster devoted to the synthesis of the complete heme uptake system also contains a gene encoding for a polypeptide that shares homologies with heme trafficking or degrading enzymes. Using complementation of an E. coli mutant strain impaired in heme degradation, we demonstrated that Hems from Bartonella henselae expressed in E. coli allows the release of iron from heme. Purified Hems from B. henselae binds heme and can degrade it in the presence of a suitable electron donor, acorbate or NADPH-cytochrome P450 reductase. Knockdown the expression of Hems in B. henselae reduces its ability to face Fe3+ induced oxidative stress.

Introduction

Bartonella species are now well established as human pathogens responsible for several emerging zoonoses [1]. Bartonella henselae (B. henselae), Bartonella quintanae (B. quintana) and Bartonella bacilliformis (B. bacilliformis) are the most medically important species although several other have also been described as pathogens [2]. B. henselae is now recognized as one of the most common zoonoses acquired from companion animals in industrialised countries [3]. The bacterium causes cat scratch disease as well as being increasingly associated with a number of other systemic disorders [4]. Most Bartonella species appear to share a similar survival cycle that involves arthropod transmission, then replication in a mammalian host. Each Bartonella species appears to be highly adapted to one or few reservoir hosts in which Bartonella causes a long lasting intracytic infection as a hallmark of infection. The bacterial persistence in erythrocyte is an original strategy for its persistence in its host and the resulting long-lasting intracytic bacteremia is considered to represent a unique adaptation to the mode of transmission by blood sucking arthropod vectors. Flagella [5], a deformation activity [6], and a locus containing sldt and altt genes [7] were shown to be important for erythrocytes invasion by B. bacilliformis. Also, the importance of the TvsTSS for erythrocytes invasion was demonstrated for B. triboceron [8] and B. bovis [9]. The erythrocytes invasion can also be proposed as a strategy for Bartonella species to get heme that is absolutely required for the growth of B. henselae. It was also shown that addition of heme in an iron depleted medium supported the growth of B. henselae, thus showing the use of heme as an iron source [10]. Analysis of the complete genomic sequences of B. quintanae and B. henselae supports the absolute requirement for heme, since these two Bartonella species do not contain genes encoding for heme biosynthesis [11]. All the sequenced Bartonella genomes indicate that all these bacteria do not encode for siderophile biosynthesis and a complete iron Fe3+ transport system. Only genes sharing strong homologies with all the components of a Fe3+ uptake system already characterized in Francisella tularensis [12], and Photobacterium damselae [13] are present in Bartonella genomes. Bartonella genomes encode for a complete heme transport system that was shown to be active in the presence of high heme concentration for B. quintanae [14]. In the cluster of genes devoted to heme uptake, one gene, Hems, shares homology both with heme degrading enzymes and heme trafficking enzymes. The activity of heme degrading enzymes is required for the release of iron from heme after its transportation into the cytosol [15]. Different activities allowing the release of iron from heme have been characterized already. The more common, is a heme oxygenase first put in evidence in Comamonetes phylotypes [16]. Other bacterial heme oxygenases, that provoke the release of iron by degrading heme in bacteria were also characterized in Gram negative and Gram positive bacteria such as Pseudomonas aeruginosa.
Materials and Methods

Bacterial strains and plasmid

Bacterial strains and plasmids used in this study are listed in Table 1.

Table 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>E.coli strains</td>
<td></td>
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</tr>
<tr>
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<td>F' plaΔIC530-3Crip</td>
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<tr>
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<td></td>
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<tr>
<td>FBB27 (pAM232ΔIC530)</td>
<td>F' plaΔIC530-3Crip</td>
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<tr>
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</table>

[17], or *C. tetani* [18] for a review see [15]. Other homo

degrading monooxygenases were identified in Gram positive

bacteria such as *Bacillus anthracis* [19] and Gram negative bacteria

such as *Borrelia burgdorferi* [20]. Protein tyrosinase activity

has been identified in bacteria such as *Tetanus mordax* [21],

*Streptomyces avermitilis* [21], *Streptomyces* [22], *Pseudomonas aeruginosa* [23]. Enzyme allowing the release of

iron from heme in B. haemolyticus [24]. Controversial data about

these activities is that B. haemolyticus has been shown to be

hemolytic in vitro also [24]. In this report, we investigated the

function of hemin for B. halodurans, using functional complementation,

and partial biochemical characterization. The effect of heme

knock-down was also investigated in B. halodurans.

Use of hemin as iron source: E. coli assay

Tested strains were grown at 37°C for 18 hours in M9 medium

without iron, with 0.4% glycerol as carbon source, and in

the presence of 0.2% arabinose. Cultures were checked for OD

at 600 nm, and a 100 µl sample of an overnight culture of tested

strain adjusted to OD600 = 1 was mixed with 4 ml of soft agar

and poured onto M9 plates containing 0.4% glycerol, 0.2%

arabinose, and 70 µM DIP (M9D3D). Wells (5 mm in diameter)

were cut in the agar and filled with 100 µl of 50 µM, 10 µM,

5 µM, or 1 µM of filter sterilized Hb solution. Growth around

the wells was recorded after one and two day incubation at 37°C.

All experiments were performed in triplicate.
Effect of the hemS knockdown in B. henselae

To evaluate the effect of hemS knockdown on growth of B. henselae, tested strains were grown both in Schneider's liquid medium and on CBA plates. B. henselae (pNS2Trc) and B. henselae (pNS2TrcHemS) were collected after 5 days growth on CBA plates and suspended in Schneider's medium. The OD600 of the bacterial suspension was adjusted to 0.5. Two ml of this suspension were poured into 12 wells plate and grown at 35°C in the presence of 5% CO2. The OD600 was checked at day 2, 4, 6, and 7 after inoculation. Serial dilutions of bacterial suspension were plated on CBA plates, and the colony size was evaluated after 6 and 10 days of growth at 35°C in the presence of 5% CO2.

H2O2 challenge

B. henselae (pNS2Trc) and B. henselae (pNS2TrcHemS) were grown on CBA plates during 5 days at 35°C under 5% CO2 atmosphere. 1 ml from each culture plate was suspended, and washed twice in PBS buffer. Cell suspension was then diluted to OD600 0.5. Before H2O2 challenges, serial dilutions of the tested cell suspension were spread on CBA plates (T1). For challenge assay, bacteria were incubated 30 minutes in PBS buffer in the presence of 10 mM, or 10 mM H2O2 at 35°C under 5% CO2 atmosphere. After exposure to H2O2, bacteria were washed twice in PBS buffer and several dilutions were plated on CBA plates (T1). After 15 days incubation at 35°C under 5% CO2 atmosphere, colonies were counted. Survival rate was expressed by (T1/T0)x100%. All experiments were performed in triplicate.

Genetic techniques

E. coli cells were transformed by the calcium chloride method (28). Plasmids and transfections were performed as previously described by Miller (29). Basmala cells were transformed by electroporation as previously described (29).

Nonpolar deletion of yelF in E. coli by red linear DNA gene inactivation

A nonpolar mutation that deletes the entire yelF gene was created by allelic exchange as previously described (30). Briefly, plasmid pKOPEG (an ampicillin-resistant derivative of pKOPEGa) (see Table 1) was introduced into the target strain, and electroporant cells were prepared at 30°C after induction of the λ red system mediated by pKOPEGa with 0.2% arabinose. A three-step PCR procedure was used to produce a PCR product in which the att site from pRP452 (31) is flanked by 500-bp homology arms corresponding to DNA regions located upstream and downstream from the yelF start and stop codons, respectively. The following primers were used: for the left 500-bp yelF homology arm, AmSpamyE and AmAVy6E, and for the right 500-bp yelF homology arm, AvAV6YX and AvAmSpamyE. The att site cassette (0.5 kb) was amplified from strain E. coli MG1655 and C600 (31) using the primers C600 and C601. The PCR product resulting from the three-step procedure was introduced into E. coli XLI1 blue/pKOPEGa using electroporation, and chloramphenicol-resistant dilution mutants produced by allelic exchange were selected at 37°C to eliminate the thermoresistant plasmid pKOPEGa. Correct chromosomal insertion was checked by PCR amplification using the cat primers C602 and C603 in combination with AvAmSpamyE and AmAVy6YX, respectively. The yelF:cat mutation was then introduced in strain EHEC17 (pKOPEGa) using PI transduction.

DNA manipulations

B. henselae chromosomal DNA was isolated using the Wizard Genomic DNA purification kit (Promega). Small-scale plasmid DNA preparation was performed by using a Qiagen Spin Miniprep kit (Qiagen). Restriction endonuclease digestions, and ligation were carried out according to the manufacturer’s recommendations. DNA fragments were amplified in a Hybaid PCR thermocycler using Phusion DNA polymerase (Finnzymes). Nucleotide sequencing was performed by Eurofins MWG Operon. Purification of DNA fragments from PCR reaction, restriction reaction, and agarose gels were performed using Macherey-Nagel Nucleospin® Extract II kit.

Construction of a recombinant vector expressing HemS from B. henselae

The coding region of hemS was amplified by PCR from the B. henselae chromosomal DNA using primers hemS-Shamont, containing NheI restriction site and allowing addition of a HindIII tagging at the N-terminus part of the protein, and primers hemS-Hampl containing a KpnI restriction site (Table 2). The 1070 bp PCR product was purified, digested with NheI and KpnI, and ligated with plasmid pBAD24, digested with NheI and KpnI, to give plasmid pBAD24:pNS2TrcHemS. Ligation mixture was introduced in E. coli strain XL1 blue, using calcium chloride method. Transformants were screened using PCR method with hemS-Shamont and hemS-Hampl primers. Six PCR positive clones were then sequenced.

Constructions of the vector for decreasing HemS amount in B. henselae

The coding region of hemS was amplified by PCR from the B. henselae chromosomal DNA using primers hemS-Santisensent, containing a BamHI restriction site, and hemS-Santisensent containing an Xhol restriction site (Table 2). The 1014 bp PCR product was purified, digested with BamHI and Xhol and then ligated with plasmid pNS2Trc digested with BamHI and Xhol to give pNS2TrcHemSHamont. Ligation mixtures were introduced in E. coli strain XL1 blue using calcium chloride method. Transformants were screened, using PCR method with hemS-Santisensent and hemS-Santisensent primers. Six PCR positive clones were then sequenced.

Expression and purification of HemS-His tagged protein

Scale: pJH33 (pBAD24:pNS2TrcHemS) was grown overnight at 37°C in LB medium containing 50 μg/ml Ampicillin. One liter of LB medium, containing 50 μg/ml Ampicillin, was inoculated at OD600 of 0.05 with the overnight culture. Bacteria were grown at 37°C to an OD600 of about 0.5. Ammonium at 0.2% final concentration was added and bacteria grown for an additional 4 h at 37°C. Bacteria were harvested by centrifugation for 10 min at 3000 g at 4°C, and the pellet was suspended in 20 ml binding buffer (50 mM Tris-HCl pH 8.0, 25 mM Mg0.6, 10 mM imidazole, 0.05%Tricine). Lysis of bacteria was obtained by sonication (7 s sonication followed by a 3 s pause) during 30 min. The suspension was then centrifuged at 13,000 g for 3 min at 4°C. The supernatant containing the soluble fraction was mixed with 500 μl of Ni-agarose beads (Qiagen), previously pre-equilibrated with the binding buffer, and the mixture was incubated 1 hour with gentle shaking at 4°C and purified following the manufacturer’s protocol. Purified protein was dialyzed twice against a buffer containing 50 mM Tris-HCl to eliminate any residual imidazole. The protein was estimated to be >95% pure through SDS gel electrophoresis and was stable for several months when kept at -20°C with 20% glycerol.

Heme binding assay

After migration in SDS-PAGE, the heme binding ability of HemS was investigated according to the protocol of Vargas (32). Briefly, samples were mixed with loading buffer in which no EDTT
Table 2. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Organism</th>
<th>Sequence</th>
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<td>hemS</td>
<td>B. subtilis</td>
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<td>hemSaand</td>
<td>B. subtilis</td>
<td>5’TCCCGTCGATACAGAGGAAACACCTGATCATCACTACATGATCATGACGGCAAT 3’</td>
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<td>B. subtilis</td>
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<td>Cat</td>
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<td>5’ATTCTCGATACAGAGGAAACACCTGATCATCACTACATGATCATGACGGCAAT 3’</td>
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<tr>
<td>Cat</td>
<td>Cat</td>
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<td>5’ATTCTCGATACAGAGGAAACACCTGATCATCACTACATGATCATGACGGCAAT 3’</td>
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<td>B. subtilis</td>
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Absorption spectroscopy

HemS binding assays also were carried out using absorption spectroscopy method. HemS and hem mine were respectively diluted to 100 μM and 250 μM in Tris-HCl 50 mM pH 8.0. Allophe of hemine (raising hemine concentration from 1 μM to 20 μM final concentration) were successfully added into the cuvette containing 100 μM of 10 μM HemS. Absorbance spectras from 300 nm to 700 nm were recorded 5 mins after each addition a n a 2000 nanometer spectrophotometer. Experiments were performed in triplicate.

Reaion of HemS-hemine complex with NADPH-cytochrome P450 reductase

The reaction was performed according to Zhou et al [35] and Sako et al [19]. Human cytochrome P450 oxidoreductase (Sigma-Aldrich) was added to the HemS-hemine complex (10 μM) at ratio of reductase to HemS equal to 5:1 in a final volume of 100 μl 50 mM Tris-HCl (pH 8.0). Initiation of the reaction was carried out by the addition of NADPH in 10 mM increments to a final concentration of 100 μM. The spectral changes between 300 and 700 nm were monitored after each addition. Experiments were performed in triplicate.

Reaion of HemS-hemine complex with ascorbate

Ascorbic acid dependent degradation of hemine was monitored spectrophotometrically as previously described [35]. HemS-hemine complex (19 μM) in 50 mM Tris-HCl (pH 8.0) was incubated with ascorbic acid (10 mM), and the spectral changes between 300 and 700 nm were recorded every 1 min. Experiments were performed in triplicate.

Antibodies preparation

250 μl of an emulsion containing purified HemS (10 μg), B. subtilis (10 μg) adherent (Sepic France Farm) (10 μl) and complete with NaCl 0.9% were inoculated twice (with 1 month interval) by subcutaneous route in C57Bl6 mice (Charles River). Two weeks after the second inoculation, 250 μl blood samples were collected each 3 week using retro orbital bleeding method. Blood samples were centrifuged twice (5,000 rpm, 5 min) and sera were stored at −20°C. Before use, unspecific antibodies were removed by incubating the immune serum with E. coli cell extract 1 hour at 4°C and centrifugation 10 min at 10,000 rpm. The supernatant was then used as serum.

Protein analysis by Electrophoresis

Proteins were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis [56], followed by Coomassie blue staining.

Immunoblot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting for detecting the decrease expression of HemS in B. subtilis were performed as follows: B. subtilis (pS2Tec) and B. subtilis (pSSTec+hemS 3A) were harvested after 5 days of growth on CBA plates. Proteins contained in 20 μg of each sample were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C Extra, GE Healthcare) according to Towbin et al [35]. Non-specific binding sites were blocked with 5% skim milk in TBS-Tween 2000 (0.05%). The immunoblot was probed with polyclonal mouse sera raised against recombinant HemS (1:2000), followed by a 1:10,000 dilution of a rabbit anti-mouse IgG alkaline phosphatase-conjugated secondary antibody (Sigma ref. A4312). The binding of antibodies to HemS was revealed using chemiluminescence reagents BCIP/NBT solution following the manufacturer’s instructions (Sigma).

Protein assay

The concentration of the protein was determined by BCA Assay protein Quantification Kit (ThermoFisher).
Statistical analysis

Data was expressed by mean ± standard errors of the means. The statistical analysis was performed using GraphPad Prism 5 software for Windows. Statistical significance of the data was assessed by use of the Student’s t-test. A value of P<0.05 was considered significant.

Results

HemS from B. henselae is able to complement E. coli mutants impaired in iron release from heme

Bartonella genomes contain a gene encoding for HemS or HemU that could likely be involved in the release of iron from heme. These proteins, which are 341 AA to 347 AA in size, share 72% to 77% identity. Searching for homologues of these proteins in bacterial genomes showed that these proteins share homology with numerous polypeptides annotated as heme degrading or heme trafficking enzymes. When searching for homologues with functionally characterized heme degrading enzymes and heme trafficking enzymes, HemS/HemU from B. henselae shared 55% to 62% identity with some of these polypeptides (Fig. 1). Analysis of the identical regions did not allow predicting that HemS/HemU from B. henselae were heme degrading enzymes or heme trafficking proteins. To attempt defining the HemS activity from B. henselae, its structural gene was amplified using a forward primer designed to add a 6XHis-tag at its N-terminus, and cloned in plasmid pBAD24. The recombinant plasmid pBAD24::hemS was introduced in E. coli strain F21028::pBAD24::hemS (pBAD::hemS) to check for complementation ability. This E. coli strain, similarly to strain F1927 (pAM::AraR), which cannot grow on an iron depleted medium since it is impaired in catechol oxidase biosynthesis ([37], when heme is added on an iron depleted medium, strain F1927 (pAM::AraR) can grow, due to the presence of the HsdR heme transporter from Shewanella marcescens contained in plasmid pMMoK (30). Heme is transported through the outer membrane by HsdR, and the deacetylation activity of HsdR and YieN allows the release of iron required for growth [24]. In E. coli strain F21028::pBAD24::hemS::pBAD::hemS (pBAD::hemS), the deacetylation activity is absent and consequently, heme dependent growth was abolished. When hemS from B. henselae, was expressed in strain F21028 (pBAD24::yieN::pBAD::hemS::pBAD::hemS) it restored the heme dependent growth on an iron depleted medium (Fig. 2). This result clearly indicates that HemS activity allows the release of iron from heme in vivo.

Expression and Purification of HemS His-tagged protein

To produce and purify the recombinant HemS protein from E. coli, plasmid pBAD24::hemS was introduced into strain JP313. To check for amounts of HemS in E. coli strain JP313, SDS gel electrophoresis (PAGE) was used to compare protein extracts of the strain JP313 (pBAD24::hemS::pBAD::hemS) and JP313 (pBAD24). A supplementary sample band of 40 KDa was observed only for the strain containing the recombinant plasmid pBAD24::hemS::pBAD::hemS grown in the presence of arabinose (data not shown). In strain JP313 (pBAD24::hemS::pBAD::hemS), grown in the presence of 0.2% arabinose, His-tagged HemS was expressed as a soluble protein. The protein was purified by Ni-nitroaffine affinity chromatography, yielding a distinct protein (59% pure) migrating at ~40 KDa as a distinct band on SDS-PAGE (Fig. S1). This size was found to be in accordance with the predicted size.

HemS can bind heme specifically in vitro

To test if pure HemS can bind heme specifically in vitro, a standard method, which has already used for detected heme binding of cytochrome C, was used. Pure HemS (4 µg) and BSA (5 µg) were run in two SDS-PAGE gels. One gel was stained with coomassie brilliant blue R (Fig. 3A). Another gel was transferred to a nitrocellulose filter to perform heme blotting and subsequent detection by ECL method. Pure HemS was able to bind heme (Fig. 3B). The latter result underlines the specificity of the HemS heme binding. Also, a crude extract obtained from strain JP313 pBAD24::hemS (data not shown) Taken together, these data demonstrate that HemS from B. henselae is able to bind heme in vitro.

HemS binds heme with a 1:1 stoichiometry

The binding of heme by HemS was also assessed spectrophotometrically. The spectral properties of heme changed when bound to a protein giving a specific Soret band. The spectrum of HemS-heme complex showed a peak at 411 nm (Fig. 4A). Titration of 10 µM HemS solution with increasing amount of heme was used to check for the HemS heme binding properties (Fig. 4B). The absorption at 411 nm increase leveled off at about 10 µM heme, showing a 1:1 stoichiometry of heme to HemS (Fig. 4B).

HemS can degrade heme in vitro

Various monochrome-protein complexes like heme oxygenases (35), and other biochemically characterized heme degrading enzymes (60), 269 were shown to degrade heme in vitro in the presence of electron donorn like, ascorbate or Cytochrome NADPH-cytochrome P450 reductase. We thus tested whether HemS was able to degrade heme in vitro first in the presence of ascorbate or Cytochrome NADPH-cytochrome P450 reductase. The HemS-heme complex was incubated with ascorbic acid (5 mM), and the spectral changes between 300 and 700 nm were recorded every 1 min. As shown in Fig. 3A, the disappearance of the Soret band was nearly complete 3 min after addition of ascorbate. In the absence of ascorbate, the Soret band was stable at least for more than 30 min (data not shown). In a second experiment, HemS dependent heme degradation was measured spectrophotometrically using human cytochrome P450-NADPH as the electron donor. Cytochrome P450 reductase was added to HemS-heme complex and heme degradation was initiated by adding NADPH and the spectral changes between 300 and 700 nm were recorded. The Soret band decreased after each addition of NADPH, and disappeared after addition of 100 µM NADPH (Fig. 5B). The Soret band of HemS-heme complex did not change if NADPH or cytochrome P450 were added alone into the mixture (data not shown). Heme degradation did not occur in the mixture containing only cytochrome P450-heme-NADPH (data not shown). All these data demonstrate that HemS is able to degrade heme in vitro through an enzymatic dependent process that requires addition of electron donors.

The hemS knockdown increased B. henselae sensitivity to hydrogen peroxide

As shown above, when expressed in E. coli, HemS was able to provoke the release of iron from heme. Biochemical results strengthen this observation. The question arises whether HemS activity is required for B. henselae. According to the conclusions provided by analysis of the genome content of B. henselae, we
null
Figure 2. Functional complementation of the E. coli mutants impaired in iron release from heme. E. coli strains FB3.27 pAMhOR (pBAD34) (A), FB3.27 pBAD34 CmlP (pAMhOR) (pBAD34) (B) and FB3.27 pBAD34 CmlP (pAMhOR) (pBAD34-hemS) (C) were tested for the use of heme as an iron source on iron depleted M63 0.3% Glu 0.4%, Ada 0.2%, Dib 70 µM Spc 0.5 µM. Growth around the walls containing 1 μM, 5 μM, 10 μM, or 50 μM Hb were performed as described in "Materials and Methods". Growth around the walls was assayed by visual turbidity in the agar. These pictures were taken after 48 hours of growth at 37°C. Experiment was repeated three times. A representative result is presented. doi:10.1371/journal.pone.0037630.g002

Figure 3. HemS heme blunting. After SDS gel electrophoresis, one gel was stained with comassie brilliant blue R. Another gel was transferred to a nitrocellulose filter to do heme blunting and detected by ECL. (A) Coomassie blue staining. Lane 1, 5 μg BSA; Lane 2, 4 μg HemS. (B) Heme binding. Lane 1, 5 μg BSA; Lane 2, 4 μg HemS. Experiment was performed in triplicate and a single representative experiment is presented. doi:10.1371/journal.pone.0037630.g003

was decreased in strain B. lusolus (pNSZTcromS) as compared with strain B. lusolus (pNSZTc). Using western blot experiments performed with anti-HemS mouse antibodies. As seen in Fig. 6B, the level of HemS in strain B. lusolus (pNSZTcromS) was lower than in strain B. lusolus (pNSZTc). Strains B. lusolus (pNSZTc) and B. lusolus (pNSZTcromS) were then tested for growth on both CBA plates and in Schneidere's medium. Our results showed that the knock down of HemS in strain B. lusolus (pNSZTcromS) did not significantly decrease its growth ability. Thus, the level of HemS in B. lusolus (pNSZTcromS) is sufficient to support a normal growth on CBA plates and in Schneidere's medium.

