



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

Ma Feng Liu

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THESIS

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

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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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Abbreviation

AP	Alkaline Phosphatase
BA	Bacillary Angiomatosis
BadA	Bartonella adhesion A
Beps	<i>Bartonella</i> -translocated effector proteins
BSA	Bovine Serum Albumin
BrpA	<i>Bartonella</i> repetitive protein A
CD	Cluster of Differentiation
CFU	Colony Forming Unit
CM	Cytoplasmic membrane
Cn	collagen
CSD	Cat scratch disease
Dip	2, 2'dipyridyl
ECM	Extracellular matrix molecules
ELISA	Enzyme-linked immunosorbent assay
Fn	fibronectin
gltA	Citrate synthase gene
Gly	glycerol
Hb	hemoglobin
Hbps	Heme binding proteins
HUVEC	human umbilical vein endothelial cells
Ial	Invasion-associated locus
IE	infective endocarditis
IFA	indirect immunofluorescence assay
IL	Interleukine
IM	Inner membrane
Irr	iron response regulator
Mb	Mega base pair
OD	Optical Density
OM	Outer membrane
Omp	Outer membrane protein
Opa	Opacity associated protein
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PH	peliosis hepatic
qRT-PCR	quantitative Real-time reverse transcription PCR
RirA	Rhizobial iron regulator A
ROS	Reactive oxygen species
T4SS	Type IV Secretion System
TAA s	Trimeric autotransporter adhesins
Vomps	Variably expressed outer membrane proteins
VEGF	Vascular endothelial growth factor
YadA	Yersinia adhesion A

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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).

<i>Bartonella</i> species	Reservoir	Vector	Human disease(s)
Human-specific species			
<i>B. bacilliformis</i>	Human	Sandfly	Carrión's disease: Oroya fever and verruga peruana
<i>B. quintana</i>	Human	Body louse, cat flea, tick	Trench fever, endocarditis, bacillary angiomatosis
Zoonotic species			
<i>B. alsatica</i>	Rabbit	Unknown	Endocarditis, lymphadenitis
<i>B. clarridgeiae</i>	Cat	Cat flea	Cat-scratch disease
<i>B. elizabethae</i>	Rat	Unknown	Endocarditis, neuroretinitis
<i>B. grahamii</i>	Mouse, vole	Rodent flea	Neuroretinitis
<i>B. henselae</i>	Cat, dog	Cat flea	Cat-scratch disease, endocarditis, bacillary angiomatosis, bacillary peliosis, neuroretinitis, bacteremia with fever
<i>B. koehlerae</i>	Cat	Unknown	Endocarditis
<i>B. rochalimae</i>	Fox, raccoon, coyote	flea	Bacteremia with fever
<i>B. tamiae</i>	Rat (?)	Mite (?)	Bacteremia with fever
<i>B. vinsonii</i> subsp. <i>arupensis</i>	Mouse	Tick (?)	Bacteremia with fever Endocarditis (?)
<i>B. vinsonii</i> subsp. <i>berkhoffii</i>	Dog	Tick (?)	Endocarditis
<i>B. washoensis</i>	Ground squirrel	Unknown	Myocarditis, endocarditis (?)
Animal-specific species			
<i>B. birtlesii</i>	Mouse	Unknown	Unknown
<i>B. bovis</i> (= <i>B. weissii</i>)	Cattle/cat	Unknown	Unknown
<i>B. capreoli</i>	Roe deer	Unknown	Unknown
<i>B. chomelii</i>	Cattle	Unknown	Unknown
<i>B. doshiae</i>	Vole	Unknown	Unknown
<i>B. peromysci</i>	Deer, mouse	Unknown	Unknown
<i>B. phoceensis</i>	Rat	Unknown	Unknown
<i>B. rattimassiliensis</i>	Rat	Unknown	Unknown
<i>B. schoenbuchensis</i>	Roe deer	Unknown	Unknown
<i>B. talpae</i>	Vole	Unknown	Unknown
<i>B. taylorii</i>	Mouse, vole	Rodent flea	Unknown
<i>B. tribocorum</i>	Rat	Unknown	Unknown
<i>B. vinsonii</i> subsp. <i>Vinsonii</i>	Vole	Unknown	Unknown