HemS can degrade heme and thus can prevent its accumulation in the cytoplasm. Consequently, HemS could prevent the deleterious effects of heme accumulation in the cytoplasm. Therefore, a decrease of HemS level in the cytoplasm could induce heme accumulation and lead to a higher sensitivity to oxidative stress. Such protecting activity against heme toxicity was demonstrated for ShgS that was evidenced to promote heme utilization in Shigella dysenteriae. In this bacterium, shgS disruption did not increase sensitivity to hydrogen peroxide [43]. Shigella dysenteriae genome (http://www.ncbi.nlm.nih.gov/Genome?term=Shigella%20dysenteriae%20strains) contains genes encoding for KatG, AhpC, and AhpF. AhpC, AlkR, Hydroperoxide Reductase, that are involved in heme peroxidative degradation [44]. Also, OxyR that regulates the response to H$_2$O$_2$-induced oxidative stress is present [44]. Analysis of the Bartonella genomes indicated that these bacteria would not be able to face oxidative stress using canonical pathways since many genes involved in oxidative stress response are not present. Homologs of genes encoding for hydrogen peroxide degrading enzymes like KatG and KatE, AlpG, and AlpF, AlkR, AlkC, Hydroperoxide Reductase, Dps and OxyR are absent in Bartonella genomes (http://www.ncbi.nlm.nih.gov/protein?term=alkB%20Bartonella%20strains). Nevertheless, a previous report showed that Bartonella henselae was able to remain quiescent after a 30 minutes exposure to 1 mM H$_2$O$_2$ [45]. This latter result strongly suggested that cellular activities allow this bacterium to face oxidative stress generated by exposure to H$_2$O$_2$. HemS, that is involved in heme disruption, could be an actor of this defense against oxidative stress. Therefore, we tested the effect of hemS knock down on the ability of B. lusolus to face a 30 minutes exposure to 1 mM and 10 mM hydrogen peroxide.
After exposure to 1 mM H$_2$O$_2$, the survival was about 50% for both strains. After exposure to 10 mM H$_2$O$_2$, the survival was about 20% for the control strain B. subtilis (pNS741). With strain B. subtilis (pNS741:HemS), the survival was decreased by threefold (Fig. 7). This result showed that lowering HemS levels in B. subtilis enhanced its sensitivity to H$_2$O$_2$.

**Discussion**

In this report, we investigated the function of HemS in B. subtilis. Homologs of this protein are present in all the *Bacillus* genomes that have been sequenced. This underlines the importance of its function for these *Alphaproteobacteria*. Predicting an important role for HemS in *Bacillus* is mainly driven by the fact that these bacteria use heme as an iron source. The use of heme as iron source requires its transport through the outer membrane, the periplasm, and the inner membrane. The release of iron from heme inside the cytoplasm requires an enzymic activity [15]. An iron release activity from heme was demonstrated in many bacteria, using physiological tests. Some of these activities were also biochemically demonstrated and characterized. In most cases, the release of heme was the consequence of the protoheme ring degradation [15]. Heme oxygenases first characterized in *Corynebacterium diphtheriae* [39], releases heme by degrading heme to forming 3,5-dihydroxybenzoate, CO and free iron. Other heme oxygenases that degrade heme and release iron in the presence of a reducing agent were characterized first, in *Bacillus anthracis* [19]. Members of these two classes of heme oxygenases were identified in various Gram-negative and Gram-positive bacteria.

**Figure 5.** HemS-dependent degradation of heme. (A): 10 mM final concentration of ascorbate was added to the HemS-heme complex (10 µM). The spectral changes from 300–700 nm were recorded every 1 min. (B): Cytochrome P450 reductase was added to 10 µM HemS-heme complex with a 0.95 molar ratio and heme degradation was initiated by adding NADPH 10 µM increments to a final concentration of 100 µM. The spectra were recorded from 300–700 nm after each addition. All experiments were performed in triplicate and a single representative experiment is presented.

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Finally, a demetallation activity was shown to be responsible for the release iron from heme in E. coli. In this latter case, the release of iron occurs without breakage of the tetrapyrryl skeleton [24]. For some other bacteria, like Yersinia enterocolitica, the heme degrading activity was demonstrated, but the reaction products were not characterized. This was the case for HemS from Yersinia enterocolitica [21] or CadS from E. coli O157:H7 [46]. Other proteins involved in the use of heme, and sharing significant sequence homology with heme degrading enzymes, were characterized in Yersinia pestis [47] or in Y. pestis p66 [48]. For HemS from B. lanceolata, both complementation abilities and biochemical assays show that HemS can degrade heme. Similarly with IdoG from Reseda nutans, purified HemS from B. lanceolata can degrade heme in the presence of a reducing agent. During the heme degradation in the presence of NADPH-cytochrome P450 reductase, a Soret band of the heme-HemS complex only decreased in intensity. This was not the case for HemoO from Clostridium botulinum since the Soret band wavelength of the heme-HemS complex also vary during the heme degradation in the presence of NADPH-cytochrome P450 reductase [30]. Heme degradation enzymes of the IdoG family were characterized in Azospirillum brasilense [16]. The heme degradation in Azospirillum brasilense produces biliverdin [20]. In Brucella melitensis the product of heme degradation by Bm10706 was not characterized [20]. Similarly with Bm10706 from Brucella melitensis [20], HemS from B. lanceolata and B. bistortae (data not shown) exhibits a Soret band at 411 nm in the presence of heme, thus suggesting a similar environment for heme. Our study however does not show the products, which would be useful to distinguish true free radicals from peroxide-coupled oxidation. HemS knockdown only provoked a slight slowdown growth effect when bacteria were grown on blood plates. When bacteria were grown in Schneider medium, no growing effect was observed.

The more striking effect related to HemS knockdown was to decrease the ability of B. lanceolata to sustain exposure to hydrogen peroxide. This result suggests that HemS could be an actor of the pathway used by B. lanceolata to face oxidative stress. Since classical pathways used to face oxidative stress characterized in E. coli are not present in B. lanceolata, we hypothesized that B. lanceolata develop alternative strategies to face oxidative stress encountered in erythrocytes or macrophages and during vesculation by the ceciles Chromatophilus, fibroblasts, and in the ileal gut, HB and heme concentration are high [16]. Thus, HemS may be proposed to have a dual role for B. lanceolata, since its heme degrading activity allows iron supplying and the control of heme homeostasis.

Supporting Information

Figure S1 Purification of H5-tagged HemS. Purification of the His-tagged HemS protein was achieved by Ni-agarose purification followed by gel filtration. Purified protein (5 μg) was
run on 12% sodium SDS-PAGE and stained with Coomassie blue. Like MIV, molecular weight markers. Line 1: purified Hem5 (115).

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Author Contributions
Conceived and designed the experiments: FH. Performed the experiments: FH. Analyzed the data: FH. Contributed reagents/materials/analysis tools: FH. Wrote the paper: FH. FH.

References

**Conclusion**

Although one heme acquisition system, that of HutA of *B. quintana*, has been characterized as a heme transporter (Parrow et al., 2009), the fate of heme in the cytoplasm and the mechanisms used for iron release are unknown. We are seeking to identify the function of HemS as a heme-degrading protein for the following reasons:

1) Heme is the sole iron source for *Bartonella*;
2) HemS is located in the Hut locus devoted to heme uptake;
3) Numerous functions of HemS homologues in other bacterial species contribute to heme trafficking, heme degrading and DNA binding.

Recently, identification of YfeX and EfeB in *E. coli K12* as a protein for releasing iron from heme provided a research model for analyzing the function of HemS *in vivo* (Letoffe et al., 2009). Like heme oxygenase PigA of *P. aeruginosa*, HemS of *B. henselae* is able to complement the *E. coli K12 yfeX efeB* double mutant, suggesting that HemS is able to release iron from heme *in vivo*. Further investigation *in vitro* in the presence of a suitable electron donor also showed that HemS exhibited a heme-degrading activity, as observed spectrophotometrically. However, we failed to detect the product of heme degradation: biliverdin produced by heme oxygenase or PPIX produced by YfeX or EfeB. Moreover, overexpression of HemS in *E. coli* did not produce a colored bacterial culture as a result of biliverdin formation from degradation of heme by other heme oxygenases. We therefore hypothesized that HemS degrades heme to produce staphylobilin produced by the IsdG family protein. This hypothesis was supported by the fact that IsdG orthologs have been characterized in the *Alphaproteobacteria Bradyrhizobium japonicum* and *Brucella melitentis* (Puri
and O'Brian, 2006). However, HemS of *Bartonella* do not have any similarity with the heme-degrading protein of *B. melitentis* or *B. japonicum*. Homologues of *B. henselae* HemS are mainly present in *Bartonella* species with high identity (70%-79%) and other α-, β- and γ-proteobacteria with low identity (40%-48%). Thus, it is also possible that HemS-degraded heme produces uncharacterized compounds. Further experiments will seek to identify products of heme degradation by HemS and to reveal the functions of products in *Bartonella*.

To search for the function of HemS in *B. henselae*, we first tried to knock out HemS. Although many attempts were made, we failed to knock it out. One possibility is that HemS is essential for *Bartonella*, a hypothesis supported by the fact that heme is the only source of iron and that the activity of HemS is crucial in this process. However, knockdown HemS of *B. henselae* had only a slight effect upon growth. One explanation is that a low expression level of HemS provides enough iron for growth. The most striking effect of HemS knockdown is a decrease in *B. henselae* ability to face exposure to hydrogen peroxide. HemS could be involved in the pathway used by *Bartonella* to face oxidative stress. This is consistent with the observation that many genes involved in the classical pathway used by *E. coli* to face oxidative stress are not present in *Bartonella* genomes. We hypothesized that HemS has peroxidase activity responsible for iron release from heme, or that the product of heme degradation produced by HemS acts as an antioxidant, like the products of vertebrate heme catabolism. For example, in eukaryotes, biliverdin is transformed into bilirubin to protect cells against oxidative damage. Thus, we proposed that HemS
plays a dual role in heme degrading and the oxidative stress response in *Bartonella*.

This work established the role of *B. henselae* HemS in heme iron utilization and in confronting oxidative stress, processes required for survival of *Bartonella* and its infection cycle. Thus, our results should help to develop a novel target for anti-infection and anti-transmission strategies in *Bartonella*. 
PAPER TWO

Heme binding proteins of *Bartonella henselae* are required when undergoing oxidative stress during cell and flea invasion

*(Submitted to PLoS One)*

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State of the art concerning outer membrane heme binding proteins of *Bartonella*

1. Outer membrane heme binding proteins in Gram-negative bacteria

Outer membrane heme binding proteins have been found and characterized in some Gram-negative bacteria, including *Haemophilus influenzae* (Lee, 1992), *Neisseria meningitidis* (Lee, 1994), *Brucella ovis* (Delpino et al., 2006), *Porphyromonas gingivalis* (Olczak et al., 2008) and *Bartonella quintana* (Carroll et al., 2000). Lee (1992, 1994) identified an outer membrane heme binding protein with a molecular weight of 39.5 kDa in *H. influenzae* (Lee, 1992) and two outer membrane heme binding proteins of molecular masses 97 and 50 kDa in *N. meningitides* using heme affinity chromatography (Lee, 1994). These proteins were detected only under conditions of iron limitation (Lee, 1994). Later, it was shown that a monoclonal antibody against the 97 kDa heme binding protein inhibited heme-dependent growth of *N. gonorrhoeae* in a concentration-dependent manner (Lee and Levesque, 1997). These outer membrane proteins from *H. influenzae* and *Neisseria* have not been further investigated. In *B. ovis*, Omp31, a 31 kDa outer membrane protein, was identified as a heme binding protein through heme blotting (Delpino et al., 2006). *E. coli* expressing recombinant Omp31 exhibited a heme binding phenotype, suggesting that recombinant Omp31 exhibited an exposed surface when expressed in *E. coli* (Delpino et al., 2006). Incubation of *B. ovis* with antibodies directed against Omp31 decreased its heme binding activity. This suggested that Omp31 plays an important role in the heme binding capacity of *B. ovis* (Delpino et al., 2006). The expression level of Omp31 was increased by iron limitation. However, this increase in expression
did not occur when the iron-depleted medium was supplemented with heme, suggesting that Omp31 plays a role in heme utilization. Later, it was shown that the Omp31 mutant was more susceptible to hydrogen peroxide than its parental wild type strain (Caro-Hernandez et al., 2007). In *P. gingivalis*, HmuY was identified as a membrane-associated heme binding lipoprotein (Olczak et al., 2006; Olczak et al., 2008; Wojtowicz et al., 2009). The *hmuY* gene encodes for a 23 kDa protein without significant similarity to any other protein (Wojtowicz et al., 2009). HmuY is present as a homodimer under heme-depleted conditions, but as a tetramer under heme-repleted conditions (Olczak et al., 2008). It was proposed that the tetramer form would protect heme from host scavengers (Wojtowicz et al., 2009). Gene *hmuY* is located in one operon containing *hmuR*, which encodes for an outer membrane heme receptor and four uncharacterized genes (Olczak et al., 2008). Under heme-restricted conditions, expression of *hmuY* is increased (Olczak et al., 2008). The *hmuY* mutant was shown to grow more slowly and to bind lower amounts of heme and hemoglobin than the wild type strain (Olczak et al., 2008). Complementation of an *E. coli hemA* mutation showed that only cells expressing both HmuY and HmuR can grow in the presence of heme (Olczak et al., 2008). It was proposed that HmuY binds heme at the cell surface as an initial heme receptor and heme is then passed to the TonB-dependent outer membrane heme receptor HmuR (Olczak et al., 2008). This mechanism was further investigated by determination of the crystal structure of HmuY loaded with heme (Wojtowicz et al., 2009). Once bound by HmuR, heme would be transported into the periplasm.
2. The finding of heme binding proteins in *Bartonella* and their structure

*Bartonellae* genomes encode for 3 to 5 heme binding proteins (Minnick and Battisti, 2009) presumed to be involved in the heme utilization process. Heme binding proteins of *Bartonella* are a group of porin-like outer membrane proteins that contain a β-barrel structure and lack similarity with known heme receptors (Minnick et al., 2003). Heme binding protein A was the first such binding protein to be identified in *B. quintana* based on the heme binding phenotype (Carroll et al., 2000). Subsequently, using sequence analysis, the same group identified four additional heme binding proteins in *B. quintana* (Minnick et al., 2003). In *B. quintana*, genes *hbpCAB* form a cluster, *hbpD* and *hbpE* are located elsewhere in the chromosome (Minnick et al., 2003). At present, there are three *hbps* in *B. bacilliformis* and four in *B. henselae* (Harms and Dehio, 2012). Multiple sequence alignment of the heme binding proteins of *B. henselae* reveals a high degree of amino acid sequence conservation (Fig. 1). Heme binding protein family members of *B. henselae* share 49-56% amino acid identity. Each protein contains a predicted signal peptide and a terminal phenylalanine.

*hbpA, hbpC* and *hbpD* genes are 840, 831, and 825 bp in length, respectively. They encode for immature proteins with 29.9 kDa, 29.956 kDa and 30.2 kDa masses, respectively. The *hbpB* gene is 1, 176 bp in length due to a ~300 bp insert near the center of its open reading frame. The *hbpB* gene encodes for an immature protein of 41.4 kDa. Secondary structure predictions suggested that heme binding protein family members are β-barrels located in the outer membrane and contain eight transmembrane domains (Fig. 1).
Fig 1. Multiple sequence alignment of the predicted proteins encoded by the \textit{hbp} gene family of \textit{B. henselae}. Identical residuals are shaded in black. Predicted $\beta$-strand transmembrane domains are boxed and numbered. Secretory signal sequence cleavage sites are indicated by an arrowhead.
The predicted heme binding protein three-dimensional model structure of *B. henselae* shows that HbpA, C and D exhibit a “pore-like” structure (Fig 2).

![HbpA](image1)

**HbpA**

![HbpB](image2)

**HbpB**

![HbpC](image3)

**HbpC**

![HbpD](image4)

**HbpD**

**Fig 2.** Three-dimensional models of *B. henselae* HbpA (region: 42aa-279aa), HbpB (region: 278aa-391aa), HbpC (region: 42aa-276aa) and HbpD (region: 41aa-274aa) were predicted by the ModBase Model.

BLAST search revealed that the closest homologs of heme binding proteins of *B. henselae* in the databases are *Brucella* OMP31 (Vizcaino et al., 1996) and *A. tumefaciens* OMP25 (Goodner et al., 2001). Sequence identities of HbpA with other characterized heme binding proteins range from 30% (OMP25) to 32% (*Brucella* OMP31). OMP25 of *A. tumefaciens* was identified as an immunogenic surface protein.
(Goodner et al., 2001). However, the functions of these proteins have not been fully elucidated (Goodner et al., 2001). BLASTp searches with heme binding proteins also generated numerous “hits” for *Neisseria* opacity (Opa), although the overall sequence identity value between heme binding proteins and Opa is only about 25%. It was shown that Opa plays a critical role in *Neisseria* adherence and entry into the host epithelial cell (Weel et al., 1991). In addition to adherence, Opa plays a role in immunomodulation, including inhibition of T-lymphocyte activation and proliferation and B-cell antibody production (Sadarangani et al., 2011; van Putten et al., 1998).

### 3. Heme binding proteins cannot transport heme

Recombinant HbpA of *B. quintana* was shown to be able to bind heme *in vitro* (Carroll et al., 2000). However, it did not confer a heme binding phenotype *in vivo* when expressed in *E. coli* (Carroll et al., 2000). The very low expression level of HbpA in *E. coli* and its misfolding were hypothesized to explain this result (Carroll et al., 2000). Pretreatment of *B. quintana* with HbpA antibody inhibited heme binding (Carroll et al., 2000). Subsequently, Zimmermann et al., (2003) identified a prominent heme binding protein Pap31 (HbpA), through a heme binding blot performed with membrane proteins from *B. henselae*. They showed that expressing Pap31 in an *E. coli* K12 *hemA* mutant strain restored its growth when heme was added at 30 μM and above (Zimmermann et al., 2003). Growth restoration was claimed to be related to heme transport activity of HbpA. However, the level of Pap31 was very low, since only the use of a monoclonal antibody enabled detecting this protein (Zimmermann et al., 2003). Moreover, heme binding ability and a β-barrel porin-like structure does not
equate with competence in directly transporting heme. Complementation assays were performed in liquid medium and at high heme concentrations. Under such conditions, the onset of a spontaneous mutant could explain the growth observed (Olczak et al., 2008). Thus, the activity of HbpA as a heme transporter was questioned by other authors. Recently, it was claimed that HbpA of B. quintana was not able to complement the E. coli hemA mutant strain in the presence of heme (Minnick and Battisti, 2009). Complementation assays using the E. coli hemA mutant strain on solid medium in the presence of different heme concentrations also showed that HbpA cannot transport heme (F. Biville personal communication). Moreover, Bartonella genomes encode for a complete heme uptake system (see chapter 2). The functions of heme binding proteins of Bartonella in the heme uptake process remain unknown, although many hypothetical functions have been discussed (Battisti et al., 2006; Battisti et al., 2007; Minnick and Battisti, 2009; Parrow et al., 2009; Roden et al., 2012). It was proposed that the putative role of heme binding proteins was to accumulate heme around the bacteria (Minnick and Battisti, 2009). First, this heme accumulation would facilitate the heme uptake process and serve as a heme reservoir (Battisti et al., 2006). Secondly, such heme accumulation around the bacteria might protect against oxidative stress produced during invasion of endothelial cells (Rydkina et al., 2002; Rydkina et al., 2010) or replication inside the arthropod gut (Graca-Souza et al., 2006) using its intrinsic peroxidase activity (Battisti et al., 2006). Finally, it was suggested that heme accumulation by heme binding proteins decreased the oxygen level around the bacteria similarly to different rhizobia (Battisti et al., 2006; Battisti et
al., 2007; O'Brian, 1996; Wittenberg et al., 1986). No experimental data have been obtained to validate these hypotheses.

4. Heme binding proteins and interactions with the infected host

Outer membrane proteins of Gram-negative bacteria play an important role in the interaction between bacteria and the infected host. Some of them are prime targets of the host humoral response (Chenoweth et al., 2004a). Heme binding proteins (HbpA, HbpC, HbpD and HbpE) of *B. quintana* and heme binding proteins (HbpA, HbpC, HbpD) of *B. henselae* have been identified as predominant outer membrane proteins (Boonjakuakul et al., 2007; Chenoweth et al., 2004a; Eberhardt et al., 2009; Li et al., 2011; Rhomberg et al., 2004; Roden et al., 2012; Saisongkorh et al., 2010). Previously, Taye et al. (2005) had identified Pap31 (HbpA) of *B. bacilliformis* as a dominant antigen, using western blot analysis of patient sera after whole cell lysate separated on a two-dimensional gel (Taye et al., 2005). ELISA assay showed that recombinant Pap31 exhibited both high sensitivity and specificity with patient serum, suggesting that recombinant Pap31 could be used for diagnosis of *B. bacilliformis* infection (Taye et al., 2005). Later, it was shown in western blot that recombinant Pap31 (HbpA) of *B. henselae* can be recognized by rabbit anti-live *B. henselae* serum. This suggested that Pap31 (HbpA) of *B. henselae* provokes an immune reaction in rabbits (Dabo et al., 2006a). Likewise, Boonjakuakul et al. (2007) identified the predominant *B. quintana* outer membrane antigen using two-dimensional immunoblotting of sera of *B. quintana*-infected patients. Among the outer membrane antigens, HbpE was most frequently recognized by patient sera, implying that HbpE of *B. quintana* may be a
candidate diagnostic antigen (Boonjakuakul et al., 2007). Recently, Saisongkorh et al. (2010) identified candidate serodiagnostic proteins for bartonellosis caused by *B. henselae*. It was shown that Pap31 (HbpA) was immunoreactive with high antibody titer infective endocarditis (IE) sera, but not with low antibody titer cat scratch disease (CSD) sera (Saisongkorh et al., 2010).

However, discrepancies exist in results. Chenoweth et al. (2004), via two-dimensional immunoblotting, identified *B. henselae* outer membrane proteins that reacted with infected cat sera. They did not find any heme binding protein that reacted with the cat sera (Chenoweth et al., 2004a). Consistent with this result, other authors failed to detect antibodies against heme binding proteins of *B. henselae* with patient sera (Eberhardt et al., 2009). Recently, using a large set of cat sera and protein microarray, no antibodies were detected against heme binding proteins of *B. henselae* (Vigil et al., 2010). These inconsistent results might be explained as follows: 1) *Bartonella* is well adapted to invasion and long-term survival in its natural host (Chenoweth et al., 2004a) and thus does not provoke a predominant immune reaction in the reservoir host; 2) the different experimental procedures used, including various means of preparing the outer membrane, along with the different sera titers, may have led to this discrepancy (Saisongkorh et al., 2010).

In addition to provoking an immune reaction in the infected host, outer membrane proteins also mediate important steps in pathogenesis, such as adhesion, invasion, intracellular survival and replication (Dehio, 2004). HbpB of *B. tribocorum* was found to be essential for establishing bacteremia in a *B. tribocorum* rat model.
(Saenz et al., 2007), suggesting that HbpB is an important factor in pathogenesis in bartonellosis. Initiation of *Bartonella* infection requires a wound to enter the skin. Extracellular matrix (ECM) molecules were reported to be involved in this process as a target of *Bartonella* infection (Dabo et al., 2006b). It has been demonstrated that adhesion to ECM molecules, such as fibronectin (Fn), collagen (Cn) and heparin-sulfate-containing proteoglycans, is a critical step in the process of bacterial invasion into the host cell, particularly for wound-associated infection (Dziewanowska et al., 1999; Joh et al., 1999; Unkmeir et al., 2002). Dabo et al., (2006b) showed that *B. henselae* can bind to ECM proteins (Fn, Cn), and pretreatment of endothelial cells with anti-Fn antibodies resulted in a significant reduction in bacterial adherence, suggesting that Fn were exposed at the surface of endothelial cells (Dabo et al., 2006b). Furthermore, they identified major Fn binding proteins in the *B. henselae* outer membrane, including Omp89, Omp43 and Pap31 (HbpA) (Dabo et al., 2006b). Later, that same group showed that Pap31 (HbpA) can bind endothelial cells in a dose-dependent manner. Moreover, adherence of *B. henselae* to endothelial cells was inhibited by anti-Pap31 antibodies in a dose-dependent manner, indicating that *Bartonella* adhesion to endothelial cell involves an interaction with Pap31 (HbpA)-Fn (Dabo et al., 2006a). After entry into endothelial cells, *B. henselae* was shown to survive in a specialized non-endocytic membrane-bound vacuole (Kyme et al., 2005), and this structure was claimed to prevent bacterial fusion with lysosomes of endothelial cells (Kyme et al., 2005). However, dead *B. henselae* were unable to delay fusion with lysosomes, suggesting that bacterial viability is required
for intracellular trafficking of *B. henselae* (Kyme et al., 2005). Screening of the *B. henselae* transposon mutant library for mutants that cannot delay lysosomal fusion demonstrated that *hbpD* disruption significantly reduced viability inside endothelial cells. The latter result suggested that the *hbpD* mutant was unable to escape phagosome maturation (Kyme et al., 2005).

5. Regulation of expression of heme binding proteins in *Bartonella*

To survive and proliferate, *Bartonella* must confront various environments in the host and vector. In mammals, free heme is rare (Baker et al., 2003) and the blood oxygen concentration is low (5% compared to 21% in the atmosphere) (Battisti et al., 2006). In contrast, in arthropod vectors, there exists a toxic heme level in the arthropod gut (Graca-Souza et al., 2006; Oliveira et al., 1999; Vaughan and Azad, 1993) and the oxygen concentration is high. Regulation of expression of heme binding proteins encoding genes was thus investigated based on conditions mimicking the natural host and vector for *B. quintana* (Battisti et al., 2006). Heme binding proteins were divided into two subgroups based on their regulatory pattern under conditions of varying heme concentrations, temperature and oxygen concentrations. The first group contained *hbpB* and *hbpC*, overexpressed under conditions mimicking the arthropod environment (high heme concentration, 30°C) (Table 1). Strikingly, transcription of *hbpC* was increased 108-fold at 30°C compared to 37°C (Battisti et al., 2006). The transcription level of *hbpB* and *hbpC* was higher at high heme concentrations (2.5 to 5 mM). The authors suggested that HbpB and HbpC might play a critical role in an abundant heme environment such as that of the arthropod gut (Battisti et al., 2006).
The second group contained \textit{hbpA}, \textit{hbpD} and \textit{hbpE} that were overexpressed under conditions mimicking the mammal environment (low heme concentrations and 37°C) (Battisti et al., 2006) (Table 1). Transcription of \textit{hbpA}, \textit{hbpD} and \textit{hbpE} was increased at low heme concentrations (0.05 mM) at 37°C, whereas transcription of \textit{hbpB} and \textit{hbpC} remained relatively unchanged (Battisti et al., 2006). The authors proposed that \textit{hbpA}, \textit{hbpD} and \textit{hbpE} are required when the heme concentration is low, as in the mammal reservoir host (Battisti et al., 2006). Consistent with this subgroup division, the first subgroup of \textit{hbpB} and \textit{hbpC} was upregulated during infection of endothelial cells, while \textit{hbpA} and \textit{hbpD} were downregulated (Quebatte et al., 2010). Expression levels of all heme binding proteins decreased when the oxygen concentration decreased to bloodstream-like conditions (5%) (Battisti et al., 2006) (Table 1). Thus, it was hypothesized that heme binding proteins do not play an important role inside erythrocytes (Harms and Dehio, 2012).

Heme- or iron-related transcription regulators (Irr, Fur, RirA) (see chapter two) were examined for their effect on \textit{hbp} gene expression (Battisti et al., 2007). The expression profiles of these transcription factors were verified under “louse-like” (30°C, high heme) and human “bloodstream-like” (5% O\textsubscript{2}, low heme) conditions using qRT-PCR. Under “bloodstream-like” (5% O\textsubscript{2}, low heme) conditions, \textit{irr} and \textit{fur} expression was slightly increased, whereas \textit{rirA} expression slightly decreased (Table 1). Under “louse-like” (30°C, high heme) conditions, \textit{irr} expression decreased (Battisti et al., 2007). Transcription of \textit{fur} and \textit{rirA} could not be detected at 30°C, but a slight increase occurred under abundant heme conditions (Table 1). Finally, a
>5-fold decrease in *irr* expression was observed at 30°C compared to 37°C. The latter result suggested that Irr might repress *hbpC* expression (Battisti et al., 2007).

To further investigate the relationship between *irr* and the *hbp* family, an Irr-overexpressing *B. quintana* strain was constructed and the transcription level of the *hbp* family was evaluated under normal growth conditions (37°C, 21% CO₂, 0.15 mM heme). Irr overexpression in *B. quintana* led to repression of *hbpB* and *hbpC* expression and an increase in *hbpA, hbpD* and *hbpE* expression (Battisti et al., 2007). Since “blood-stream-like” stimuli (37°C, low heme) resulted in significant increases in *hbpA, hbpD* and *hbpE* expression levels (Battisti et al., 2006), it was suggested that the strain overexpressing Irr exhibits a “blood-stream-like” expression pattern of *hbp* genes (Battisti et al., 2007). The relationship between Irr and *hbp* expression was also studied under “louse-like” conditions (30°C, 21%CO₂) (Battisti et al., 2007).

**Table 1.** Expression regulation profiles of heme binding proteins, Irr, Fur and RirA of *B. quintana* at different concentrations of oxygen, temperature and heme used to for growth (Battisti et al., 2006; Battisti et al., 2007).

<table>
<thead>
<tr>
<th>Regulator</th>
<th>30°C Compared to 37°C (0.15 mM heme)</th>
<th>5% Compared to 21% O₂ (37°C, 0.15 mM heme)</th>
<th>0.05 mM Compared to 0.15 mM heme</th>
<th>5 mM Compared to 0.15 mM heme</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hbpA</em></td>
<td>No difference</td>
<td>Decreased</td>
<td>Elevated</td>
<td>No difference</td>
</tr>
<tr>
<td><em>hbpB</em></td>
<td>No difference</td>
<td>Decreased</td>
<td>No difference</td>
<td>Elevated</td>
</tr>
<tr>
<td><em>hbpC</em></td>
<td>Significantly elevated</td>
<td>Decreased</td>
<td>No difference</td>
<td>Elevated</td>
</tr>
<tr>
<td><em>hbpD</em></td>
<td>No difference</td>
<td>Decreased</td>
<td>Elevated</td>
<td>No difference</td>
</tr>
<tr>
<td><em>hbpE</em></td>
<td>No difference</td>
<td>Decreased</td>
<td>Elevated</td>
<td>No difference</td>
</tr>
<tr>
<td><em>irr</em></td>
<td>Significantly reduced</td>
<td>Slightly elevated</td>
<td>Slightly elevated</td>
<td>Slightly reduced</td>
</tr>
<tr>
<td><em>fur</em></td>
<td>No difference</td>
<td>Slightly elevated</td>
<td>Slightly elevated</td>
<td>Slightly elevated</td>
</tr>
<tr>
<td><em>rirA</em></td>
<td>No difference</td>
<td>Slightly reduced</td>
<td>Slightly reduced</td>
<td>Slightly elevated</td>
</tr>
</tbody>
</table>
Irr overexpression blocked the sharp increase in *hpbC* expression provoked when the temperature decreased to 30°C (see above). This indicated that Irr inhibits *hpbC* transcription (Battisti et al., 2007). In *B. quintana*, *rirA* and *fur* expression was increased when *irr* was overexpressed. This suggested that Irr regulates *fur* and *rirA* expression (Battisti et al., 2007). Furthermore, the relationship between *fur*, *rirA* and the *hbp* family was investigated (Battisti et al., 2007). There were no remarkable changes observed in expression of the *hbp* family when Fur was overexpressed in *B. quintana* under normal growth conditions (Battisti et al., 2007). It was hypothesized that Fur did not play an important role when Irr was present in *B. quintana*, as in other *Rhizobiales* (Johnston et al., 2007; Wexler et al., 2003). *RirA* overexpression in *B. quintana* leads to repression of *hpbB* and *hpbC* expression, and the increase in *hpbA*, *hpbD* and *hpbE* expression is analogous to a “blood-stream-like” expression pattern of *hbp* genes under normal growth conditions (Battisti et al., 2007). Finally, the authors found that Irr binds the *hpbC* promoter element (TTTTTACTACAGAT) referred to as the “H-box”. This H-box is highly conserved in *Bartonella hbp* genes and other open reading frames, including five members of a six-member family of co-hemolysin autotransporters (Battisti et al., 2007).
Introduction

Persistence inside erythrocytes was believed to be a strategy enabling *Bartonellae* species to obtain heme, an absolute requirement for their growth. *Bartonellae* are able to rapidly shift between heme-limited conditions in the mammal host and heme-replete conditions in the arthropod gut (Battisti et al., 2006). Thus, *Bartonellae* must not only be able to obtain heme for growth when it is scarce, but must also be able to protect themselves from heme-mediated toxicity when it is abundant.

*Bartonellae* genomes encode for a complete heme transport system shown to be active in the presence of high heme concentrations in *B. quintana* (Parrow et al., 2009). In addition to a classical heme uptake system, *Bartonellae* genomes also encode three to five heme binding proteins located in the outer membrane. The regulation pattern leading to expression of *hbp* genes was thus investigated in *B. quintana*. Based on their regulatory patterns, *hbp* genes were divided into two groups. The first contained *hbpB* and *hbpC*, overexpressed under conditions that mimic the gut arthropod environment (abundant heme concentrations and low temperature, high O₂ concentrations) (Battisti et al., 2006). The authors suggested that HbpB and HbpC play a critical role in the arthropod gut. Transcription of *hbpA*, *hbpD* and *hbpE* was increased at low heme concentrations (0.05mM) at 37°C (Battisti et al., 2006). The authors suggested that HbpA, HbpD and HbpE are required when the free heme concentration is low, as in blood circulation of the mammal host.

Various reports demonstrated that HbpA of *B. henselae*, which shares homology
with Opa of *N. meningitides*, plays a role in the endothelial cell adhesion process (Dabo et al., 2006a). HpbB of *B. tribocorum* was shown to be required for establishing long-term bacteremia in a rat model, but its role remains unidentified (Saenz et al., 2007). Hpbc of *B. henselae* was recently shown to be a heme-detoxifying protein (Roden et al., 2012). Finally, HpbD of *B. henselae* was hypothesized to be required for survival in endothelial cells (Kyme et al., 2005). However, the functions of heme binding proteins in heme utilization, cell colonization and arthropod transmission remain unknown.

In the present report, we investigated the activity of four *B. henselae* heme binding proteins (HbpA, B, C, D) using both homologous and heterologous (*E. coli*) models. In *E. coli*, the ability to bind Congo red/heme was investigated *in vivo* and *in vitro*. In *B. henselae*, *hbp* knockdown was investigated for determining growth ability, the oxidative stress response and the capacity to invade and survive in endothelial cells. In addition, these mutants were tested for their ability to develop in the *B. henselae* arthropod vector *Ctenocephalides felis*. 
Heme binding proteins of *Bartonella henselae* are required when undergoing oxidative stress during cell and flea invasion

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Running title: Function of heme binding proteins in *B. henselae*

Keywords: *Bartonella* / Heme binding protein/ heme uptake/ oxidative stress/endothelial cell invasion/ flea transmission
Abstract

*Bartonella* are hemotropic bacteria responsible for emerging zoonosis. These heme auxotroph alphaproteobacteria must import heme for their growth, since they cannot synthesize it. To import exogenous heme, *Bartonella* genomes encode for a complete heme uptake system enabling transportation of this compound into the cytoplasm and degrading it to release iron. In addition, these bacteria encode for four or five outer membrane heme binding proteins (Hbps). The structural genes of these highly homologous proteins are expressed differently depending on oxygen, temperature and heme concentrations. These proteins were hypothesized as being involved in various cellular processes according to their ability to bind heme and their regulation profile. In this report, we investigated the roles of the four Hbps of *Bartonella henselae*, responsible for cat scratch disease. We show that Hbps can bind heme *in vitro*. They are able to enhance the efficiency of heme uptake when co-expressed with a heme transporter in *Escherichia coli*. Using *B. henselae* Hbp knockdown mutants, we show that these proteins are involved in defense against the oxidative stress, colonization of human endothelial cell and survival in the flea.
Introduction

*B. henselae* is now recognized as one of the most common zoonoses acquired from animal companions in industrialized countries [1]. The bacterium causes cat scratch disease as well as a number of other syndromes associated with tumoral proliferation of endothelial cells [2]. Most *Bartonella* species appear to share a similar natural cycle that involves arthropod transmission, followed by exploitation of a mammalian host. Each *Bartonella* species appears to be highly adapted to one or several reservoir hosts in which it causes long-lasting intra-erythrocytic bacteremia as a hallmark of infection. Before colonizing erythrocytes, the bacteria need to replicate and become competent in a primary niche [3] such as endothelial cells, although other nucleated cells might constitute part of the primary niche [4]. Bacterial persistence in erythrocytes is an original strategy, and is considered an adaptation to the mode of transmission by bloodsucking arthropod vectors. The invasion of erythrocytes might also be a strategy for *Bartonella* species to obtain heme, an absolute requirement for growth [5]. Not all of the already sequenced *Bartonella* genomes contain heme biosynthesis genes [6]. Moreover, these genomes do not encode for siderophore biosynthesis or a complete iron Fe^{3+} transport system. Only genes sharing strong homology with all compounds of an Fe^{2+} already characterized in *Yersinia pestis* [7] and *Photorhabdus luminescens* [8] are present in *Bartonella* genomes. Moreover, *Bartonella* genomes encode for a complete heme transport system shown to be active in *Bartonella quintana* [9].

Analysis of *Bartonella* genomes sequenced to date clearly shows the absence
of numerous genes proven to be required for *E. coli* in order to face oxidative stress [10]. Genes coding for polypeptides involved in degradation of hydrogen peroxide, like catalase and peroxidase [11], methyl sulf dioxide reduction (MsrA and MsrB) [12] and oxidative stress response regulation (SoxR, OxyR) [13] [14], are not contained in *Bartonella* genomes. Based on the above information relating to *Bartonella* genome contents, it can be hypothesized that *Bartonella* are highly sensitive to oxidative stress. However, hydrogen peroxide challenges performed with *B. bacilliformis* clearly show that this bacterium can efficiently face exposure to 1 mM H$_2$O$_2$ for 30 min [15]. Moreover, the lifestyle of *Bartonella* suggests that these bacteria must face oxidative stress after the blood meal of the arthropod vector [16]. *Bartonella* genomes are small in size, 1.2 to 2 M bases. In spite of their reduced size, they encode for three to five heme binding proteins (Hbps) [17]. Comparison of *B. quintana* Hbps (HbpA, HbpB, HbpC and HbpD) shows that three of these proteins are close in size and peptide sequences. The HbpA, HbpC and HbpD sequences of these approximately 30 kDa polypeptides are nearly 53% identical. HbpB can be distinguished from other Hbps, as this polypeptide is about 11 kDa bigger than the other Hbps and contains a central part not present in other Hbps from *B. quintana* [17].

To survive and multiply, *Bartonella* are forced to encounter various environments in their hosts and vectors. In mammals, free heme is rare [18] and the blood oxygen concentration is low (5% compared to 21% in the atmosphere) [19]. In contrast, in arthropod vectors, toxic level heme is found in the gut and the oxygen
concentration is high [16]. The regulation pattern of *hbp* gene expression was thus investigated in *B. quintana* [19]. Under all conditions tested, *hbpA* was more strongly expressed than other *hbp* genes [19]. Based on their regulatory pattern, *hbp* genes were divided into two groups. The first contained *hbpB* and *hbpC*, over expressed under conditions that mimic the gut arthropod environment (high heme concentration and low temperature, high O$_2$ concentration). The transcription level of *hbpB* and *hbpC* was higher at high heme concentrations (2.5 to 5 mM) [19]. Those authors suggested that HbpB and HbpC play a critical role in the arthropod gut [19]. The transcription of *hbpA*, *hbpD* and *hbpE* was increased at low heme concentrations (0.05 mM) at 37°C. The authors suggested that HbpA, HbpD and HbpE are required when the free heme concentration is low, such as in blood circulation in the mammalian host. However, it was recently shown that transcription of *B. henselae hbpA* is also significantly increased at 28°C, suggesting that HbpA could protect *B. henselae* from heme toxicity in the arthropod gut [20].

The function of HbpA was first investigated in *B. henselae*. Controversial results excluded a direct role for HbpA in the heme uptake process [21], [22]. Various reports demonstrated that HbpA of *B. henselae*, which shares homology with Opa from *Neisseria meningitides* [23], plays a role in the endothelial cell adhesion process [24] [25]. HbpB of *B. tribocorum* was shown to be required for establishing long-term bacteremia in a rat model, but its role has not been elucidated [26]. HpbC of *B. henselae* was recently identified as a heme detoxifying protein [20]. Finally, it was suggested that HbpD of *B. henselae* is required for survival in
endothelial cells [27].

In this report, we investigated the activity of four Hbps (HbpA, B, C, D) of *B. henselae* using both homologous and heterologous (*E. coli*) models. In *E. coli*, the ability to bind Congo red and heme was investigated, respectively, *in vivo* and *in vitro*. In *B. henselae*, *hbp* knockdowns were checked for their growth ability, oxidative stress response and capacity to invade and survive within endothelial cells. Moreover, these mutants were tested for their ability to develop within the *B. henselae* arthropod vector *Ctenocephalides felis*. 
Materials and methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. Bovine hemoglobin (Hb) and 2, 2’-dipyridyl (Dip) were obtained from Sigma Chemical. Heme was dissolved immediately before use in 0.02 M NaOH. Hb was dissolved in 100 mM NaCl. Heme and Hb solutions were filter-sterilized with 0.20 µm Millipore filters for bacterial growth experiments. E. coli strains were grown on LB medium (Sigma), M63 minimal medium, aerobically at 37°C [28] or on Congo red plates. M63 medium was supplemented with 0.4% glycerol (Gly) as carbon source. Solid media and soft agar respectively contained 1.5% or 0.7% Difco agar. Congo red plates consisted of solid BHI medium (Difco) supplemented with Congo red dye (0.02% final concentration). Iron-depleted medium was obtained with the addition of Dip at an 80 µM final concentration. Antibiotics were added to the following final concentrations (µg ml⁻¹): ampicillin (Amp), 50; kanamycin (Km), 50; and spectinomycin (Spc), 50. Arabinose (Ara) was added to a final concentration of 0.02%, 0.2% or 0.4% for induction of the Para promoter. B. henselae was grown on a Columbia blood agar (CBA) plate containing 5% defibrinated sheep blood (Biomérieux; ref 43041) or in Schneider’s medium (Gibco) supplemented with 10% fetal calf serum [29] at 35°C under a 5% CO₂ atmosphere. For flea infection assays, Bartonella strains were collected after 5 days of growth on
CBA plates and suspended in PBS buffer. The bacterial suspension was diluted with PBS to obtain a cell density of $1.98 \times 10^8$ bacteria/ml. The survival of bacteria in PBS buffer was not significantly decreased after 24 h storage at room temperature.

**Congo red binding assay.** Tested strains containing pBAD derivatives expressing Hbps were grown overnight at 37°C in LB medium containing 50 μg/ml ampicillin. Two ml of LB medium containing 50 μg/ml ampicillin were inoculated to an OD$_{600}$ of 0.05 with overnight culture and grown at 37°C. Expression was induced at an OD$_{600}$ of about 0.6 for 2 h by adding arabinose (0.4% final concentration). Cultures were diluted, plated on Congo red plates and incubated at 37°C for 24 h.

**E. coli heme-dependent growth assays.** Tested strains were grown for 18 h in M63 medium without iron with 0.4% glycerol as carbon source, and in the presence of 0.02% arabinose. Cultures were checked for OD at 600 nm and adjusted to OD$_{600}$ =1. A 100 μl sample of the bacterial suspension was mixed with 4 ml of soft agar. The mixture was poured onto M63 plates containing 0.4% glycerol, 0.02% arabinose and 80 μM Dip (M63D). Wells (5 mm in diameter) were cut in the agar and filled with 100 μl of 50 μM, 10 μM, 5 μM, or 1 μM of filter-sterilized Hb solution. Growth around the wells was recorded after 2-day incubation at 37°C. All experiments were performed in triplicate.

**Physiological characterization of hbp knockdown strains.** To evaluate the effect of
*hbp* knockdown on growth of *B. henselae*, tested strains were grown both in liquid Schneider’s medium and on CBA plates. *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::hbpsAS were collected after 5 days of growth on CBA plates and suspended in Schneider’s medium or phosphate buffered saline (PBS). For growth in Schneider’s medium, the OD$_{600}$ of the bacterial suspension was adjusted to 0.05. Five ml samples of this suspension were poured into 6-well plates and grown at 35°C in the presence of 5% CO$_2$. OD$_{600}$ was checked at days 2, 4, 5 and 7 after inoculation. For growth on CBA plates, serial dilutions of bacterial suspension in PBS were plated on CBA plates and colony size was evaluated after 6 and 10 days of growth at 35°C in the presence of 5% CO$_2$. All experiments were performed in triplicate.