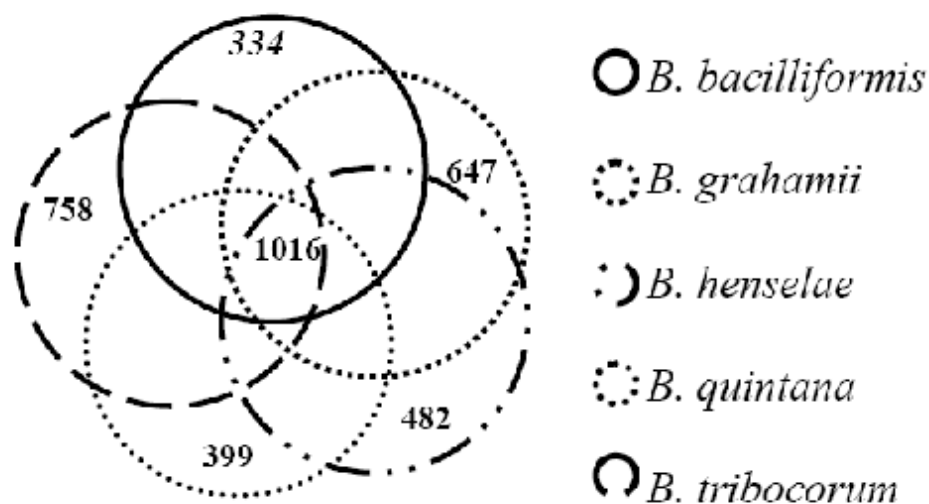
1. Analysis of *Bartonellae* genomes

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.

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



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 3. Essential *E. coli* genes not present in *Bartonellae* genomes.

Gene	Pathway
<i>csrA</i>	Pleiotropic regulatory protein for carbon source metabolism
<i>Can</i>	Carbonic anhydrase 2
<i>hemA</i>	Heme biosynthesis
<i>hemB</i>	Heme biosynthesis
<i>hemC</i>	Heme biosynthesis
<i>hemD</i>	Heme biosynthesis
<i>hemG</i>	Heme biosynthesis
<i>hemH</i>	Heme biosynthesis
<i>hemL</i>	Heme biosynthesis
<i>folB</i>	Tetrahydrofolate biosynthesis
<i>folK</i>	Tetrahydrofolate biosynthesis
<i>thiL</i>	Thiamine biosynthesis
<i>fldA</i>	Isoprenoid biosynthesis
<i>Dxr</i>	Ubiquinone biosynthesis
<i>ispU</i>	UPP biosynthesis
<i>lpxH</i>	Lipid A biosynthesis
<i>kdsC</i>	Lipid A biosynthesis
<i>plsB</i>	Phospholipid biosynthesis
<i>mreB</i>	Murein biosynthesis
<i>mreD</i>	Murein biosynthesis
<i>mreC</i>	Murein biosynthesis
<i>rlpB</i>	Lipopolysaccharide assembly
<i>lptA</i>	LPS export
<i>ftsB</i>	Essential cell division protein
<i>ftsL</i>	Essential cell division protein
<i>ftsN</i>	Essential cell division protein
<i>secM</i>	Secretion
<i>mukF</i>	Chromosome partitioning
<i>mukE</i>	Chromosome partitioning
<i>mukB</i>	Chromosome partitioning
<i>dnaT</i>	DNA replication
<i>Orn</i>	Oligoribonuclease
<i>yceQ</i>	Uncharacterized protein
<i>yfiO</i>	Outer membrane assembly lipoprotein
<i>ygjD</i>	Uncharacterized protein
<i>yrbK</i>	Uncharacterized protein
<i>yrfF</i>	Uncharacterized protein
<i>yihA</i>	GTP binding protein

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 burnetii* (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).