**H$_2$O$_2$ challenge.** *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::hbpsAS were grown on CBA plates for five days at 35°C under a 5% CO$_2$ atmosphere. Bacteria collected from one plate were suspended and washed twice in PBS buffer. The cell suspension was then diluted to OD$_{600}$ 0.5. Before H$_2$O$_2$ challenge, several dilutions of the tested cell suspension were spread on CBA plates ($T_0$). For the challenge assay, bacteria were incubated 30 min in PBS buffer in the presence of 1 mM and 10 mM H$_2$O$_2$ at 35°C under a 5% CO$_2$ atmosphere. After exposure to H$_2$O$_2$, bacteria were washed twice in PBS buffer and several dilutions plated onto CBA plates ($T_1$). After 15-day incubation at 35°C under a 5% CO$_2$ atmosphere, colonies were counted. Survival rate was expressed by ($T_1$/$T_0$) X100%. All experiments were performed in triplicate.
**Endothelial cell culture and invasion assay.** Endothelial cell line Ea.hy 926 resulting from a fusion of HUVEC and lung carcinoma cell line A549 were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum decomplemented by heating 30 min at 56°C before use. When required, kanamycin was added at a 50 μg/ml final concentration. Cells were incubated at 37°C in humidified 5% CO₂ and cultured every 7 days using 0.025% trypsin and 1 mM EDTA in Hanks’ balanced salt solution [30]. Endothelial cells were seeded in 24-well plates at a density of 10⁴ cells/well. After 6 days, cell number was estimated as 1.5×10⁵/well. *Bartonella* strains harvested from CBA plates after 5-day growth at 35°C under a 5% CO₂ atmosphere were washed twice in modified DMEM buffer and then resuspended in the same medium. Bacterial number was adjusted to 3×10⁴/ml (1OD was estimated at 6.6×10⁸/ml). For cell invasion assays, medium in the well was removed and 0.5 ml of modified DMEM containing 1.5×10⁴ colony-forming units (CFUs) of the *B. henselae* tested strains were added to the well to obtain 0.1 multiplicity of infection (m.o.i). After remaining for 1 h at 37°C, 1.5 ml of modified DMEM medium was supplemented in the well. The number of bacteria was controlled by plating several dilutions on CBA plates, and CFUs were determined after 15 days of growth (T₀). Mixtures were incubated at 37°C at 5% CO₂ for 24 h. After 24 h, the number of viable bacteria was determined by plating serial dilutions of mixtures on CBA plates, and CFUs were determined after 15 days growth (T₂₄). After 24 h, bacterial viability was nearly 100%. The intracellular bacterial population was quantified by the gentamicin protection assay as described by Mehock [31]. Briefly, DMEM with
gentamicin (final concentration 250 μg/ml) was added to the mixture assay and removed after 2 h at 37°C in the presence of 5% CO₂. A control *B. henselae* bacterial suspension showed no survival after 2 h exposure to gentamicin (250 μg/ml). After removing modified DMEM medium containing gentamicin, endothelial cells were then washed three times with modified DMEM medium to remove residual antibiotic. Endothelial cells were then collected after 4 min incubation with 400 μl trypsin at 37°C. After centrifuging at 12,000 rpm, cells were suspended in 1 ml of sterile water and disrupted using a 1 ml syringe and a 0.4 mm×20 mm needle and 5 pushes [32]. Microscopic controls revealed that, after 5 pushes, all cells were lysed. Cell lysates were supplemented with 100 μl 10×PBS to overcome osmotic lysis. The number of viable bacteria was determined by plating lysates on CBA plates. After 15 days of incubation at 35°C under a 5% CO₂ atmosphere, colonies were counted (Tᵢ). The invasion rate was expressed as \((Tᵢ/T₀)\times100\%\). Each assay was performed in double wells and all experiments were performed in triplicate.

**Survival assay in endothelial cells.** To perform survival assays of *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::hbpsAS in endothelial cells, the monolayer contained in mixtures challenged for gentamicin killing was washed three times and incubated in modified DMEM medium at 37°C and 5% CO₂ for 24 and 48 h. Cells were treated as described above and the number of viable bacteria was determined by plating the lysates on CBA plates. After 15-day incubation at 35°C under a 5% CO₂ atmosphere, colonies were counted (Tₛ₂₄ or Tₛ₄₈). The survival rate was expressed as
(TS24/T1 or TS48/T1) × 100%. Each assay was performed in double wells and all experiments were performed in triplicate.

**Flea maintenance and supply.** Strain *Ctenocephalides felis (C. felis) (Siphonaptera: Pulicidae)* originating from a wild strain harvested from a cat has been maintained under laboratory conditions since 1990.

**Feeding of C. felis with B. henselae pNS2Trc- or B. henselae pNS2Trc::hbpsAS-infected blood.** Dog blood used in all experiments was obtained from 3 beagles from the Ectoparasite Laboratory of the National Veterinary School in Toulouse, France. The absence of *Bartonella* spp. in the blood of these dogs was confirmed by PCR. Lithium heparin–coated vacutainer tubes (Venosafe, Terumo Europe) were used to draw blood by venipuncture. Blood functional complement was deactivated by maintaining blood samples at room temperature for 2 h after the blood test and before storing them at 4°C. Blood samples were stored less than 48 h at 4°C. When required, kanamycin was added to blood at a 50 μg/ml final concentration. Kanamycin was previously determined to have no effect on *C. felis* feeding, viability or egg production. A total of 500 unfed fleas (males and females aged between 8-10 days) were placed in a plexiglas box in contact with a glass feeder closed at the bottom by a parafilm membrane. To stimulate flea blood-feeding, a constant temperature (38.5°C) was maintained by a water-jacket circulation system through the glass feeder. For blood infection, 500 μL of bacterial suspension at a concentration of
approximately $1.98 \times 10^8$ bacteria/ml in PBS were added to 5 ml of blood. Viability of *Bartonella* in blood was about 100% after 2-h incubation. Blood was complemented by bacterial suspension for the first two days of feeding. Then, fleas were fed with uninfected dog blood for the next 8 days. Every 24 h, the glass feeder was cleaned, a new parafilm membrane was stretched and blood was changed. At the same time, flea feces were collected. All samples were stored at -20°C until PCR analysis. Ethanol 70% was added to flea feces samples.

**Genetic techniques.** *E. coli* cells were transformed by the calcium chloride method [33]. *Bartonella* cells were transformed by electroporation as previously described [34].

**DNA manipulations.** A small-scale plasmid DNA preparation was performed using a QIAprep Spin Miniprep kit (Qiagen). Restriction, modification, and ligation were carried out according to the manufacturer’s recommendations. DNA fragments were amplified in a Hybaid PCR thermocycler using Phusion DNA polymerase (Finnzymes). Nucleotide sequencing was performed by Eurofins MWG Operon. Purification of DNA fragments from the PCR reaction, restriction reaction or agarose gels was performed using the Macherey-Nagel NucleoSpin® Extract II kit.

**Construction of a recombinant vector expressing Hbps of *B. henselae.*** Complete *B. henselae hbpA, hbpB, hbpC and hbpD* genes with a C-terminal Histag (6 His) were amplified by PCR from *B. henselae* chromosomal DNA using primers
HbpABhamont and HbpABhaval, HbpBBhamont and HbpBBhaval, HbpCBhamont and HbpCBhaval or HbpDBhamont and HbpDBhaval, respectively (Table 2). The fragments amplified (890 bp for \textit{hbpA}, 881 bp for \textit{hbpC} and 875 bp for \textit{hbpD}) were purified and digested with NheI and KpnI. The \textit{hbpB} fragment (1235bp) was purified and digested with NheI and Hind III. Then, fragments were ligated with pBAD24 plasmid digested with NheI and KpnI or NheI and Hind III. Ligation mixtures were introduced into CaCl$_2$-competent \textit{E. coli} strain XL1 Blue cells and transformants were selected on LB plates containing ampicillin. Clones were screened by the PCR method with corresponding primers. Six PCR-positive clones were then sequenced.

\textbf{Construction of the vector for decreasing the amount of Hbp in \textit{B. henselae}.} The entire coding region of \textit{hbpA}, \textit{hbpB}, \textit{hbpC} and \textit{hbpD} was amplified by PCR from the \textit{B. henselae} chromosomal DNA using primers hbpAantisensamt and hbpAantisensavl, hbpBantisensamt and hbpBantisensavl, hbpCantisensamt and hbpCantisensavl or hbpDantisensamt and hbpDantisensavl, respectively (Table 2). The PCR product (846 bp for \textit{hbpA}, 1,182 bp for \textit{hbpB}, 837 bp for \textit{hbpC} and 831 bp for \textit{hbpD}) was purified, digested with BamHI and XbaI and then ligated with plasmid pNS2Trc digested with BamHI and XbaI. Ligation mixtures were introduced into CaCl$_2$-competent \textit{E. coli} strain XL1 Blue cells. Transformants were screened by PCR with corresponding primers. Six PCR-positive clones were then sequenced.

\textbf{Extraction of DNA from flea feces.} DNA was extracted from flea feces using the
Nucleospin tissue kit according to the manufacturer’s instructions (NucleoSpin® Tissue, Macherey-Nagel). The quantity of biological material used for DNA extraction was about 30-40 mg of feces. Flea feces were incubated overnight to 56°C for the pre-lysis step. For all samples, the final elution volume was 100 μL. The concentration of DNA extraction in all samples was measured using a nanodrop spectrophotometer (NanoDrop 2000, Thermo Scientific).

**Detection of *B. henselae* DNA from flea feces.** DNA of *B. henselae* was detected by amplification of a 1,052 bp fragment containing *B. henselae bh02390* gene using primers bh2390fo, and bh2390re (Table 2). DNA of *C. felis* was detected by amplification of a fragment of *C. felis* 18S rDNA using primers Cf18Sf and Cf18Sr (Table 2) [35]. Amplifications were performed with at least 20 ng of DNA extract for flea feces. Each reaction was conducted in a total volume of 20 μL with 0.5 μM of each primer, 200 μM of each dNTP, 4 μL of 5× PCR buffer G/C and 0.02U/μl of Taq DNA polymerase (Phusion® High-Fidelity DNA Polymerase, Thermo scientific). The PCR program was as follows: an initial denaturation step for 30 s at 98°C, followed by 40 cycles of denaturation for 10 s at 98°C, annealing for 30 s at 55°C and extension for 1 min at 72°C, and a final extension step at 72°C for 7 min.

**Expression and purification of recombinant His-tagged Hbps.** Strain XL1-Blue pBAD24::hbpA, XL1-Blue pBAD24::hbpB, XL1-Blue pBAD24::hbpC and
XL1-Blue pBAD24::hbpD were grown overnight at 37°C in LB medium containing 50 μg/ml ampicillin. Then, 200 ml of LB medium containing 50 μg/ml ampicillin was inoculated to an OD$_{600}$ of 0.05 with the overnight culture and grown at 37°C. Expression was induced at an OD$_{600}$ of about 0.6 for 2 h by adding arabinose (0.4% final concentration). Bacteria were harvested by centrifugation for 10 min at 3,000 g at 4°C, and the pellet was suspended in 20 ml binding buffer (50 mM Tris-HCl, 8M urea, 0.05% triton, pH 8.0). Lysis of bacteria was obtained by incubation at room temperature with rotation for 3 h. The suspension was then centrifuged at 13,000 g for 30 min at 4°C. The supernatant containing the soluble fraction was mixed with 200 μl of Ni-agarose beads (Qiagen) according to the manufacturer’s instructions. Purified protein was dialyzed twice against a buffer containing 50 mM Tris-HCl to eliminate any residual imidazole and urea. The protein was stable for at least one month when kept at -80°C with 20% glycerol.

**Heme binding assay in vitro.** For heme blotting, proteins were separated on standard 12.5% SDS gels followed by electrophoretic transfer to nitrocellulose membranes according to the protocol of Vargas [36]. Briefly, samples were mixed with loading buffer to which no DTT was added and samples were not boiled before electrophoresis. 1 μg of HbpA, HbpB, HbpC and HbpD or 3 μg of BSA was separated on 12.5% SDS-PAGE. One gel was stained with Coomassie brilliant Blue R. Another gel was transferred to nitrocellulose using the general methods of Towbin et al. [37]. The heme binding blot was done according to the protocol of Carroll et al. [22].
Briefly, the resulting blots were rinsed with Tris-buffered saline containing 0.1% Tween 20 (TBST; 10 mM pH 8.0 Tris-HCl containing 150 mM NaCl and 0.1% Tween 20) three times for 30 min and subsequently probed for 1.5 h with TBS containing heme (10^{-6}M) at room temperature. Nitrocellulose was washed three times for 30 min with TBS-Tween 20 (0.1%) at room temperature. Heme was visualized via its intrinsic peroxidase activity [38] using enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia, Piscataway, N.J.). Hbp bands were visualized by exposing the blot to autoradiographic film (Labscientific, Livingston, N.J.).

**Antibody preparation.** 200 μl of an emulsion containing purified HbpB (10 μg) and ISA 61 VG adjuvant (Seppic) (120 μl), completed with NaCl 0.9%, were inoculated twice (at a 1-month interval) subcutaneously into C57B6 mice (Charles River) (Ethics Committee Anses/ENVA/UPEC agreement n°:14/06/2011-1). Two weeks after the second inoculation, 200 μl blood samples were collected every 3 weeks via retro-orbital bleeding. Blood samples were centrifuged twice (3,600 rpm 5 min) to obtain serum which was stored at -20°C. Before use, non-specific antibodies were removed by incubating the immune serum with *E. coli* cell extract for 1 h at 4°C and centrifugation for 10 min at 8,000 rpm. The supernatant was then used as serum.

**Protein analysis by electrophoresis.** Proteins were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis [39], followed by Coomassie Blue staining.
**Immunoblot analysis.** Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting for detecting decreased expression of HbpA in *B. henselae* were performed as follows. Tested strain biomass was collected after 5 days of growth on CBA plates, suspended in PBS buffer and centrifuged. Bacterial pellets calculated to contain 40 μg of proteins were suspended in loading buffer and heated for 5 min at 100°C. Proteins were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C Extra, GE Healthcare) according to Towbin *et al* [37]. Non-specific binding sites were blocked with 5% skim milk in TBS-Tween 20(0.05%). The immunoblot was probed with polyclonal mouse sera raised against recombinant HbpB (1:200), followed by a 1:1,000 dilution of a rabbit anti-mouse IgG horseradish peroxidase[HRP]-conjugated secondary antibody (cell signaling). Visualization of the HRP signal was performed using enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia, Piscataway, N.J). HbpA bands were visualized by exposing the blot to autoradiographic film (Labscientific, Livingston, NJ, USA).

**Protein assay.** The concentration of the protein was determined using the BC assay protein quantitation kit (Interchim).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5 software for Windows. Statistical significance of the data was ascertained by use of Student’s *t* test. A value of *P* <0.05 was considered significant.
Results

Hbps are able to bind Congo red when expressed in E. coli. To determine that Hbps of B. henselae can bind heme, we first checked for Congo red binding activity, since it had been shown that Hbps can also bind Congo red [40]. Moreover, it had already been shown that expressing a Congo red binding protein on the outer membrane of E. coli conferred a Congo red binding phenotype [41]. We transformed E. coli XL1-Blue with plasmids pBAD24, pBAD24::hbpA, pBAD24::hbpB, pBAD24::hbpC and pBAD24::hbpD to determine whether hbp genes can produce a Congo red binding phenotype. Strain XL1-Blue with plasmid pBAD24 did not confer a Congo red phenotype when grown on the plate with 0.2% arabinose (Fig 1B). However, strains expressing HbpA, B, C and D formed a red colony on the plate with 0.2% arabinose (Fig 1 C, D, E, F). Without arabinose, neither XL1-Blue pBAD24 nor XL1-Blue pBAD24::hbpA, XL1-Blue pBAD24::hbpB, XL1-Blue pBAD24::hbpC, XL1-Blue pBAD24::hbpD, conferred a Congo red binding phenotype (data not shown). Taken together, these data showed that Hbps can bind Congo red and confirmed that Hbps were exposed on the cell surface when expressed in E. coli.

Recombinant HbpA, HbpB, HbpC and HbpD can specifically bind heme in vitro.

To produce and purify recombinant HbpA, B, C, D from E. coli, we amplified their structural gene using B. henselae chromosomal DNA as template and primers allowing addition of a six His-tag at the C-terminal of the protein. The fragments were cloned into the pBAD24 plasmid as described in Materials and methods. Plasmids pBAD24::hbpA, pBAD24::hbpB, pBAD24::hbpC and pBAD24::hbpD were
introduced into strain XL1-Blue. To check for quantities of Hbps in *E. coli* strain XL1-Blue, SDS gel electrophoresis (SDS-PAGE) was used to compare protein extracts of strain XL1-Blue pBAD24::*hbps*<sub>his</sub> and XL1-Blue pBAD24. A supplementary visible band was observed on SDS page gel for XL1-Blue derivatives harboring plasmid pBAD24::*hbpA*, pBAD24::*hbpB*, pBAD24::*hbpC* or pBAD24::*hbpD* when grown in the presence of 0.4% arabinose (Fig 1A). Recombinant Hbp proteins were purified by Ni-agarose affinity chromatography as described in Materials and methods. The size of purified Hbp proteins corresponded well to those predicted from their sequences.

To test whether pure Hbps can specifically bind heme *in vitro*, a standard method already used for detecting heme binding of cytochrome C was used [36]. Pure HbpA, HbpB, HbpC, HbpD and BSA were separated on two SDS-polyacrylamide gels. One gel was then stained with Coomassie brilliant Blue R (Fig. 2A). Another gel was transferred to a nitrocellulose filter to perform heme blotting and ECL detection (Fig. 2B). Pure Hbps were able to bind heme added at 10⁻⁶M concentration (Fig. 2B). In contrast, under our assay conditions, BSA was unable to bind heme (Fig. 2B). The latter result underlines the specificity of the proteins that bind heme. Taken as a whole, we conclude that when purified, all Hbps can bind heme *in vitro*.

**Hbps increase the efficiency of heme uptake mediated by HemR from *Serratia marcescens* in *E. coli***. Conflicting results concerning the heme transport activity of HbpA encouraged us to first check for the ability of Hbps to transport heme using *E.
coli hemA mutant complementation. Our results demonstrated that Hbps from *B. henselae* did not act as heme transporters (Supplement table 1). These results are at variance with those obtained for Pap31 (HbpA) from *B. henselae* [21], but are in agreement with those obtained for HbpA of *B. quintana* [9] and *Bartonella birtlesii* (Biville F, unpublished data). We checked whether Hbps could modulate the efficiency of the heme uptake process. We first verified the effect of Hbps on the activity of HutA of *B. henselae* expressed in *E. coli*. HutA from *B. quintana* was shown to transport heme and, consequently, to restore growth of an *E. coli* hemA mutant. [9]. Experiments were performed in the presence of high heme concentrations in liquid medium. The weak growth restoration may have been the consequence of mutations enhancing outer membrane permeability [42]. These complementation assays failed for an *E. coli* K-12 hemA mutant expressing HutA of *B. birtlesii* when grown on heme-supplemented solid medium [43]. To avoid factual results concerning the heme transportation activity of HutA from *B. henselae*, we expressed it in an *E. coli* entF mutant that cannot grow in the presence of an iron chelator [44]. When a heme transporter is expressed in such strain, addition of heme to the medium restores growth. In order to be used as an iron source, the amount of heme required is 100 times greater than that required for its use as a heme source. Such a complementation assay had already been used to check the heme transport activity of HasR from *Serratia marcescens* [45] and also for characterization of heme-degrading enzymes [46,47]. No growth was observed in the *E. coli* entF strain expressing HutA from *B. henselae* when grown in iron-depleted medium in the presence of hemoglobin
To check for the effect of Hbps from *B. henselae* upon the heme uptake process, plasmids harboring *hbps* genes were introduced into the *E. coli entF* mutant [44] expressing a HemR heme transporter from *S. marcescens* [48]. Strains obtained were tested for growth on iron-depleted medium in the presence of hemoglobin added at different concentrations. As seen in table 3, HemR alone led to growth around the well containing hemoglobin at 50 μM in the presence of 80 μM dip. Strains expressing *hbpA*, *hbpB*, *hbpC* and *hbpD* were able to grow at lower concentrations of Hb (Table 3), suggesting that HbpA, HbpB, HbpC and HbpD increase the efficiency of the HemR-mediated heme uptake process. HbpB and HbpD were more efficient than HbpA and HbpC (Table 3). To further investigate the efficiency of heme uptake mediated by Hbps and HemR, we grew the bacteria on a minimal iron-depleted plate with 100 μM Dip in the presence of different concentrations of Hb. For the control strain only expressing HemR and strains co-expressing HbpA or HbpC and HemR, no growth was observed around the well whatever the concentration of Hb added (Table 3). The strain co-expressing HbpD and HemR showed growth only around the well containing 50 μM Hb. The strain co-expressing HbpB and HemR was able to grow around the well whatever the concentration of Hb (Table 3). Taken together, we concluded that HbpB and HbpD were more efficient than others at increasing heme uptake when expressed in *E. coli*, and thus they might play an important role at low heme concentrations. Similar results were obtained by introducing plasmids harboring *hbps* genes in an *E. coli entF* mutant expressing HasR from *S. marcescens* (data not shown). Binding of heme by Hbps
increased its concentration around the bacteria and consequently facilitated its uptake.

**Hbp activity is important for *B. henselae* growth.** Since Hbps can modulate heme uptake efficiency when expressed in *E. coli*, a similar activity was hypothesized in *B. henselae*. In that bacterium, heme serves both as heme and as an iron source [47]. Thus, abolishing or decreasing synthesis of the different Hbps in *B. henselae* could potentially affect its growth capacity. Knockout of HbpA in *B. henselae* was hypothesized as being lethal to the bacteria [20]. A preliminary unsuccessful assay in our lab to disrupt *hbpA* of *B. henselae* was in agreement with this hypothesis. To investigate the function of the four Hbps of *B. henselae* using the same genetic tool, we chose the knockdown method that had been successfully used for *B. henselae* [47], [49], [50]. We cloned *hbps* of *B. henselae* oriented in the reverse direction such that the anti-sense strand was transcribed in plasmid pNSTrc [49]. Plasmids pNS2Trc, pNS2Trc::*hbpA*AS, pNS2Trc::*hbpB*AS, pNS2Trc::*hbpC*AS and pNS2Trc::*hbpD*AS were introduced into *B. henselae* using electroporation. We first checked for the knockdown effect on the Hbp expression level in *B. henselae* using a western blot experiment. Multiple sequence alignment of Hbps of *B. henselae* revealed a high degree of amino acid sequence conservation at its N-terminal and C-terminal parts even for HbpB, which is longer than other Hbps. According to its size, HbpB can easily be distinguished from other Hbps. Mouse anti-serum against HbpB was prepared and tested for recognition of purified Hbps. Our results demonstrated that anti-HbpB antibody can recognize well-purified HbpB and HbpA. For HbpD and
HbpC, recognition by anti-HbpB antibodies was lower or absent (data not shown). Detection of HbpB expression in *B. henselae* was investigated using anti-HbpB anti-serum. We failed to detect HbpB in *B. henselae* using the western blot method. This result is in good agreement with those of proteomic analysis of outer membrane fractions of *B. henselae* and *B. quintana* [51], [52], [53], [54], [55], [56], which showed that HbpB was not detectable using this method. Since HbpA is the most abundant Hbp in *B. henselae* [52] [51], we checked its level in *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::hbpAAS using mouse antibody directed against HbpB. As seen in Fig. 3, the level of HbpA was lower in strain *B. henselae* pNS2Trc::hbpAAS than in strain *B. henselae* pNS2Trc.

The strains obtained were tested for growth on both blood agar plates and in Schneider’s medium as described in Materials and methods. After 6 and 10 days of growth on blood agar plates, colonies produced by strain *B. henselae* harboring pNS2Trc::hbpAAS, pNS2Trc::hbpBAS, pNS2Trc::hbpCAS or pNS2Trc::hbpDAS were much smaller than those formed by *B. henselae* pNS2Trc (Table 4). The *B. henselae* strain containing pNS2Trc::hbpsAS also grew more slowly than *B. henselae* pNS2Trc in Schneider’s medium (Fig 4). These results indicated that a decrease in the amount of Hbps slowed growth of *B. henselae*.

**Knockdown of Hbps increased *B. henselae* sensitivity to hydrogen peroxide**

Analyses of *Bartonellae* genomes demonstrated that numerous genes involved in the oxidative stress response were not present. However, it was shown that *Bartonella*
bacilliformis was able to successfully endure 30 min exposure to 1 mM H₂O₂ [15], suggesting that certain activities enable this bacterium to undergo oxidative stress generated by exposure to H₂O₂. It was suggested that one potential role for Hbps was to bind heme at the surface of the bacteria and provide an antioxidant barrier via heme intrinsic peroxidase activity [57]. To examine this hypothesis, we tested the effect of hbp knockdown on the ability of B. henselae to face 30 min exposure to 1 mM and 10 mM hydrogen peroxide. After exposure to 1 mM H₂O₂, survival was about 50% for both control strain B. henselae pNS2Trc and B. henselae pNS2Trc::hbpCAS. There existed slightly decreased survival capacity for B. henselae pNS2Trc::hbpAAS, B. henselae pNS2Trc::hbpBAS and B. henselae pNS2Trc::hbpCAS (data not shown). After exposure to 10 mM H₂O₂, survival was about 25 % for control strain B. henselae pNS2Trc. With strain B. henselae pNS2Trc::hbpAAS, B. henselae pNS2Trc::hbpBAS and B. henselae pNS2Trc::hbpCAS, sensitivity to hydrogen peroxide increased about 3-4-fold (Figure. 5). Decreasing HbpD levels more sharply increased B. henselae sensitivity to hydrogen peroxide (Figure. 5). These results indicated that lowering the Hbp level in B. henselae significantly increased its sensitivity to H₂O₂.

**Effect of Hbp knockdown on B. henselae capacity to invade endothelial cells.**

In mammals, reactive oxygen species (ROS) are a part of immune defenses [58]. Within cells, bacterial infection was shown to induce ROS production [59]. Thus, decreasing the ability to undergo oxidative stress is expected to decrease the ability of B. henselae to survive in endothelial cells. This promoted us to check the effect of
Hbp knockdown upon the capacity of *B. henselae* to invade human endothelial cells and to survive within them.

For endothelial cell invasion, both *B. henselae* pNS2Trc::*hbpB* as and *B. henselae* pNS2Trc::*hbpC* as exhibited the same invasion rate as control strain *B. henselae* pNS2Trc (about 2%) (Fig. 6). However, invasion rates of *B. henselae* pNS2Trc::*hbpA* as and *B. henselae* pNS2Trc::*hbpD* as decreased 3-fold compared to the control strain (Fig. 6).

For survival in the endothelial cell assay, mixtures were grown for 24 h or 48 h after the gentamicin killing assay. After 24 h in endothelial cell, cell lysates were spread on the blood plate to check viable bacterial number. Surprisingly, no bacteria were visible on the blood plate after 2-week incubation. To overcome this problem, we first grew the mixture in Schneider’s liquid medium overnight, sustaining primary isolation of *B. henselae* [29] before plating it on blood plates. Colony count after 2-week incubation showed a decrease in viable bacteria for all strains tested, though it has been claimed that *B. henselae* is able to replicate inside endothelial cell through bacterial rRNA replication [60]. Survival rates for *B. henselae* pNS2Trc::*hbps* as were much lower than those of control *B. henselae* pNS2Trc (Fig 4), after 24 h or 48 h growth in endothelial cells. To check for an effect of overnight growth in Schneider’s medium, about 600 *B. henselae* pNS2Trc or *B. henselae* pNS2Trc::*hbps* as bacteria were grown overnight in that medium and plated on blood plates for enumeration. The increase in bacterial numbers was calculated for all tested strains. The increase was about 20-30% for both *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::*hbps* as. The
differing survival rates of *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::hbps AS were not related to a growth defect in Schneider’s medium. We conclude that Hbps of *B. henselae* play an important role in survival within endothelial cells.

**Hbps are involved in multiplication of *B. henselae* in *C. felis**. The role of cat fleas (*C. felis*) in transmission of *B. henselae* was reported in northern California in the early 1990s [61]. Later, it was experimentally demonstrated that *B. henselae*-infected fleas can transmit *B. henselae* to cats [62] and that *B. henselae* can replicate in the gut of the cat flea [63]. Transmission to humans is thought to occur via a cat scratch contaminated with flea feces [63]. Inside the arthropod gut, bacteria confront oxidative stress after each blood meal [16]. Since Hbps of *B. henselae* play a protective role against H2O2-produced oxidative stress, this prompted us to check for a Hbp knockdown effect on *B. henselae* multiplication in fleas. After feeding fleas for 2 days with blood containing the bacteria, fleas were fed with blood without bacteria for another 8 days. For control strain *B. henselae* pNS2Trc, we were able to detect *B. henselae* DNA in the feces from day 1 to day 10 (Table 5). For strains with a decreased amount of HbpA, HbpB, HbpC or HbpD, no bacterial DNA could be detected after day 6. To exclude the possibility that this result was due to small amounts or to the quality of DNA from flea feces, we amplified flea 18S rDNA from day-7-to-10 flea feces samples using primers Cf18Sf and Cf18Sr. Flea 18S rDNA was detected in all of the day-7-to-10 flea feces samples (data not shown). This suggests that Hbps play an important role in multiplication of *B. henselae* in fleas.
Discussion

In this report, we investigated the role of *B. henselae* Hbps in heme utilization, the oxidative stress response, cell colonization and survival within arthropod vector *C. felis*. Previous data had shown that recombinant HbpA of *B. quintana* was able to bind heme *in vitro*, but did not confer a heme binding phenotype *in vivo* when expressed in *E. coli* [22]. Later it was claimed that HbpB of *B. quintana* did not bind heme [19]. Recently, it was shown that *hbpC* of *B. henselae*, when expressed in *E. coli*, confers a heme binding phenotype *in vivo* [20].

Our results clearly show that expression of all Hbps from *B. henselae* in *E. coli* confers a Congo red binding phenotype (Fig 1), thus suggesting that Hbps have a surface location when expressed in *E. coli*. We also demonstrate that, *in vitro*, all purified Hbps specifically bind heme. To characterize the physiological importance of Hbps, we first investigated their effect on the heme uptake process. Pap31 (HbpA) of *B. henselae* was claimed to act as a heme porin when expressed in *E. coli* [21], but conflicting data about this activity were published for HbpA of *B. quintana* [57]. Moreover, we failed to visualize any heme porin activity for Hbps of *B. henselae* when expressed in *E. coli*. Based on the above results, we hypothesized that the Hbp family of *Bartonella* could act as a heme reservoir, thus rendering it available under heme-limited conditions. This hypothesis is in good agreement with the absence of genes encoding for heme and iron storage proteins in *Bartonellae* genomes. Such heme storage activity might enhance the efficiency of the heme uptake process. We examined the effect of Hbps on the activity of heterologous heme transporters HemR
and HasR of *S. marcescens* [48], [64]. All Hbps increased heme uptake efficiency mediated by HemR and HasR. Efficiency at low heme concentrations was better with HbpB and HbpD than with HbpA and HbpC. Such differences in efficacy could be related to the differing levels of Hbps in *E. coli*. This is the case for HbpB, but not for HbpD (Fig 1). This result suggests that HbpD might be active when the heme concentration is low. This conclusion is in good agreement with the increase in *hbpD* expression when *B. quintana* was grown in the presence of low heme concentrations [19]. How heme is transferred from heme binding proteins to HemR and HasR heme transporters remains unknown.

During mammal and flea invasion, *Bartonellae* must face microenvironmental shifts, stress and the host immune defense. For example, it was shown that reactive oxygen species (ROS: O$_2^-$, H$_2$O$_2$ and OH$^-$) production is an important immune defense mechanism for mammal hosts and arthropod vectors against pathogenic bacteria [16] [65]. Recently, it was shown that ROS (H$_2$O$_2$) levels in midgut were higher (over 10 mM) in infected fleas. Antioxidant treatment prior to infection decreased ROS levels and resulted in higher *Yersinia pestis* loads [66]. An OxyR *Y. pestis* mutant showed reduced growth in fleas early after infection [66]. ROS are potentially toxic for both the host cell and pathogenic bacteria [16,67,68,69]. Host cells are protected from oxidative damage by enzymes that detoxify ROS, such as SOD, catalase, glutathione peroxidase (Gpx) and thioredoxin peroxidase, that detoxify H$_2$O$_2$ [70]. In arthropod vectors, the adaptive response to ROS has also been thoroughly investigated [16]. *B. henselae* replicates in the gut of the cat flea and is able to survive several days in flea
It was shown that the hematophagous vector has a substantial need for huge amounts of blood at each meal; digestion of hemoglobin within the gut of the vector releases large quantities of heme, which has potential pro-oxidant and cytotoxic effects if not bound to proteins [71,72]. To successfully replicate in the cat flea gut, *B. henselae* must confront toxic ROS and repair damage. Many homologues of genes involved in the oxidative stress response in *E. coli* are not present in the *B. henselae* genome. The absence of homologues of these genes suggests that *Bartonella* must possess uncharacterized mechanisms in response to oxidative stress. One actor in this oxidative stress response was recently identified as being a heme-degrading enzyme enabling release of iron from heme [47]. In this report, we demonstrate that all Hbps are required to efficiently undergo exposure to hydrogen peroxide. A role for the intrinsic peroxidase activity of heme bound to Hbps was hypothesized for *B. quintana* [19] and was shown to be the case for *Porphyromonas gingivalis* [73]. The competence of Hbps in detoxifying H$_2$O$_2$ might also be required for efficient invasion and survival in endothelial cells, major target cell types for bacterial colonization in the reservoir host(s) as well as in the infected host [74]. Bacterial infection was shown to induce ROS production in endothelial cells [59]. As a consequence, the weaker survival capacity in endothelial cells, related to knockdown of all Hbps, might be related to lower resistance to oxidative stress.

For HbpA and HbpD, we also show a weakening in the endothelial cell invasion process when their level decreases. For HbpA, this effect might be related to its Opa domain, which has been shown to be involved in entry into the host epithelial cell in
Neisseria gonorrhoeae [75]. Indeed, previous data also showed that Pap31 (HbpA) of B. henselae was able to bind endothelial cells in a dose-dependent manner, and binding was inhibited by anti-Pap31 antibodies [24]. However, HbpD involvement in the endothelial cell invasion process is striking, and can be explained by greater sensitivity to oxidative stress produced by endothelial cells, thus leading to more rapid killing of bacteria. This hypothesis is supported by the fact that the mutant of HbpD cannot delay lysosomal fusion, leading to significantly reduced viability within endothelial cells [27].

Clearance of bacteria in the flea feces can be explained by a decrease in survival within the arthropod. Our results clearly demonstrate more rapid clearance of B. henselae in the flea feces when invasion assays are performed using Hbp knockdown mutants. In addition, this more rapid clearance of bacteria in the flea feces can be attributed to decreased ability to confront oxidative stress. Thus, the ROS-detoxifying activity of Hbps also plays an important role during colonization of fleas. Taken together, we reveal the functions of Hbps in heme utilization, the oxidative stress response, cell colonization and flea transmission. The ability of Hbps to bind heme provides competence in destroying ROS, thus constituting an important immune defense system for host cell and arthropod vectors. Functional identification of Hbp families in cell interactions and flea transmission should help to develop strategies for fighting infection and transmission. It will be interesting in the future to elucidate anti-oxidant mechanisms used by other vector-borne pathogens.
Acknowledgments

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Table 1. Strains and plasmids used in this study

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<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>F' supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac' F' proAB' lacIq lacZΔM15 Tn10 (Tet&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>XL1-Blue pBAD24</td>
<td>XL1-Blue carrying pBAD24, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>XL1-Blue pBAD24::hbpA</td>
<td>XL1-Blue carrying pBAD24::hbpA, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>XL1-Blue pBAD24::hbpB</td>
<td>XL1-Blue carrying pBAD24::hbpB, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>XL1-Blue pBAD24::hbpC</td>
<td>XL1-Blue carrying pBAD24::hbpC, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>XL1-Blue pBAD24::hbpD</td>
<td>XL1-Blue carrying pBAD24::hbpD, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>FB8.27 pAM239::hemR, pBAD24</td>
<td>FB8.27 pAM239::hemR, pBAD24, Tet&lt;sup&gt;R&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>FB8.27 pAM239::hemR, pBAD24::hbpA</td>
<td>FB8.27 pAM239::hemR, pBAD24::hbpA, Tet&lt;sup&gt;R&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>FB8.27 pAM239::hemR, pBAD24::hbpB</td>
<td>FB8.27 pAM239::hemR, pBAD24::hbpB, Tet&lt;sup&gt;R&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>FB8.27 pAM239::hemR, pBAD24::hbpC</td>
<td>FB8.27 pAM239::hemR, pBAD24::hbpC, Tet&lt;sup&gt;R&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>FB8.27 pAM239::hemR, pBAD24::hbpD</td>
<td>FB8.27 pAM239::hemR, pBAD24::hbpD, Tet&lt;sup&gt;R&lt;/sup&gt;, Spc&lt;sup&gt;R&lt;/sup&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Bartonella strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. henselae Houston-1</td>
<td>Houston-1, ATCC 49882&lt;sup&gt;T&lt;/sup&gt;</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>B. henselae pNS2Trc::hbpA&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>B. henselae carrying pNS2Trc::hbpA&lt;sub&gt;AS&lt;/sub&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>B. henselae pNS2Trc::hbpB&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>B. henselae carrying pNS2Trc::hbpB&lt;sub&gt;AS&lt;/sub&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>B. henselae pNS2Trc::hbpC&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>B. henselae carrying pNS2Trc::hbpC&lt;sub&gt;AS&lt;/sub&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>B. henselae pNS2Trc::hbpD&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>B. henselae carrying pNS2Trc::hbpD&lt;sub&gt;AS&lt;/sub&gt;, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pBAD24</td>
<td>pBR322 araC, arabinose-inducible promoter, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pAM239::hemR</td>
<td>pAM239 carrying hemR from Serratia marcescens</td>
<td>[76]</td>
</tr>
<tr>
<td>pBAD24::hbpA</td>
<td>pBAD24 carrying hbpA from B. henselae, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD24::hbpB</td>
<td>pBAD24 carrying hbpB from B. henselae, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD24::hbpC</td>
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<td>This study</td>
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<tr>
<td>pBAD24::hbpD</td>
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<td>This study</td>
</tr>
<tr>
<td>pNS2Trc</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNS2Trc::hbpA&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>pNS2Trc carrying anti-sense hbpA&lt;sub&gt;AS&lt;/sub&gt;, km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNS2Trc::hbpB&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>pNS2Trc carrying anti-sense hbpB&lt;sub&gt;AS&lt;/sub&gt;, km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNS2Trc::hbpC&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>pNS2Trc carrying anti-sense hbpC&lt;sub&gt;AS&lt;/sub&gt;, km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNS2Trc::hbpD&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>pNS2Trc carrying anti-sense hbpD&lt;sub&gt;AS&lt;/sub&gt;, km&lt;sup&gt;R&lt;/sup&gt;</td>
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Table 2. Primers used in this study

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<tr>
<th>Primer</th>
<th>Gene</th>
<th>Organism</th>
<th>Sequence</th>
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<tr>
<td>HbpABhamont</td>
<td>hbpA</td>
<td>B. henselae</td>
<td>5’ CTAGCTAGCAGGAGGAATTCACCATGAATAAAAATCTTTAATGA 3’</td>
</tr>
<tr>
<td>HbpABhaval</td>
<td>hbpA</td>
<td>B. henselae</td>
<td>5’ CGGGGTACCTCAGTGTTGGTGGTGGTGGTAATTTTGCTACACCAACACCG 3’</td>
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<tr>
<td>HbpBBhamont</td>
<td>hbpB</td>
<td>B. henselae</td>
<td>5’ CTAGCTAGCAGGAGGAATTCACCATGAATACGAAACGTTTAATAACAG 3’</td>
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<tr>
<td>HbpBBhaval</td>
<td>hbpB</td>
<td>B. henselae</td>
<td>5’ ATCCCCGAAGCTTTATATGTGTTGGTGGTGGTGGTAATTTTGTAAGCGACAC 3’</td>
</tr>
<tr>
<td>HbpCBhamont</td>
<td>hbpC</td>
<td>B. henselae</td>
<td>5’ CTAGCTAGCAGGAGGAATTCACCATGAATACGAAACGTTTAATAACAG 3’</td>
</tr>
<tr>
<td>HbpCBhaval</td>
<td>hbpC</td>
<td>B. henselae</td>
<td>5’ CGGGGTACCTCAGTGTTGGTGGTGGTGGTAATTTTGTAAGCGACACAC 3’</td>
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<tr>
<td>HbpDBhamont</td>
<td>hbpD</td>
<td>B. henselae</td>
<td>5’ CTAGCTAGCAGGAGGAATTCACCATGAATACGAAACGTTTAATAACAG 3’</td>
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<tr>
<td>HbpDBhaval</td>
<td>hbpD</td>
<td>B. henselae</td>
<td>5’ CGGGGTACCTCAGTGTTGGTGGTGGTGGTAATTTTGTAAGCGACACAC 3’</td>
</tr>
<tr>
<td>hbpAantisensamt</td>
<td>Antisens</td>
<td>hbpA</td>
<td>B. henselae 5’ CCCGGATCCTTAGAATTTTGTAAGCTACACC3’</td>
</tr>
<tr>
<td>hbpAantisensavl</td>
<td>Antisens</td>
<td>hbpA</td>
<td>B. henselae 5’ CCCGGATCCTTAGAATTTTGTAAGCTACACC3’</td>
</tr>
<tr>
<td>hbpBantisensamt</td>
<td>Antisens</td>
<td>hbpB</td>
<td>B. henselae 5’ CCCGGATCCTTAGAATTTTGTAAGCGACACC3’</td>
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<tr>
<td>hbpBantisensavl</td>
<td>Antisens</td>
<td>hbpB</td>
<td>B. henselae 5’ CCCGGATCCTTAGAATTTTGTAAGCGACACC3’</td>
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<td>hbpCantisensamt</td>
<td>Antisens</td>
<td>hbpC</td>
<td>B. henselae 5’ CCCGGATCCTTAGAATTTTGTAAGCGACACC3’</td>
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<tr>
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<td>Antisens</td>
<td>hbpC</td>
<td>B. henselae 5’ CCCGGATCCTTAGAATTTTGTAAGCGACACC3’</td>
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<td>Antisens</td>
<td>hbpD</td>
<td>B. henselae 5’ CCCGGATCCTTAGAATTTTGTAAGCGACACC3’</td>
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<td>hbpDantisensavl</td>
<td>Antisens</td>
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<td>B. henselae 5’ CCCGGATCCTTAGAATTTTGTAAGCGACACC3’</td>
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<td>bh2390fo</td>
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<td>B. henselae</td>
<td>5’ GGGATGGTGGAATTTTGTAAGC 3’</td>
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<tr>
<td>bh2390re</td>
<td>bh2390</td>
<td>B. henselae</td>
<td>5’ CCAAAACGGCAAAAGAC 3’</td>
</tr>
<tr>
<td>Cf18Sf</td>
<td>18S rDNA</td>
<td>C. felis</td>
<td>5’ TGTCACCAGTTGGACTTGGG 3’</td>
</tr>
<tr>
<td>Cf18Sr</td>
<td>18S rDNA</td>
<td>C. felis</td>
<td>5’ GTTTTCAGGCTCCCTGTACC 3’</td>
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Table 3. The effect of Hbps on HemR-dependent heme uptake.

*E. coli* strains FB8.27 pAM239::hemR pBAD24, FB8.27 pAM239::hemR pBAD24::hbpA, FB8.27 pAM239::hemR pBAD24::hbpB, FB8.27 pAM239::hemR pBAD24::hbpC and FB8.27 pAM239::hemR pBAD24::hbpD were tested for efficiency of heme utilization as an iron source in iron-depleted medium M63 (Gly 0.4%, Ara 0.02%, Dip 80 or 100 μM, Spc, Amp). Growth around the wells containing 1 μM, 5 μM, 10 μM, or 50 μM Hb was as described in Materials and methods. After 48 h of growth, the diameter of the zone of turbidity around the well was measured in quadruplicate for each plate and the mean diameter was calculated. Results are expressed as mean±SD of the diameter (in cm) obtained for the three plates. NM: Not measurable.

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Diameter of the halo (cm) (Dip 80 μM)</th>
<th>50 μM</th>
<th>10 μM</th>
<th>5 μM</th>
<th>1 μM</th>
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<tbody>
<tr>
<td>FB8.27 pAM::hemR (pBAD24)</td>
<td>1.82±0.015</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
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<tr>
<td>FB8.27 pAM::hemR (pBAD24::hbpA)</td>
<td>2.23±0.04</td>
<td>1.78±0.03</td>
<td>1.48±0.09</td>
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<td>NM</td>
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<tr>
<td>FB8.27 pAM::hemR (pBAD24::hbpB)</td>
<td>2.77±0.11</td>
<td>2.37±0.08</td>
<td>2.31±0.09</td>
<td>1.84±0.1</td>
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<tr>
<td>FB8.27 pAM::hemR (pBAD24::hbpC)</td>
<td>2.09±0.04</td>
<td>1.64±0.03</td>
<td>1.42±0.08</td>
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<tr>
<td>FB8.27 pAM::hemR (pBAD24::hbpD)</td>
<td>2.55±0.10</td>
<td>1.92±0.09</td>
<td>1.68±0.09</td>
<td>1.22±0.06</td>
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</table>

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Diameter of the halo (cm) (Dip 100 μM)</th>
<th>50 μM</th>
<th>10 μM</th>
<th>5 μM</th>
<th>1 μM</th>
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<tr>
<td>FB8.27 pAM::hemR (pBAD24)</td>
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<tr>
<td>FB8.27 pAM::hemR (pBAD24::hbpA)</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>FB8.27 pAM::hemR (pBAD24::hbpB)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>FB8.27 pAM::hemR (pBAD24::hbpC)</td>
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<td>-</td>
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<tr>
<td>FB8.27 pAM::hemR (pBAD24::hbpD)</td>
<td>+</td>
<td>-</td>
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</table>
Table 4. Effect of hbps knockdown on growth of *B. henselae* on blood plates.