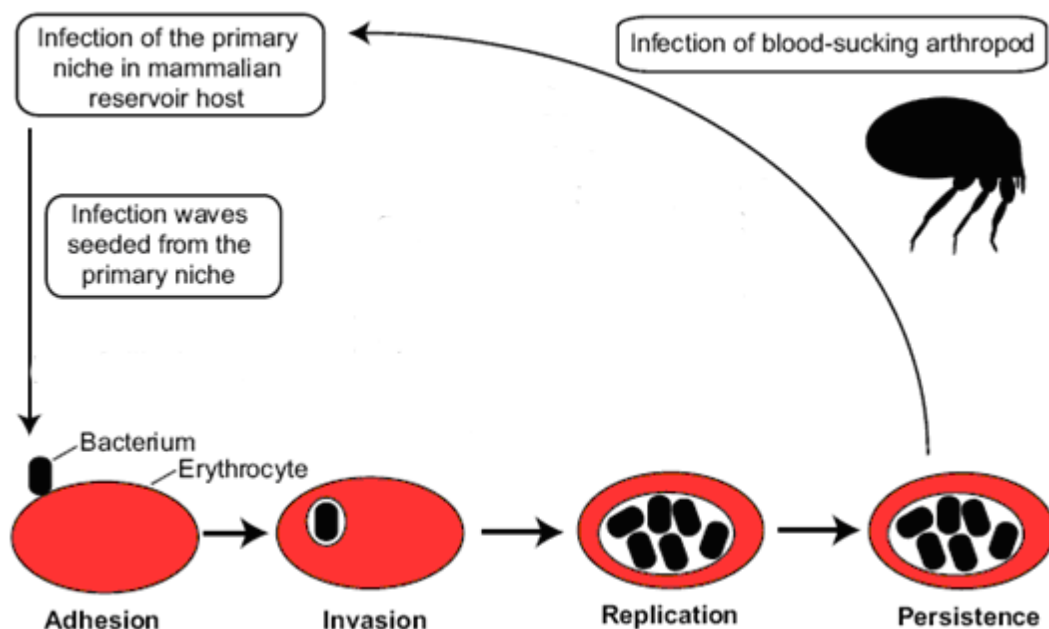


Fig. 2 Model of the infection cycle of *Bartonella* in their reservoir host. Adapted from (Pulliainen and Dehio, 2012).

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 (Dramsı 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 anti-flagellin 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).

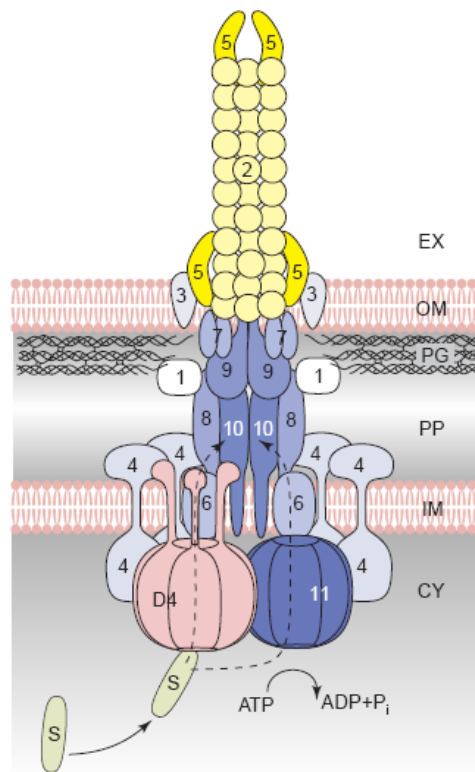


Fig 3. Model of VirB/VirD4 T4SS machinery. T4SS machinery spans the inner and outer membranes of Gram-negative bacteria. This transporter is able to secrete substrates from the bacterial cytoplasm directly into the cytoplasm of infected host cells and into the extracellular milieu. Adapted from (Schroder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

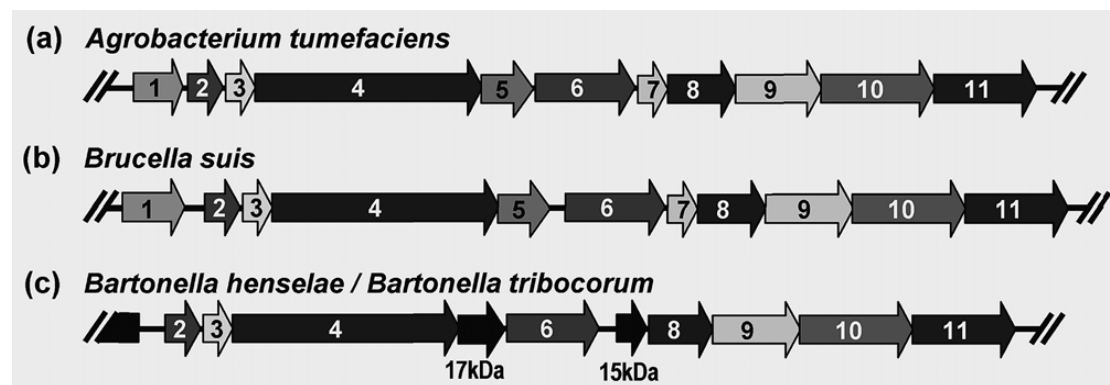


Fig 4. The VirB-like type IV secretion systems of (a) *Agrobacterium tumefaciens*, (b) *Brucella suis* and (c) *Bartonella henselae* and *Bartonella tribocorum*. Homologous genes are represented in the same number. Adapted from (Seubert et al., 2002).

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).

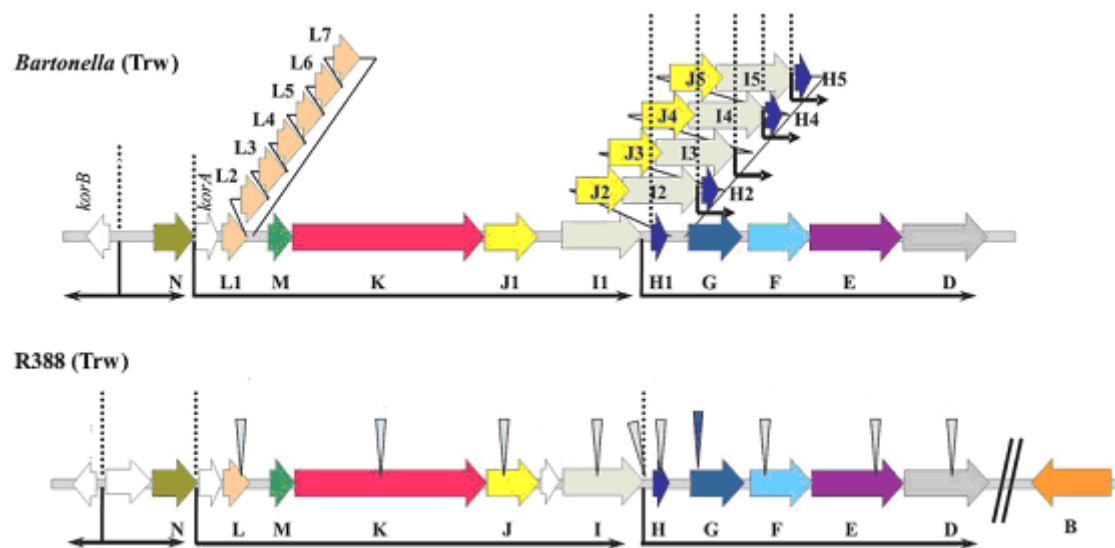


Fig 5. Organization of *B. tribocorum* Trw (Seubert et al., 2003) and R388-Trw(de Paz et al., 2005; Seubert et al., 2003). Homologous genes have the same color. *B. tribocorum* presents seven *TrwL* repetitions and five *TrwJ-TrwI-TrwH* repetitions. Adapted from (de Paz et al., 2005; Seubert et al.,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