For the growth test on CBA plates, strains *B. henselae* (pNS2Trc), *B. henselae* (pNS2Trc::*hbpA*::AS), *B. henselae* (pNS2Trc::*hbpB*::AS), *B. henselae* (pNS2Trc::*hbpC*::AS) and *B. henselae* (pNS2Trc::*hbpD*::AS) were collected after 5 days of growth on CBA plates and suspended in PBS buffer to obtain about 10^3 CFU ml^-1. Two-hundred microliters of cell suspension were plated on the CBA plate. Colony sizes were measured after 6 and 10 days of growth at 35°C in the presence of 5% CO₂. Data are the mean diameter (mm) ± SD of 10 colonies from one representative experiment. Standard deviations were calculated using Statview software. All experiments were repeated three times. NM: not measurable

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 6</td>
</tr>
<tr>
<td><em>B. henselae</em> (pNS2Trc)</td>
<td>0.58±0.12</td>
</tr>
<tr>
<td><em>B. henselae</em> (pNS2Trc::<em>hbpA</em>::AS)</td>
<td>NM</td>
</tr>
<tr>
<td><em>B. henselae</em> (pNS2Trc::<em>hbpB</em>::AS)</td>
<td>NM</td>
</tr>
<tr>
<td><em>B. henselae</em> (pNS2Trc::<em>hbpC</em>::AS)</td>
<td>NM</td>
</tr>
<tr>
<td><em>B. henselae</em> (pNS2Trc::<em>hbpD</em>::AS)</td>
<td>NM</td>
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</tbody>
</table>
Table 5. Detection of *B. henselae* DNA from flea feces samples using PCR.

About 500 fleas were first feed with blood containing 500 μl bacteria (2 × 10^8/ml) for 2 days and then fed uninfected blood for 8 days. Flea feces were collected every day. DNA was extracted from flea feces and PCR was performed as described in Materials and methods.

<table>
<thead>
<tr>
<th>Flea feces samples (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. henselae</em> pNS2Trc</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. henselae</em> pNS2Trc::hbpA&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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</tr>
<tr>
<td><em>B. henselae</em> pNS2Trc::hbpB&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>-</td>
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</tr>
<tr>
<td><em>B. henselae</em> pNS2Trc::hbpC&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
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</tr>
<tr>
<td><em>B. henselae</em> pNS2Trc::hbpD&lt;sub&gt;AS&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
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</table>
Fig 1. Congo red binding assays. Strains XL1-Blue pBAD24 and XL1-Blue pBAD24 expressing Hbps were expressed for 2 h at 37°C as described in Materials and methods. Expression was evident in SDS-PAGE (A). Line 1: XL1-Blue pBAD24, lines 2 to 5: XL1-Blue pBAD24 expressing HbpA, B, C, D, respectively (20 μg). After plating on Congo red plates supplemented with 0.2% arabinose, strains were grown for 20 h at 37°C. Strain XL1-Blue pBAD24 (B) formed white colonies. Strain XL1-Blue pBAD24::hbpA (C), XL1-Blue pBAD24::hbpB (D), XL1-Blue pBAD24::hbpC (E) and XL1-Blue pBAD24::hbpD (F) formed red colonies.

Fig 2. Detection of heme binding by Hbps. After SDS gel electrophoresis, one gel was stained with Coomassie brilliant Blue R. Another gel was transferred to a nitrocellulose filter to perform heme blotting and was detected by ECL as described in Materials and methods. (A) Coomassie Blue staining result. Line 1: 1 μg HbpA, line 2: 1 μg HbpB, line 3: 1 μg HbpC, line 4: 1 μg HbpD, line 5: 3 μg BSA. (B) Heme binding results. Line 1: 1 μg HbpA, line 2: 1 μg HbpB, line 3: 1 μg HbpC, line 4: 1 μg HbpD, line 5: 3 μg BSA.

Fig 3. Detection of HbpA expression levels in B. henselae pNS2Trc and B. henselae pNS2Trc::hbpA AS by immunoblotting.

40 μg samples of B. henselae pNS2Trc (line 1) and B. henselae pNS2Trc::hbpA AS (line 2) and a 100 ng sample of purified his-tagged HbpA (line 3) were loaded on SDS-PAGE. After electrophoresis, one gel was stained with Coomassie brilliant Blue
Fig 4. Effect of *hbps* knockdown on growth of *B. henselae* in Schneider’s medium.

Strains *B. henselae pNS2Trc* and *B. henselae pNS2Trc::hbpsAS* were cultured in Schneider’s medium, collected after 5-day growth on CBA blood plates and suspended in Schneider’s medium. The bacterial suspension was used to inoculate Schneider’s medium at an OD$_{600}$ of 0.05. Cultures were grown at 35°C in the presence of 5% CO$_2$ and OD$_{600}$ was measured on days 2, 4, 5 and 7 after inoculation. All experiments were repeated three times.

**Fig. 5. Hbp knockdown decreases the ability of *B. henselae* to undergo exposure to H$_2$O$_2$.**

*B. henselae pNS2Trc* and *B. henselae pNS2Trc::hbpsAS* were challenged with 10 mM H$_2$O$_2$ as described in Materials and methods. Experiments were performed in triplicate; survival rates were expressed as described in Materials and methods. (*P<0.05, **P<0.01 compared to *B. henselae pNS2Trc*).

**Fig 6. Effect of Hbp knockdown on endothelial cell invasion.**

Invasion of endothelial cells by *B. henselae pNS2Trc* and *B. henselae
pNS2Trc::hbps_{AS}. Cells were mixed with bacteria at 0.1 m.o.i. After 24 h, infected cell were treated with gentamicin to kill extracellular bacteria and lysates were plated on the CBA blood plate to determine the number of intracellular bacteria. Invasion was calculated using the equation provided in Materials and methods. (**P<0.005 compared to \textit{B. henselae} pNS2Trc)

**Fig 7. Effect of Hbp knockdown on survival of \textit{B. henselae} in endothelial cells.**

Survival of \textit{B. henselae} pNS2Trc and \textit{B. henselae} pNS2Trc::hbps_{AS} in endothelial cell. After gentamicin killing, infected cell were grown for 24 h or 48 h in modified DMEM medium. Lysates were incubated in Schneider’s medium overnight. Then lysates were collected by centrifuge and plated on CBA blood plates to determine the number of intracellular bacteria. The survival rate was calculated using the equation provided in Materials and methods. (*P<0.05, **P<0.01, ***P<0.005 compared to \textit{B. henselae} pNS2Trc)
Fig 1. Congo red binding phenotype of XL1-Blue (pBAD24) (B), XL1-Blue (pBAD24::hbpA) (C), XL1-Blue (pBAD24::hbpB) (D), XL1-Blue (pBAD24::hbpC) (E) and XL1-Blue (pBAD24::hbpD) (F).
Fig 2. Detection of Hbp binding to heme.
Fig 3. Detection of the HbpA expression level in *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::*hbpA*AS by immunoblotting.
Fig 4. Effect of hbp knockdown on growth of *B. henselae* in Schneider’s medium.
Fig 5: Hbp knockdown decreases *B. henselae* capacity to face H$_2$O$_2$ challenge.
Fig 6: Effect of Hbp knockdown on endothelial cell invasion.
Fig 7: Effect of Hbp knockdown on survival of *B. henselae* in endothelial cells.
Supplement Table 1

Heme acquisition of an *E. coli* hem*A* strain expressing the *B. henselae* Hbps protein.

*C600ΔhemA* is incapable of the synthesis of Δala, a heme precursor that is necessary for bacterial growth in an aerobic environment on LB rich medium. Because *E. coli* does not have a heme transport system, in the presence of extra cellular heme does not permit bacterial growth unless such a transport system is introduced. *E. coli* strains *C600 Δ hemA pAM::hasR* (positive control), *C600 Δ hemA pBAD24* (negative control), *C600ΔhemA pBAD24* expressing Hbps were tested for heme transport activity in LB plates (Ara 0.02%). Wells were cut in the agar and filled with 100 μl of 10⁻⁴M, 10⁻⁵M or 10⁻⁶M of filter-sterilized Hb or heme solution. Growth was recorded around the wells after 48-hours incubation at 37°C. This growth assay was used with success to visualize heme acquisition by the heme transporter HasR of *Serratia marcescens* (*S. marcescens*). *C600ΔhemA pBAD24* expressing Hbps have no any growth was observed.

<table>
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</thead>
<tbody>
<tr>
<td>Heme10⁻⁴M</td>
<td>+</td>
<td>-</td>
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<tr>
<td>Heme10⁻⁵M</td>
<td>+</td>
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<td>Heme10⁻⁶M</td>
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<td>Hb 10⁻⁵M</td>
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<td>Hb 10⁻⁶M</td>
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</tbody>
</table>
Supplement Table 2

Iron acquisition of an *E. coli* strains FB8.27 expressing the *B. henselae* HutA protein.

*E. coli* strains FB8.27 pAM239::hemR (positive control), FB8.27 pBAD24 (negative control) and FB8.27 pBAD24::hutA were tested for heme utilization as an iron source in iron-depleted medium M63 (Gly 0.4%, Ara 0.02%, Dip 80, Spc or Amp). Wells were cut in the agar and filled with 100 μl of 50 μM, 10 μM, 5 μM, 1 μM of filter-sterilized Hb solution. Growth was recorded around the wells after 48-hours incubation at 37°C.

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Hb 50 μM</th>
<th>Hb 10 μM</th>
<th>Hb 5 μM</th>
<th>Hb 1 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>FB8.27 pAM::hemR</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FB8.27 pBAD24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FB8.27 pBAD24::hutA</td>
<td>-</td>
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</tr>
</tbody>
</table>
Conclusion

In the present report, we investigated the function of heme binding proteins in heme utilization, the oxidative stress response, endothelial cell colonization and flea transmission. For heme utilization, we first showed that heme binding proteins are able to bind Congo red \textit{in vivo} and heme \textit{in vitro}. We next showed that heme binding proteins of \textit{B. henselae} cannot transport heme when expressed in the \textit{E. coli hemA} mutant. We then sought to determine whether heme binding proteins affect HutA-mediated heme uptake activity when co-expressed in \textit{E. coli}. We failed to reconstitute the activity of HutA as a heme transporter in \textit{E. coli}, although it has been claimed that HutA of \textit{B. quintana} can transport heme when expressed in an \textit{E. coli hemA} mutant in the presence of high heme concentrations (Parrow et al., 2009). We thus examined the effect of heme binding proteins on the heme uptake activity of heme transporter HemR of \textit{S. marcescens} when expressed in \textit{E. coli}. Our results clearly showed that heme binding proteins increase HemR-mediated heme transport when expressed in \textit{E. coli}. We hypothesized that expression of heme binding proteins increased the heme concentration surrounding the bacteria, thus facilitating heme transport activity.

In order to reside in endothelial cells and fleas, \textit{B. henselae} must confront the oxidative stress provoked by bacterial infection (Rydkina et al., 2002; Zhou et al., 2012). However, many homologues of genes involved in the oxidative stress response in \textit{E. coli} are not present in \textit{Bartonellae}. The fact that \textit{B. bacilliformis} was able to successfully endure 30 min exposure to 1 mM H$_2$O$_2$ (Coleman and Minnick, 2003) implies that \textit{Bartonellae} can efficiently sustain oxidative stress using an
uncharacterized pathway. It was proposed that heme binding proteins might be a way of facing oxidative stress using the intrinsic peroxidase activity of heme (Battisti et al., 2006). Therefore, we first checked whether heme binding proteins are involved in an oxidative stress response by decreasing the expression of heme binding proteins in *B. henselae*. Our results showed that HbpA, HbpB, HbpC and HbpD knockdown decreases the capacity of *B. henselae* to endure oxidative stress induced by exposure to H$_2$O$_2$.

Since heme binding proteins were shown to be involved in oxidative stress *in vitro*, it was reasonable to hypothesize that heme binding protein knockdown expression in *B. henselae* might affect invasion and survival in endothelial cells and flea gut. We first verified the effect of heme binding protein knockdown upon invasion of *B. henselae* into endothelial cells and survival within them. We showed that HbpA and HbpD are involved in endothelial cell invasion, and that all heme binding proteins are involved in survival of *B. henselae* inside endothelial cell. Involvement of HbpA in endothelial cell invasion had already been described (Dabo et al., 2006a). The Opa domain of HbpA, which has been shown to be involved in entry into host epithelial cells in *Neisseria gonorrhoeae* (Weel et al., 1991), lends support to this conclusion. For HbpD, its involvement in the endothelial cell invasion process is striking. The higher sensitivity to oxidative stress produced by endothelial cells in response to bacterial invasion might explain our observation. This hypothesis is also supported by the fact that the *B. henselae* *hbpD* mutant cannot delay lysosomal fusion, thus leading to reduced viability inside endothelial cells (Kyme et al., 2005).
In subsequent experiments, we also showed that decreased expression of heme binding proteins affected the ability of \textit{B. henselae} to survive inside the flea. Taken together, these results demonstrate that the anti-oxidative stress response activity of heme binding proteins is important in the infection cycle of \textit{B. henselae}. 
Discussion and perspectives

To accomplish their infection cycle, *Bartonella* must overcome two main hurdles: meeting the necessary nutrient requirements and confronting oxidative stress immune defenses produced by hosts in their resident niches.

To meet nutrient requirements, heme is one of the most important elements in growth of *Bartonella*. The latter cannot synthesize this compound, which is also the main iron source. Although it is one component of the heme acquisition system, HutA of *B. quintana* has been characterized as a heme transporter (Parrow et al., 2009), though the fate of heme in the cytoplasm is unclear. Moreover, the function of outer membrane proteins able to bind heme has remained unidentified. In the present report, our data clearly show that HemS can degrade heme *in vivo* and *in vitro*.

We established that heme binding proteins are able to bind Congo red *in vivo* and heme *in vitro*. We failed to observe heme transport activity when expressing heme binding proteins of *B. henselae* in *E. coli*. In contrast, we showed that heme binding proteins increase heme uptake efficiency when co-expressed with a heterologous heme transporter in an *E. coli* model strain. *Bartonella* species have an arthropod vector infection cycle. In the gut of the arthropod, high heme levels are present and are toxic. In contrast, in the mammalian host, available heme is scarce. *Bartonella* genomes do not encode for bacterioferritin, ferritin-like heme or iron storage proteins. It has been proposed that heme binding proteins function as a heme reservoir on the surface of *Bartonella* (Battisti et al., 2006). Here we propose that heme binding proteins increase the heme concentration around the bacteria in the arthropod vector,
thus facilitating *Bartonella* use of heme after exploitation in the mammalian host. An intriguing parallel occurs in the flea-borne agent of bubonic plague *Yersinia pestis*, where the outer membrane is the primary site of exogenous heme storage (Hinnebusch et al., 1996). Future work should focus on the mechanism by which heme is transferred from heme binding proteins to the heme transporter.

Compared to other pathogens that also lack heme synthesis, the heme requirement of *Bartonella* is 100-fold higher than that of *Porphyromonas gingivalis* and 1,000-fold higher than that of *Haemophilus influenzae* under aerobic iron-replete conditions (Liu et al., 2006; Myers et al., 1972; White and Granick, 1963). It was hypothesized by other authors that a high heme requirement consists not only of metabolic nutrients but also of mediators of metabolic homeostasis (Battisti et al., 2006), where heme may function as a defense mechanism against ROS, or else exogenously generate a decreased oxygen environment for the bacteria (Battisti et al., 2006).

It is generally agreed that *Bartonella* species are aerobic (Birtles et al., 1995). During aerobic respiration, superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are naturally generated, and superoxide dismutase and catalase/peroxidase are normally used for intracellular detoxification (Messner and Imlay, 1999). Moreover, it was shown that ROS production is an important immune defense mechanism of the mammal host and arthropod vector against pathogenic bacteria (Graca-Souza et al., 2006). As a consequence, *Bartonellae* must possess enzymes to prevent and repair oxidative stress damages.
Many homologues of genes involved in the oxidative stress response in *E. coli* are not present in *Bartonella* genomes (Table 1).

**Table 1. Genes involved in oxidative defense in *E. coli* and *Bartonella*.**

<table>
<thead>
<tr>
<th>Function</th>
<th><em>E. coli</em></th>
<th><em>B. henselae</em></th>
<th><em>B. quintana</em></th>
<th><em>B. bacilliformis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ros degradation</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>sodA</em></td>
<td>Manganese superoxide dismutase</td>
<td>+</td>
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<tr>
<td><em>sodB</em></td>
<td>Iron superoxide dismutase</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td><em>sodC</em></td>
<td>Copper-zinc superoxide dismutase</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td><em>katG</em></td>
<td>Hydroperoxidase I</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>katE</em></td>
<td>Hydroperoxidase II</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>ahpC</em></td>
<td>Alkyl hydroperoxide reductase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>ahpF</em></td>
<td>Alkyl hydroperoxide reductase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>dps</em></td>
<td>Non-specific DNA binding protein</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>acrA</em></td>
<td>ROS excretion</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td><em>acrB</em></td>
<td>ROS excretion</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>ROS damage repair</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>gorA</em></td>
<td>Glutathione reductase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>grxA</em></td>
<td>Glutaredoxin 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>msrA</em></td>
<td>Methionine sulfoxide reductase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>msrB</em></td>
<td>Methionine sulfoxide reductase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>trxB</em></td>
<td>Thioredoxin reductase</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>zwf</em></td>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td><em>fpr</em></td>
<td>Ferrodoxin reductase</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>nfo</em></td>
<td>Endonuclease IV</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>xthA</em></td>
<td>Exonuclease III</td>
<td>+</td>
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</table>

|------------|-----------------|-----------------|-----------------|-----------------|

It was thus hypothesized that *Bartonella* use an uncharacterized pathway to confront oxidative stress. We found that HemS was involved in the oxidative stress response of *B. henselae in vivo*. *B. henselae* HemS is the first identified heme-degrading protein shown to be involved in the oxidative stress response. However, HemS knockdown in *B. henselae* did not have any effect on the ability to survive inside endothelial cells (data not shown), where ROS are produced in response to bacterial invasion. One possible explanation is that HemS destroys only endogenous H$_2$O$_2$. This hypothesis was supported by the absence of a strategy for endogenous H$_2$O$_2$ detoxification, according to genome analysis (Table 1). For heme binding proteins, we showed that *B.
*henselae* was more sensitive to H$_2$O$_2$ exposure when heme binding proteins showed decreased expression. In addition, the heme blotting technique that we used to identify heme binding proteins relies on the intrinsic peroxidase activity of heme. These results strongly support the hypothesis that heme binding proteins may serve as an antioxidant barrier in *Bartonella*. Similarly, it was shown that *P. gingivalis* stores heme on its surface. Stored heme can function as an antioxidant due to its intrinsic peroxidase activity (Smalley et al., 2000) and might also exclude oxygen from the cell (Smalley et al., 1998). *Bartonella* are members of the order *Rhizobiales*. Many rhizobia form a symbiotic relationship with their legume host plant by fixing atmospheric nitrogen in root nodules. For nitrogen fixation to occur, a microaerophilic environment must be established for the bacteria. This is accomplished by plant-generated leghemoglobin, a molecule similar to hemoglobin binding to the rhizobial surface to shield the bacteria from oxygen (Appleby et al., 1983). Considering the close relationship of *Bartonella* and rhizobia, it has been proposed that heme binding is a common strategy used by members of this order to decrease oxygen in the environment (Battisti et al., 2006).

Interestingly, when compared to *B. henselae* HbpA, B, C knockdown mutants, the *B. henselae* HbpD mutant appears to be more sensitive to H$_2$O$_2$. We hypothesized that HbpD might be more efficient at confronting oxidative stress in *B. henselae*. To further understand the role of heme binding proteins in *B. henselae* physiology, we checked for the effect of *hbps* knockdown on the *B. henselae* capacity to invade erythrocytes and endothelial cells. We also investigated the effect of *hbps* knockdown
on the *B. henselae* capacity to survive in the flea.

Concerning erythrocyte invasion, we did not see any effect when expression of heme binding proteins was decreased. Consistently, it has been shown that all *hbp* transcripts significantly decrease when *B. quintana* is grown at an O$_2$ concentration (5%) that simulates the human bloodstream (Battisti et al., 2006). It was proposed that bacteria grown at 5% oxygen encounter fewer ROS; thus, fewer heme binding proteins are necessary to combat ROS (Battisti et al., 2006). Recently, it was proposed by other authors that heme binding proteins did not play a role in the bloodstream (Harms and Dehio, 2012).

For endothelial cell invasion, we showed that *B. henselae* less efficiently invaded endothelial cells when HbpA or HbpD expression was decreased. It has already been shown that recombinant HbpA of *B. henselae* is able to adhere to endothelial cells (Dabo et al., 2006a) and this result is easily explained by the presence of an “Opa” domain in HbpA. The Opa protein was shown to be involved in entry into the host epithelial cell in *Neisseria gonorrhoeae* (Weel et al., 1991). The involvement of HbpD in the endothelial cell invasion process is more striking and could be explained by greater sensitivity to oxidative stress produced by endothelial cells, thus leading to more rapid killing of bacteria. This hypothesis needs to be further confirmed experimentally.

We also clearly showed that all heme binding proteins are involved in survival of *B. henselae* inside endothelial cell. The weaker survival capacity in endothelial cells, related to knockdown of all heme binding proteins, might be related to weaker
resistance to oxidative stress. It has already been shown that the HbpD mutant of *B. henselae* is attenuated in intracellular survival in endothelial cells because it can not delay lysosomal fusion (Kyme et al., 2005). How heme binding proteins might modify the intracellular signal cascade needs to be further experimentally demonstrated.

Many arthropod vectors, such as sandflies, body lice and fleas have been implicated as vectors in *Bartonella* transmission. These arthropod vectors acquire blood meal several times daily (Graca-Souza et al., 2006), whereupon erythrocytes are hemolyzed almost immediately. As a result, the bacteria are exposed to waves of potentially toxic heme and ROS with each blood meal (Graca-Souza et al., 2006). Since we showed that *B. henselae* heme binding proteins against H$_2$O$_2$ produced oxidative stress damage, it was reasonable to hypothesize that Hbp knockdown expression affects colonization of *B. henselae* in fleas. Here, our results showed that *B. henselae* heme binding protein knockdown mutant strains were eliminated more quickly in flea feces than the wild type, suggesting decreased ability of heme binding protein knockdown mutant strains to colonize fleas. This result was supported by profiles of regulation of heme binding proteins. It was shown that the expression of HbpC of *B. quintana* increased 108-fold at the louse-like temperature (30°C) than at 37°C. Recently, it was also shown that *hbpA* transcript expression is significantly increased in *B. henselae* at 28°C compared to 37°C (Roden et al., 2012), suggesting that HbpA and HbpC might affect arthropods. In any case, all heme binding proteins
are related to survival in the flea, probably due to the anti-oxidant activity of heme binding proteins.

To accomplish the infection cycle, *Bartonella* must be able to survive and replicate in the different niches they encounter, namely the arthropod vector, the primary niche (endothelial cells) and erythrocytes. As we have shown in this report, heme binding proteins of *B. henselae* play an important role in its two resident microniches: endothelial cells and fleas. The anti-oxidant activity of heme binding proteins might be crucial for survival in these two resident micro-niches. In summary, we conclude that heme binding proteins of *Bartonella* play an important role in virulence and the infection cycle. A better understanding of the function of the heme binding protein family should help us to develop strategies for fighting infection and transmission. However, other factors might also be involved in the *Bartonella* oxidative stress response. Further experiments are necessary to elucidate this question.

As described above, the oxidative stress response plays an important role in the infection cycle of *Bartonellae*. This led us to attempt to identify other actors in the *B. henselae* oxidative stress response. Genome analysis showed that *Bartonellae* encode for homologues of YfeABCD from *Yersinia pestis* and SitABCD from avian pathogenic *E. coli* that had been characterized as Fe$^{2+}$ and Mn$^{2+}$ transporters (Anjem et al., 2009; Sabri et al., 2006). It was shown that manganese contributes to protection against oxidative stress as a cofactor in a number of enzymes in bacteria (Kehres and Maguire, 2003) and might also contribute directly to degradation of hydrogen peroxide (Horsburgh et al., 2002). It was shown that disruption of manganese
transporters increases sensitivity to hydrogen peroxide in avian pathogenic *E. coli* (Sabri et al., 2006). To identify the function of YfeABCD in *B. henselae*, we first checked for the effect of YfeAB knockdown upon the ability to face exposure to hydrogen peroxide. Our results clearly demonstrated that *yfeAB* knockdown strongly increases the sensitivity of *B. henselae* to hydrogen peroxide exposure. A weaker effect was observed with *yfeA* knockdown (Figure 1). Consequently, we searched for the effects of *yfeA* and *yfeAB* knockdown on the ability of *B. henselae* to survive inside endothelial cells and fleas. Our results showed that *yfeAB* knockdown decreased the ability of *B. henselae* to survive inside both endothelial cells and fleas (Fig 2 and Table 2). Thus, manganese transport can be proposed as an actor in the oxidative stress response and, consequently, survival inside cells and fleas in *B. henselae*. Further experiments should investigate whether the YfeABCD transporter of *B. henselae* is involved in manganese transport and anti-oxidant activity in an *E. coli* model strain.
Fig. 1: YfeA and YfeAB knockdown decreases the ability of *B. henselae* to face exposure to H$_2$O$_2$.

![Graphs showing survival of *B. henselae*](image)

**Fig 2:** Effect of YfeA and YfeAB knockdown on survival of *B. henselae* in endothelial cells.

**Table 2:** Effect of YfeA and YfeAB knockdown on survival of *B. henselae* in fleas. *B. henselae* DNA was detected from flea feces samples using PCR.

<table>
<thead>
<tr>
<th>Flea feces samples (days)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. henselae pNS2Trc</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. henselae pNS2Trc::yfeAAS</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. henselae pNS2Trc::yfeABAS</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tbody>
</table>

In the present report, we identified three actors, HemS, heme binding proteins and YfeABCD, involved in the oxidative stress response in *B. henselae*. Oxidative stress is caused by exposure to reactive oxygen intermediates such as superoxide
anion ($O_2^-$), hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (HO •) (Storz and Imlay, 1999). The sources of oxidative stress mainly include the following: 1) ROS, endogenously produced by aerobic metabolism (Storz and Imlay, 1999). $O_2^-$ and $H_2O_2$ are formed whenever molecular oxygen chemically oxidizes electron carriers (Fig 3). Reduced flavoproteins have been implicated in this process in $E. coli$ (Storz and Imlay, 1999).

![Fig. 3 The redox state of oxygen with standard reduction potential. Adapted from (Imlay, 2008).](image)

2) Plants, other microorganism and animals that exploit ROS to attack bacterial competitors (Storz and Imlay, 1999). In the case of Bartonella, it is generally agreed that Bartonella species are aerobic (Birtles et al., 1995). Thus, superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) are naturally generated. During its infection cycle, Bartonella must survive in its arthropod vector and primary niche, the endothelial cell, presumed to produce ROS to face bacterial invasion (Graca-Souza et al., 2006; Rydkina et al., 2002; Rydkina et al., 2010). Based on our results, we proposed that HemS and YfeABCD are mainly involved in destroying endogenous $H_2O_2$ produced by aerobic bacterial metabolism. Heme binding proteins could degrade exogenous $H_2O_2$ encountered in the flea gut and during endothelial cell invasion. Since all Bartonella species have a similar life cycle, these genes might have a conserved function in this genus.
ROS attack most macromolecules, including nucleic acids, cysteine and methionine protein residues and lipids (Ezraty et al., 2005). To protect against damage caused by oxidative stress, bacteria possess not only a number of antioxidant enzymes, but also repair activities such as DNA and protein repair. However, Bartonella genomes do not encode for methionine sulfoxide reductase activity (MsrA and MsrB) (Table 1), which makes methionine oxidation a reversible process. Here we proposed that Bartonella do not need these genes to repair protein, or else they use another means of repairing methionine sulfoxide. Oxidative stress responses coordinated by specific regulators ensure bacterial survival during episodic exposure to exogenous ROS or to ROS generated as a consequence of normal respiration (Chiang and Schellhorn, 2012). In E. coli, gene responses to oxidative stress are regulated mainly by three regulators, OxyR, SoxR and RpoS (Chiang and Schellhorn, 2012). OxyR controls a regulon of almost 40 genes which protects the cell from hydrogen peroxide toxicity in E. coli (Chiang and Schellhorn, 2012). SoxR is a regulator of resistance to superoxide radicals (Tsaneva and Weiss, 1990), while RpoS is a stationary phase general stress response regulator (Lange and Hengge-Aronis, 1991). However, Bartonella genomes also do not encode for these regulators. For regulation of heme binding proteins, it has been suggested that, when Bartonella grow in an arthropod vector where the temperature is lower and the heme concentration is higher, heme binding proteins are upregulated by temperature and heme concentration to face oxidative stress. When Bartonella grow in erythrocytes where the oxygen concentration is lower, heme binding proteins are downregulated by the oxygen
concentration, corresponding to less oxidative stress. For regulation of HemS, it has been shown that hemS is regulated, at least in part, by Irr (Parrow et al., 2009). Free intracellular iron is easily oxidized by H₂O₂ to form the reactive hydroxyl radical via the Fenton reaction. Therefore, homeostatic control of free intracellular iron levels is important for minimizing oxidative stress. The Fur repressor is the principal regulator of iron homeostasis. For the transporter YfeABCD, we found that a Fur box is present upstream from the yfeABCD promoter. Thus, we proposed that YfeABCD was regulated by Fur. Also, it has already been shown that the iron-containing superoxide dismutase gene sodB was positively regulated by Fur (Niederhoffer et al., 1990). The manganese-containing superoxide dismutase gene sodA was negatively regulated by Fur in E. coli (Touati, 2000). Since iron homeostasis and the oxidative stress response are connected via regulatory interactions, we proposed that other Bartonella genes involved in oxidative stress, such as sodB, were regulated by the iron regulators Fur, Irr and RirA.
SUPPLEMENTARY PAPER

Identification of a novel nanoRNase in *Bartonella*

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Introduction

Comparative genomics is the reference tool for identifying essential biological functions. However, there is no one-to-one correspondence between structure and function, and comparison of 1,000 prokaryotic genomes has recently shown that there is no protein coding gene common to all prokaryotes. Thus, direct experimental approaches must be used to identify essential enzymes when they have no common ancestor. A case in point is the activity of nanoRNases, the enzymes that degrade RNA leftovers remaining after the action of RNases on long RNA molecules, in particular, messenger RNAs. These very short RNA oligos (3-5 mer) have been termed ‘nanoRNAs’ to distinguish them from the longer microRNAs. Among RNases with the ability to degrade nanoRNA are Orn of *Escherichia coli* and NrnA and NrnB of *Bacillus subtilis* (Fang et al., 2009). Orn homologs are widely present in Actinobacteria, Betaproteobacteria and Gammaproteobacteria. The homologs of NrnA or NrnB are widely present in Chlorobi/Bacteroidetes, Firmicutes and Deltaproteobacteria. Neither *Alphaproteobacteria* nor *Cyanobacteria* contain Orn, nor do NrnA or NrnB homologues. In the class of *Alphaproteobacteria*, numerous genera belonging to the *Rhizobiales* order contain plant symbionts (Stacey et al., 2006). Other members of the *Rhizobiales* order, like *Brucellaceae* and *Bartonellaceae*, have been reported to be pathogenic for mammals (Guerra, 2007), (Minnick and Battisti, 2009). The genus *Bartonella* comprises a unique group of facultative intracellular bacteria that use hemotrophism and arthropod transmission as a mammalian parasitism strategy (Chomel et al., 2009). *Bartonella* genomes sequenced to date are
short: 1.4 to 1.9 mega bases in size (Alsmark et al., 2004), and are the smallest of the
Rhizobiales order.

In the present report, we identified a functional Orn analog in the
Alphaproteobacteria family member B. birtlesii. This protein, BA0969 (now renamed
NrnC), was identified by screening a genomic library of B. birtlesii for genes that
complement a conditional lethal orn mutant in E. coli. NrnC was characterized both in
vitro and in vivo in E. coli and B. henselae.
Identification of a novel nanoRNase in Bartonella

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In Escherichia coli, only one essential oligoribonuclease (Om) can degrade oligoribonucleotides of five residues and shorter in length (nanoRNA). In Bacillus subtilis, NrmA and NrmB, which do not show any sequence similarity to Om, have been identified as functional analogues of Om. Sequence comparisons did not identify om, nrmA or nrmB homologues in the genomes of the Chlamydia/Cyanobacteria and Alphaproteobacteria family members. Screening a genomic library from Bartonella brisei, a member of the Alphaproteobacteria, for genes that can complement a conditional om mutant in E. coli, we identified BA0968 (NmcC) as a functional analogue of Om. NmcC is highly conserved (more than 80% identity) in the Bartonella genomes sequenced to date. Biochemical characterization showed that this protein exhibits oligo RNA degradation activity (nanoRNase activity). Like Om from E. coli, NmcC is inhibited by micromolar amounts of 5’-phosphoadenosine 5’-phosphate in vitro. NmcG homologues are widely present in genomes of Alphaproteobacteria. Knock down of nmcG decreases the growth ability of Bartonella henselae, demonstrating the importance of nanoRNase activity in this bacterium.

INTRODUCTION

Comparative genomics is the reference tool to identify essential biological functions, yet comparing genome sequences is not always successful. Indeed, there is no one-to-one correspondence between structure and function, and it has recently been shown that comparing 1000 prokaryotic genomes leaves no protein coding gene in common to all prokaryotes (Lageen et al., 2010). Direct experimental approaches must be used to identify essential enzymes when they have no common ancestor. A case in point is the activity of nanoRNases, the enzymes that degrade RNA remaining after the action of processive Rnas on long RNA molecules, in particular miRNAs. Investigation of RNA degradation in prokaryotes showed that different sets of enzymes can be devoted to this process in different bacteria. In the Gram-negative model bacterium Escherichia coli, close to 20 Rnas have been identified so far (Arralano et al., 2010). In this organism, the essential RNase E initiates the degradation of RNA endonucleolytically (Cordouy, 2007), and other RNases exhibiting a 3’> 5’ activity complete RNA degradation. RNase II produces 3’- to 5mers or 4- to 5mers (Cheng & Deutscher, 2002) as end products of degradation, whereas RNase R produces 2- to 3mers (Cheng & Deutscher, 2002; Vincent & Deutscher, 2006). The complete degradation of mRNAs requires the activity of oligoribonuclease (Om) (Yu & Deutscher, 1995), the only E. coli exoribonuclease able to degrade 2- to 5mers (Zhang et al., 1998). These very short RNA oligos have been termed ‘nanoRNAs’ to distinguish them from the longer microRNAs (Mechold...
et al., 2007). Orn activity was shown to be essential in E. coli (Ghosh & Deutscher, 1999). When accumulated, nanoRNA can be used to prime transcription initiation and alter global gene expression (Goldman et al., 2011; Nickels & Dove, 2011). NanoRNase activity of Orn and its human homologue, Sfn, was shown to be inhibited by 3'-phosphodiamidate 5'-phosphate (pAp) in vitro (Mehdof et al., 2006). Among the RNases with the ability to degrade nanoRNA are NmA and Nrbh from Bacillus subtilis (Fang et al., 2009). Similarly to Orn from E. coli, these enzymes strongly prefer nanoRNA as substrate over longer RNA molecules. NmA homologues are widely present in bacterial species that do not have a homologue of Orn. In addition to its activity on nanoRNA, NmA can degrade pAp (Mehdof et al., 2007). In contrast to an earlier report on the NmA homologue of Streptococcus mutans (Zhang & Biswas, 2009), bifunctionality seems to be common among NmA homologues (Postic et al., 2012). NanoRNases from Gamma-proteobacteria and A−T-rich Firmicutes, in particular, have widely divergent structures while displaying the same activity (Fang et al., 2009). In Clostridiales, Cytophagales and Alphaproteobacteria, no counterparts of the enzymes of either the Gamma-proteobacteria or the Firmicutes were readily identified from the available genome sequences. In the class Alphaproteobacteria, numerous genera belonging to the order Rhizobiales contain plant symbionts (Stacey et al., 2006). Other members of the Rhizobiales, such as Brucellaceae and Bartonellaceae, are described as pathogenic for mammals (Guerra, 2007; Minnick & Batisse, 2009). The genus Bartonella comprises a unique group of facultative intracellular bacteria that use haemophagous and arthropod transmission as a mammalian parasitic strategy (Chomel et al., 2009). Among the 25 species identified to date (Guptill, 2010), 10 were shown to be human pathogens. *Bartonella* species are recognised to be responsible for emerging zoonoses (Breitschwerdt et al., 2010). Genomes of *Bartonella* species sequenced to date are short, 1.4–1.9 Mbp in size (Alemark et al., 2004), and are the smallest of the order Rhizobiales. A search for genes with homology to genes that are required for growth of *E. coli* revealed that homologues of 38 of these essential *E. coli* genes are not present in *Bartonella* genomes. In particular, no counterparts of either the Gamma-proteobacteria or the Firmicutes nanoRNases were readily identified from the *Bartonella* genome sequences. Here we identify a functional Orn analogue in the *Alphaproteobacteria* family member *Bartonella henselae*. This protein, BACT0690 (now renamed NmC), was identified by screening a genomic library of *B. henselae* for genes that complement a conditional lethal orn mutant in *E. coli*. NmC was characterized both in vitro and in vivo in *E. coli* and *B. henselae*.

**METHODS**

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>F− rpsE46 hsdR17 recA1 endA1 gyrA46 thi rP16 lacI16 lacZAM15 Thr10 (TeC)</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pIP313</td>
<td>araD159 M15::Tn5 pgl150 thrB3501 (lacQ139 del72 prf25 Δmar174</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pUM341</td>
<td>CIP10230, araA under control of Pmar:: TorCkK</td>
<td>Mehof et al. (2007)</td>
</tr>
<tr>
<td>pUM358</td>
<td>CIP10230, araA under control of Pmar:: TorCkK</td>
<td>Mehof et al. (2007)</td>
</tr>
<tr>
<td>pUM146 (pUM408)</td>
<td>CIP10230, araA under control of Pmar:: TorCkK</td>
<td>Mehof et al. (2007)</td>
</tr>
<tr>
<td><em>B. henselae</em></td>
<td>PyrC</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Ampk Cpmk</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pIP408</td>
<td>As pBAD4 (Geurman et al., 1995). Orn with C-terminal His-tag, Ampk</td>
<td>Mehof et al. (2006)</td>
</tr>
<tr>
<td>pBAD24</td>
<td>pAM32 araC, arabinose-inducible promoter, Ampk</td>
<td>Guaux et al. (1995)</td>
</tr>
<tr>
<td>pCSkA</td>
<td>Knk</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>pBAD24::nmC</td>
<td>pBAD24 carrying nmC from <em>B. henselae</em>, Ampk</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD24::nmC-gs</td>
<td>pBAD24 carrying <em>B. henselae</em> nmC synthetic gene with C-terminal His-tag</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD24::nmC-ch</td>
<td>pBAD24 carrying nmC from <em>B. henselae</em>, Ampk</td>
<td>This study</td>
</tr>
<tr>
<td>pCSkA::nmC</td>
<td>pCSkA carrying arabinose pmC, Knk</td>
<td>This study</td>
</tr>
<tr>
<td>pCSkA::nmC-ch</td>
<td>pCSkA carrying arabinose pmC, Knk</td>
<td>This study</td>
</tr>
</tbody>
</table>

http://www.mic.sgmjournals.org
of 250 ng ml\(^{-1}\) for induction of the Pna14 promoter. B. brevis and B. amarele were grown on sheep blood agar plates. B. breve was plated on MRS agar, and 24 h later colonies were identified by their morphology. Cultures were grown on sheep blood agar plates, B. breve on MRS agar, and B. amarele on blood agar plates. B. breve and B. amarele were cultured in a 12-well plate and grown at 35 °C for 5 days. The OD\(_{595}\) of the bacterial suspension was adjusted to 0.05. Then, 2 ml of each suspension was poured into a 12-well plate and grown at 35 °C for 10 days. The OD\(_{595}\) of each suspension was recorded on days 2, 4, and 7 after inoculation.

### Genetic techniques

E. coli cells were transformed by the calcium chloride method (Maniatis et al., 1989) or electroporation (Dower et al., 1987). E. coli cells were transformed by electroporation (Ruse et al., 2003).

### DNA manipulations

B. breve genomic DNA was isolated using the Wizard Genomic DNA Purification kit (Promega). Smal-cut plasmid DNA preparation was performed by using a Qiagen Spin Miniprep kit (Qiagen). Restriction endonuclease digests and ligation were carried out according to the manufacturer’s recommendation. DNA fragments were amplified using hybrid PCR protocols using Phusion DNA polymerase (New England Biolabs). Nonsynthetic sequencing was performed by Eurofins MWG Operon. Purification of DNA fragments from PCR products, restriction reactions, or agarose gels was performed using a Machery-Nagel Nucleosep Ltd kit.

### Preparation of the B. breve genomic library

B. breve genomic DNA was submitted to partial digestion by Sau3A I. Hybridization on an 8% agarose gel, fragments of 2–3 kb in length were purified from agarose and partially filled with A and G using DNA Polymerase I, Large (Klenow) Fragment (Invitrogen). Plasmid pMCG184 was digested by SalI, purified and partially filled with C and T using DNA Polymerase I, Large (Klenow) Fragment (Invitrogen). Vector and chromosomal DNA fragments were ligated, and introduced into E. coli strain XL1-blue by electroporation.

### Construction of a recombinant vector expressing NmIC from B. breve

The complete B. breve nmc gene with a C-terminal His-tag (His-mc) was synthesized according to the E. coli codon usage (Milligen) (see Supplementary Fig. S1) and used to replace the RNA primers in the cloning of nmcB by PCR. The primers used in this study are listed in Table 1.

### Expression of the recombinant nmcC gene

Expression of the B. brevis nmcC gene was induced at 37 °C in LB medium containing 50 µg ml\(^{-1}\) of Rifampicin for 4 h by adding arabinose (0.2% final concentration). The bacterial suspension was centrifuged at 10,000 g for 5 min at 4 °C, and the pellet was suspended in 20 µl binding buffer (50 mM Tris/HCl, pH 8.0, 250 mM NaCl). Bacteria were lysed by incubation at 4 °C for 30 min in the presence of lysozyme (1 mg ml\(^{-1}\) and sonication (7 s, followed by 3 s, 3 cycles) for 15 min. The suspension was centrifuged at 13,000 g for 30 min at 4 °C. The supernatant containing the soluble fraction was mixed with 500 µl Ni-agarose beads (Qiagen) and purified following the manufacturer’s protocol. Purified protein was dialyzed twice against a buffer containing 50 mM Tris/HCl and 250 mM NaCl to remove any residual impurities. The protein was estimated to be >99% pure through SDS gel electrophoresis and was stable for several months when kept at -20 °C with 20% glycerol.

### NmcC activity assays

Activity assay was performed on a custom-made RNA fluorosensor, tagged 5' (5'CC-AAAA) as described previously (Medford et al., 2007). The optimal buffer for NmcC activity was 50 mM Tris/HCl, pH 8.0, containing 5 mM MgCl\(_2\).

### Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Gene</th>
<th>Organism</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ermBBeasiment</td>
<td>Synthesized nmc</td>
<td>B. breve</td>
<td>ATCCCAGGAGTACAGG</td>
</tr>
<tr>
<td>orf1 Bamboosyn</td>
<td>Synthesized nmc</td>
<td>B. breve</td>
<td>TGGGCTGAGGAGGAAATTGCAAAGAGTAGTTCACAG</td>
</tr>
<tr>
<td>orf1 Bbam</td>
<td>Wild-type nmc</td>
<td>B. breve</td>
<td>TGGGCTGAGGAGGAAATTGCAAAGAGTAGTTCACAG</td>
</tr>
<tr>
<td>orf1 BBam</td>
<td>Wild-type nmc</td>
<td>B. breve</td>
<td>TGGGCTGAGGAGGAAATTGCAAAGAGTAGTTCACAG</td>
</tr>
<tr>
<td>orf1 Bacillus</td>
<td>Antibiotic nmc</td>
<td>B. breve</td>
<td>C2CTCTGAGAACTGGAGGAAATTGCAAAGAGTAGTTCACAG</td>
</tr>
<tr>
<td>orf1 Bacillus</td>
<td>Antibiotic nmc</td>
<td>B. breve</td>
<td>C2CTCTGAGAACTGGAGGAAATTGCAAAGAGTAGTTCACAG</td>
</tr>
</tbody>
</table>

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or 5 mM MnCl₂. Mixtures containing 50 mM Tris/HCl pH 8.0, 5 mM MgCl₂, and 0.3 μM substrate were pre-incubated for 5 min at 37 °C. The reaction was initiated by adding 0.05 μg protein (40 mM final concentration) at 37 °C. At the indicated times (from 0.5 to 30 min), 4.5 μl reaction aliquots were added to an equal volume of sample buffer (4 x Tris-borate-EDTA (TBE), 100 mM dithiothreitol, 10% glycerol and 20 mM EDTA) to stop the reaction and stored at –20 °C. For analysis of the reaction products, 1.5 μl of sample was resolved by electrophoresis through 22% polyacrylamide (PAA) gels prepared in 2 x TBE running buffer. Fluorescent RNA oligos were visualized using a Molecular Dynamics STORM 860 in 650 nm long-pass filter mode. Data quantification was based on defining the total amount of fluorescence measured in the substrate and the reaction products for each time point and expressing the amount of each reaction product as a fraction of the total. Comparison of NrnC activity on ‘A’ or ‘G’-containing substrates was performed using Student’s t-test (two-tailed, equal variances assumed) as described previously (Yang et al., 2003). The estimated concentration of labelled substrate was 1.66 pmol μl⁻¹. Five micro- liters of substrate was first incubated for 5 min at 37 °C with 45 μl of buffer containing 50 mM Tris/HCl, pH 8.0 and 5 mM MnCl₂. Reactions were started by adding 0.5 μg NrnC A-ligase (6 μl) taken at the times indicated and added to an equal volume of loading buffer and set on ice to stop the reaction. After 3 min at 95 °C, 7 μl of the samples was resolved on a 20% PAA, 7.5% urea gel containing 2 x TBE. Labeling of the O-deacetylated marker was performed as described previously (Michold et al., 2007).

**Protein analysis by electrophoresis.** Proteins were analysed by SDS-PAGE (Laemmli, 1970) on 12% PAA-SDS gels, followed by Coomassie blue staining.

**Protein assay.** The concentration of the protein was determined using the BC Assay protein quantification kit (Interchim).

**Statistical analysis.** Data regarding colony diameter measured on blood agar plates for the B. lentus wild-type strain and the B. lentus nmc knocked-down strains were presented as mean±SD of 10 colonies. The experiments were performed three times. Statistical significance of the data was assessed by use of Student’s t-test. A value of P<0.05 was considered significant.

**Phylogenetic analysis.** The sequence of the B. lentus nrnC locus was deposited at the EMBL-EBI under accession no. HE800918. A total of 1706 completely sequenced bacterial genomes deposited before October 2011 in the EMBL-EBI database were analysed for the presence of homologues of the NrnC protein using FASTA comparisons.

**RESULTS**

**Identification of a functional analogue of Orn in B. lentus**

To search for the gene coding for a potential named RNAse, we constructed a genomic library of B. lentus. This genomic library was introduced into the conditional orn mutant of E. coli (strain UM341) by electroporation. In this strain, orn expression is under the control of the Atc-inducible promoter P<orn (Michold et al., 2007). A growth defect of this mutant strain was easily observable on LB plates without Atc. Transformants of this strain with the vector control (pBAD24) produced pinpoint sized colonies after overnight growth in the absence of Atc whereas growth of the mutant strains expressing a plasmid borne copy of orn from E. coli (pUM408) was not affected on LB plates lacking Atc (Michold et al., 2006). After introduction of the B. lentus genomic library into strain UM341, three clones exhibited normal growth on LB plates in the absence of Atc. Two clones were shown to harbour a recombinant plasmid encoding a Msch family transcriptional regulator (Michendorfer & Gonzalez, 2011) that can interfere with the P<orn promoter. One recombinant plasmid contained the structural gene coding for BA0969. This protein was annotated as an RNAase D using sequence comparisons as the exonuclease domains of RNAse D and BA0969 are 30% identical. In fact, most of the amino acids that constitute the conserved motifs, Exol, II and III (Zuo & Deutscher, 2001), and many other amino acids that are highly conserved in RNAse D family members, are present in BA0969. BA0969 can be placed into the DEDD exonuclease protein (Zuo & Deutscher, 2001). The DEDD exonuclease residues are identified by Asp22 and Glu27 in Exol, Asp84 in Exol2, and Tyr84 and Asp185 in Exol3. The DW'x(2)-RPL motif, which is the signature of the RNAse D family, is only partially conserved in BA0969 as the conserved Arg and Leu residues are missing (Fig. 1). BA0969 and E. coli Orn show some local homology in the N-terminal part of the two proteins covering approximately 35 amino acids (data not shown). This raises the possibility that this region might be involved in determining substrate specificity. We cloned orn into plasmid pBAD24 giving pBAD24::nrnC and this plasmid, as with the vector control, was introduced into strain UM341. Strain UM341 harbouring the pBAD24 vector control produced pinpoint-sized colonies on LB plates without Atc, whereas strain UM341 containing pBAD24::nrnC formed normal-sized colonies on LB plates without Atc as compared with those on LB plates that contained Atc (Fig. 2). Thus, expression of BA0969 (now renamed NrnC) could complement the lack of Orn in E. coli, suggesting that this protein is able to degrade RNA in vivo in E. coli.

**Expression and purification of recombinant NrnC from E. coli**

To produce and purify the recombinant B. lentus NrnC protein expressed in E. coli, we amplified its structural gene from B. lentus chromosomal DNA, using a reverse primer that allows the addition of a His-tag at the C-terminus of the protein. The fragment was cloned into pBAD24 plasmid. This recombinant plasmid (pBAD24::nrnC) was able to rescue the growth defect of E. coli strain UM341 in the absence of Atc (data not shown). However, when introduced into strain IP313, the amount of NrnC protein produced did not allow purification (data not shown). The G+C content of the B. lentus genome (37.8 mol%) is lower than that of
the E. coli genome (50 mol%). The different G+C content could lead to different codon usage in these two bacteria and potentially to a low expression of a B. biformis gene in E. coli.

<table>
<thead>
<tr>
<th>Red</th>
<th>NRC</th>
<th>RnD</th>
<th>RbD</th>
</tr>
</thead>
<tbody>
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<td>146</td>
<td>135</td>
<td>134</td>
<td>133</td>
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<tr>
<td>146</td>
<td>135</td>
<td>134</td>
<td>133</td>
</tr>
</tbody>
</table>

Therefore, we decided to use a synthetic ba0969 gene (Milligen) with E. coli codon usage coding for a C-terminally His-tagged protein. This synthetic gene (ba0969-s) was cloned into plasmid pBAD24, yielding plasmid pBAD24::nrnC-s. Expression of ba0969-s was able to completely restore the growth defect of the E. coli om mutant strain UM341 in the presence of arabinose (data not shown). When grown in the absence of Atc, colony sizes of the strain expressing ba0969-s were similar to those of the strain expressing E. coli om. However, complementation could not be seen when bacteria were grown in the absence of arabinose (data not shown). The amount of Nrnc in E. coli strain JP313 was investigated using SDS-PAGE comparing protein extracts of the strain containing the recombinant plasmid with those of the strain containing the empty vector. An additional 24 KDa band was observed only in extracts of the strain containing the recombinant plasmid pBAD24::nrnC-s and only when expression was induced by arabinose (data not shown).

Strain JP313 carrying plasmid pBAD24::nrnC-s expressed soluble His-tagged Nrnc protein when induced by the addition of arabinose to a concentration of 0.2%. The protein was purified by Ni-agarose affinity chromatography, yielding a protein that migrated at ~24 KDa as a distinct band on SDS-PAGE and was estimated to be at least 95% pure (Supplementary Fig. S2, available with the online version of this paper). This mass corresponds well with the predicted value from the amino acid sequence.

**NanoRNase activity of Nrnc**

First experiments to measure nanoRNase activity of Nrnc were performed in 50 mM Tris/HCl, pH 8.0, in the presence of 5 mM MgCl₂ or MnCl₂ and showed that Nrnc was able to degrade a nanoRNA 5mer (5'Cy5-AAAAA3') to define

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**Fig. 1.** E. coli RNaseD (RnD) and B. biformis Nrnc sequence alignment. This alignment was generated by CLUSTAL W. The conserved DEED motifs are indicated by a double-headed arrow and numbered. Asp25 and Glu27 in Ecol, Asp54 in Ecol, and Tyr151 and Asp155 in Ecol of Nrnc are highlighted in grey. The DW-(2)-RPL motif of RnD is underlined and highlighted in grey. Red and Nren enzyme-domains are dotted underlined.

**Fig. 2.** Complementation of the conditional E. coli om mutant by ba0969 from B. biformis. Strains UM341 containing pBAD24 (vector control), pUM408 (expressing Om from E. coli) or pBAD24::ba0969 (expressing Nrnc from B. biformis) were spread on LB agar plates containing 0.2% arabinose in the absence or in the presence of anhydrotetracycline (ATC, 250 ng ml⁻¹). The experiment was repeated three times; a representative result is presented.
the optimal conditions for nanoRNAse activity of NmC, we first measured activity on a nanoRNA 5mer (5'Cy5-AAAA3') at different pH, in 50 mM Tris/HCl buffer, and in the presence of different divalent cations (magnesium, manganese, cobalt or calcium). Our results clearly indicate that the nanoRNAse activity of NmC was higher at pH 8.0 than at pH 7.0, 7.5 and 9.0 (data not shown). Activity was similar in the presence of manganese or magnesium and considerably lower, but still appreciable, in the presence of cobalt (data not shown). We were unable to detect any activity in the presence of calcium in 50 mM Tris/HCl, pH 8.0, buffer. Therefore, subsequent experiments to measure activity of NmC were performed in 50 mM Tris/HCl, pH 8.0, and in the presence of 5 mM MgCl₂ or MnCl₂. The sequential appearance of reaction products (Fig. 3) resembled Orm-catalysed reactions (Mechold et al., 2007), and pointed to a similar reaction mechanism (Fig. 3). Under the conditions tested, the activity of NmC measured as the disappearance of substrate 5mer was 8 nmol min⁻¹ µg⁻¹ (Fig. 3). We also tested the activity of NmC on other RNA oligos, namely 5'Cy5-CCCGC3' (Fig. S3, available with the online version of this paper), RNA 3mer 5'Cy5-AAA3' was also degraded more efficiently by NmC than RNA 3mer 5'Cy5-CCG3' (Supplementary Fig. S3). In addition, quantification of the activity on different substrates revealed that, similarly to NmsB-catalysed degradation (Fang et al., 2009), RNA 3mers were a better substrate for NmC than RNA 3mers.

**NanoRNAse activity of NmC is inhibited by pAp**

In *E. coli*, pAp is generated from phosphoadenosine 5'-phosphosulfate during sulfur assimilation. In *E. coli*, pAp inhibits the activity of Orm (Mechold et al., 2006). In contrast, NmC from *B. subtilis* is able to degrade pAp in vitro and in vivo (Mechold et al., 2007). As a consequence, when expressed in *E. coli*, nmcA from *B. subtilis* can restore normal growth of a cyoQ mutant grown in the absence of cysteine (Mechold et al., 2007). We thus wondered whether *nmC* from *B. burgdorferi* could restore the growth of an *E. coli* cyoQ mutant (UM285). Complementation assays showed that expression of *nmC* did not restore the growth of *E. coli* strain UM285 in the absence of cysteine (data not shown). We investigated whether pAp could inhibit the degradation of 5'Cy5-AAA3' by NmC nanoRNAse activity, using 5'Cy5-AAA3' as substrate. As shown in Fig. 4, the presence of 50 µM pAp inhibited NmC nanoRNAse activity.

**Activity of NmC on RNA 24mer**

To check whether NmC specifically degrades nanoRNA, or is active on longer RNAs as well, we tested degradation of an RNA 24mer 5'-end labelled with [3²P]ATP. The NmC-catalysed turnover of 24mers into monomers could be roughly estimated from this experiment as 8.3 pmol min⁻¹ µg⁻¹ (Fig. 5). The activity of *B. burgdorferi* NmC on the 24mer was lower than that on nanoRNA, but was 4.1-fold higher than that of Nn1 on 24mers and 80-fold higher than that of *B. subtilis* NmA on 24mers (Mechold et al., 2007; Fang et al., 2009).

**NmC activity is important for growth in *B. henselae***

In *E. coli*, nanoRNAse activity of Orm is required for growth (Ghoos & Decker, 1999). When expressed in *E. coli*, *B. burgdorferi* nmC can complement the orm mutation. We investigated whether NmC activity was required for normal growth in *B. henselae*. Due to the lack of genetic tools, it is presently not possible to generate mutants in essential genes of *B. henselae*. However, it is possible to analyze the effect of the lack of an essential gene product in this genus via knockdown gene expression. We used pNS2 vector derivatives that had been used previously for knockdown experiments in *B. henselae* (Gillaspie et al., 2007).
In spite of numerous attempts, we failed to introduce the pNS2 vector and its derivatives into \( B. \) \( birtlesii \). Therefore, we decided to try to determine the effects of knocking down \( nmcC \) in \( B. \) \( henselae \). The primary sequence of \( NmcC \) from \( B. \) \( henselae \) is 98% identical to that of \( NmcC \) from \( B. \) \( birtlesii \). To test whether \( NmcC \) from \( B. \) \( henselae \) has nanorNase activity in vivo similar to \( NmcC \) from \( B. \) \( birtlesii \), we cloned \( nmcC \) from \( B. \) \( henselae \) into pBAD24. The plasmid expressing \( B. \) \( henselae \) \( nmcC \), pBAD24::\( nmcC \), was then introduced into \( E. \) \( coli \) strain \( UM341 \) to test for complementation of the lack of \( Cmr \). Our experiments demonstrated that expression of \( NmcC \) from \( B. \) \( henselae \) can rescue the growth defect of strain \( UM341 \) in the absence of \( Acr \) (data not shown). We cloned \( nmcC \) of \( B. \) \( henselae \) into a vector that allowed low-level expression, pNS2Amp, and a vector that allowed high-level expression, pNS2Trc (Gillisie et al., 2009). The gene was in the reverse orientation such that the antisense strand was transcribed. Plasmids pNS2Amp, pNS2Trc, pNS2Amp::\( nmcC \), and pNS2Trc::\( nmcC \) were introduced into \( B. \) \( henselae \) by electroporation. The strains obtained were tested for growth on blood agar plates and in Schneider’s medium. \( B. \) \( henselae \) containing plasmid pNS2Amp::\( nmcC \) grew (data not shown) as well as the two control strains, \( B. \) \( henselae \) (pNS2Amp) (data not shown) and \( B. \) \( henselae \) (pNS2Trc). In contrast, the \( B. \) \( henselae \) strain containing pNS2Trc::\( nmcC \) grew more slowly in Schneider’s medium (Fig. 6). The doubling time of the strain carrying pNS2Trc::\( nmcC \) (12 h) was longer than that observed for the strain carrying the vector control pNS2Trc (7 h). After 10 days of growth on blood agar plates, colonies produced by \( B. \) \( henselae \) (pNS2Trc::\( nmcC \)) were much smaller (0.65 ± 0.1 mm) than those formed by \( B. \) \( henselae \) (pNS2Trc) (0.56 ± 0.09 and 1.13 ± 0.13 mm after 6 and 10 days, respectively). These results show that a decrease in the amount of \( NmcC \) slowed the growth of \( B. \) \( henselae \), and demonstrate the importance of \( NmcC \) activity for the physiology of this alphaproteobacterium.

**Phylogenetic distribution of NmcC**

Searching for \( NmcC \) homologues in bacterial genomes sequenced to date showed that this protein is well conserved...
in all Bartonella, B. henselae Nmc was 87-91% identical to the Nmc homologues found in the six Bartonella genomes present in the databases [http://www.ncbi.nlm.nih.gov/]. In addition, we found Nmc homologues in the genomes of many other Alphaproteobacteria with identities ranging from 77% for Brucella melitensis to 43% for Candidatus peregrinatus [Supplementary Table S1, available with the online version of this paper]. Other Nmc homologues that fulfilled our criterion of varying less than 20% in size were found in Cytophaga, and were between 42 and 50% identical to Nmc [Supplementary Table S1]. Genomes of some Spirochaetes also encode Nmc homologues that are between 44 and 48% identical to Nmc [Supplementary Table S1]. Nmc homologues were absent in Beta- and Gammaproteobacteria genomes that harbour homologues of Orn [Mechold et al., 2007]. Also, Nmc homologues were absent in Chlororib Bacteriodetes and Firmi taxa that have Nmc homologues. Thus, Nmc represents a third class of nmrNase that is mainly present in Alphaproteobacteria and Cyanobacteria. According to its size, Nmc is closer to Orn from E. coli (181 aa) (Ghosh & Deutscher, 1999) than to NrnA (313 aa) (Mechold et al., 2007) and NrnB (399 aa) from B. subtilis (Yang et al., 2009). Similarity between Nmc and its functional analogues E. coli Orn and B. subtilis NrnA and NrnB was negligible, 22, 16 and 11% identity, respectively.

**DISCUSSION**

Exploring Bartonella genomes sequenced to date revealed the lack of homologues for 38 of the 302 essential genes identified in the E. coli genome. These essential genes are, in E. coli, involved in the biosynthesis of haem (tetrahydrofolate), thiamine, isoprenoid, ubiquinone, undecaprenyl-P, lipid A, phospholipid and murein. Two of these genes are involved in the biosynthesis and export of lipopolysaccharides. In addition, homologues of certain genes involved in cell division, chromosome partitioning, secretion, replication and oligonucleotide degradation are also missing in Bartonella genomes. Absence of some of the above-mentioned genes can be easily explained by the life style of Bartonella. For example, Bartonella can replicate inside erythrocytes where haem is available. The function of other genes involved in processes such as cell division, DNA replication or oligonucleotide degradation are undoubtedly assumed by functional analogues. We demonstrate here that this is the case for Orn, an RNA exonuclease that is essential for cell growth in E. coli. Screening for B. henselae genes that could complement a conditional lethal mutant of E. coli orn, we identified a gene bat0909 that encodes a polypeptide that has 30% identity to the catalytic domain of E. coli RNase D. However, the B. henselae protein is 206 aa shorter than E. coli RNase D (375 aa) (Zuo et al., 2005), and the two C-terminal domains, HRDC1 and HRDC2, are not.

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present in NmC. Despite the similarities between RNase D and NmC, it is noteworthy that RNase D from E. coli cannot replace Orn and was found to be essentially devoid of oligoribonuclease activity in vitro (Yu & Deutscher, 1995).

Biochemical characterization of NmC from B. biflexus clearly showed that this protein can degrade RNA in the presence of magnesium or manganese. In both cases, the specific activity of NmC is higher than those measured for B. subtilis OrnA and OrnB (Mechold et al., 2007).

The nanoRNAse activity of NmC is higher at pH 8.0–9.0 than at pH 7.0, similar to what was shown to be optimal for Orn from E. coli (Niyogi & Datta, 1975). As shown in this report, NmC can also degrade RNA 24mers. To our knowledge, Orn from E. coli was not tested on oligos of a similar length or longer. However, exploring the activity on 24mers we note the general trend of decreased reaction rate with increasing chain length (Datta & Niyogi, 1975). The activity of OrnA from B. subtilis on 24mer oligos was shown to be insignificant (Mechold et al., 2007).

Despite the fact that OrnB from B. biflexus is more active on RNA 24mers compared with OrnA, this activity is nevertheless three orders of magnitude lower than the activity on nanoRNA (Fang et al., 2009). For NmC, the activity on RNA 24mers (8.3 pmol μg⁻¹ 1 μg⁻¹) is 1000 times lower than the activity on nanoRNA (8 amol μg⁻¹ 1 μg⁻¹). Hence, all these enzymes strongly prefer nanoRNA over longer RNA substrates. Similar to Orn activity (Mechold et al., 2006), the nanoRNAse activity of NmC is strongly inhibited by pAp. In contrast, the nanoRNAse activity of NmC is not sensitive to pAp at low concentrations. However, pAp is a substrate for NmC and as such pAp can compete with nanoRNA for degradation by NmC. In agreement with this in vitro activity, NmC can restore the growth of an E. coli strain impaired in pAp degradation due to the lack of a functional CysQ and thus enable strain to grow in the absence of cystine (Mechold et al., 2007). NmC from B. biflexus cannot restore growth of the E. coli cysQ mutant, and hence pAp is not a substrate for this enzyme.

NmC is present and well conserved in the genomes of Bartonella species. In agreement with this observation, we show that the NmC homologue from B. henselae could equally complement an E. coli orn mutant, pointing to conserved function of NmC homologues.

ORN is essential in E. coli and its function cannot be assumed by any other RNase. In contrast, growth of B. subtilis is not affected in the absence of OrnA and OrnB, and it seems that other RNases can substitute for their function (Fang et al., 2009). Comparison of growth rates of B. henselae carrying pNSTTr::ΔnmCΔD (doubled time 12 h), and the corresponding vector control (doubled time 7 h) shows that knocking down nmC in B. henselae slows growth. This result suggests that a full RNAse activity of NmC is important for optimal growth in this Bartonella species and that other RNases cannot take over this function. Hence, similar to E. coli, in Bartonella only one enzyme, here NmC, might be responsible for nanoRNA degradation.

NmC homologues can be found in the genomes of numerous alphaproteobacteria with identities in the range 48.8–77%. Additional NmC homologues are present in Cyanobacteria genomes and are between 45.9 and 51.2% identical to NmC. Members of the genera Alphaproteobacteria and Cyanobacteria do not contain Orn, or OrnA or OrnB homologues. We propose that NmC homologues fulfill the function of degrading RNA in these organisms. Thus NmC represents a new, third member of the family of proteins that are specialized in degrading nanoRNA.

As reported here, 38 of the 302 essential E. coli genes do not have homologues in Bartonella genomes. The essential functions of these gene products may therefore be encoded by functional analogues of these genes. Here, we identify one of these functional analogues. Similarly, it might be possible to identify further functional analogues of essential E. coli genes by screening a genomic library of Bartonella species for genes that complement the corresponding conditional E. coli mutants. This would contribute to a better understanding of the physiology of Bartonella, which encounter various environments during their invasion of erythrocytes and endothelial cells, or during their presence inside arthropod guts and larvae.

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Conclusion

By screening a genomic library from *B. birtlesii* through complementation assays of the *E. coli* orn mutant, we identified an Orn functional homolog in *B. birtlesii* (now named NrnC). This Orn functional homolog is well conserved (more than 80% identity) in already sequenced *Bartonella* genomes. Further biochemical characterization revealed that NrnC exhibits nanoRNA degradation activity in the presence of magnesium and manganese and is able to bind pAp, but it is not able to complement the auxotrophy of an *E. coli* cysQ mutant impaired in pAp degradation. The nanoRNase activity of NrnC is strongly inhibited by pAp. NrnC can also degrade longer RNA of 24 mers, but with less efficiency. According to its activity, NrnC is close to the Orn characterized in *E. coli*.

Knockdown of *nrnC* significantly decreases the growth capacity of *B. henselae*, thus demonstrating the importance of NrnC activity in this bacterium. Sequence comparisons revealed that the gene encoding for the homologue of NrnC is present in the genome of numerous *Alphaproteobacteria*, with identities in the range of 48.8% to 77%. Additional NrnC homologues are present in Cyanobacteria genomes and are between 45.9% and 51.2% identical to NrnC.

In summary, we have identified a new nanoRNase family in *Bartonella*. Among the 302 genes characterized as essential in *E. coli*, 38 do not have homologues in *Bartonella* genomes. The essential functions of these gene products may be encoded by functional analogues. Here we report identification of one of these functional analogues. Similarly, it might be possible to identify further functional analogues of essential genes missing in the *B. henselae* genome by screening a genomic library of
this organism using complementation assays of *E. coli* mutants. This would contribute to a better understanding of the physiology of *Bartonella*, which must confront varying types of environment during their infection cycle.
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