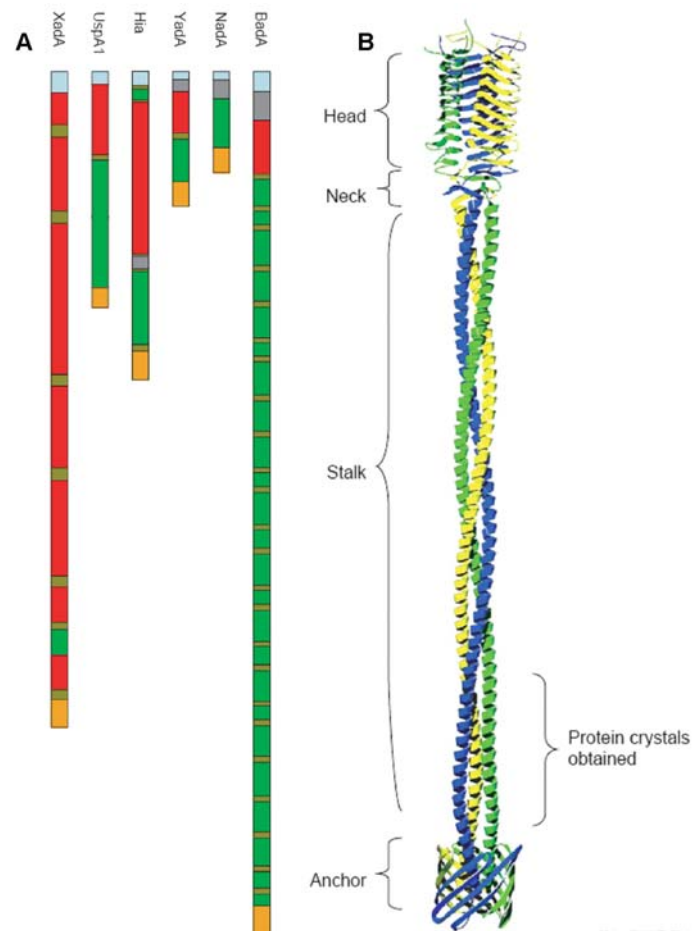


Fig 6. Domain organization of representative trimeric autotransporter adhesins (A) and full model structure of YadA (B). Head domains are shown in red, stalk in green,

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 *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 *Bartonella* species investigated thus far (O'Rourke et al., 2011), suggesting that there exists a conserved role for them in *Bartonella* pathogenicity (Gilmore et al., 2005; Zhang et al., 2004). In the case of *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 *B. quintana* lacking expression of *vomp* did not induce VEGF secretion (Schulte et al., 2006) and the *vomp* mutant strain of *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 *virB* operon in *B. henselae* (Padmalayam et al., 2000). Subsequently, the VirB/VirD4 type IV system was widely studied. The VirB/VirD4 type IV system of *B. henselae* is able to transport *Bartonella* effector proteins (Beps) BepA to BepG, which are encoded downstream from the *virB/virD4* locus of *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)- κ B, 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, potentially 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; Kruszevska and Tylewska-Wierzbanska, 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).

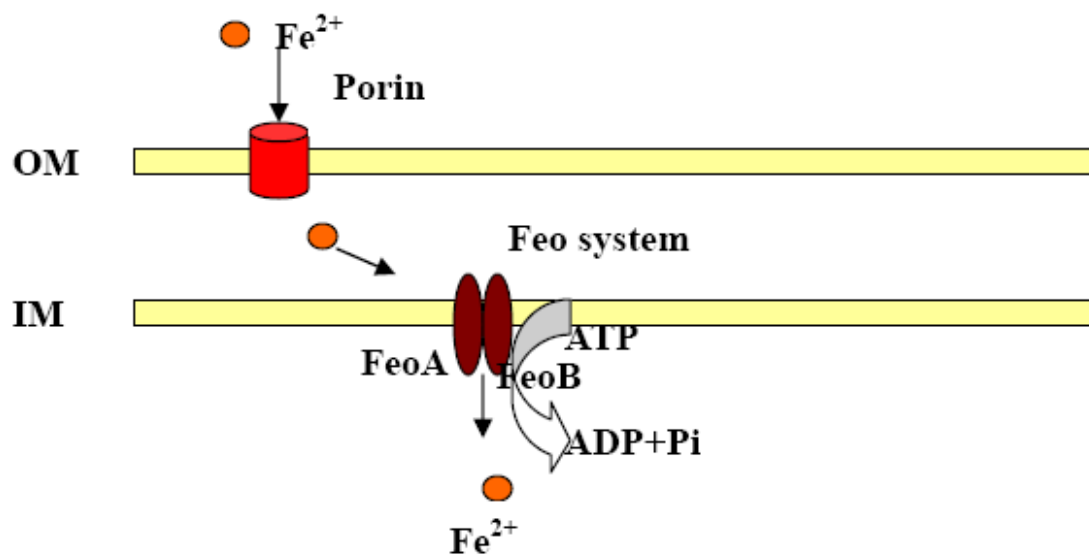


Fig.1 The Fe²⁺ transport system of *E. coli*. Fe²⁺ can pass through the porin freely and is transported by the Feo system into the cytoplasm.

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).

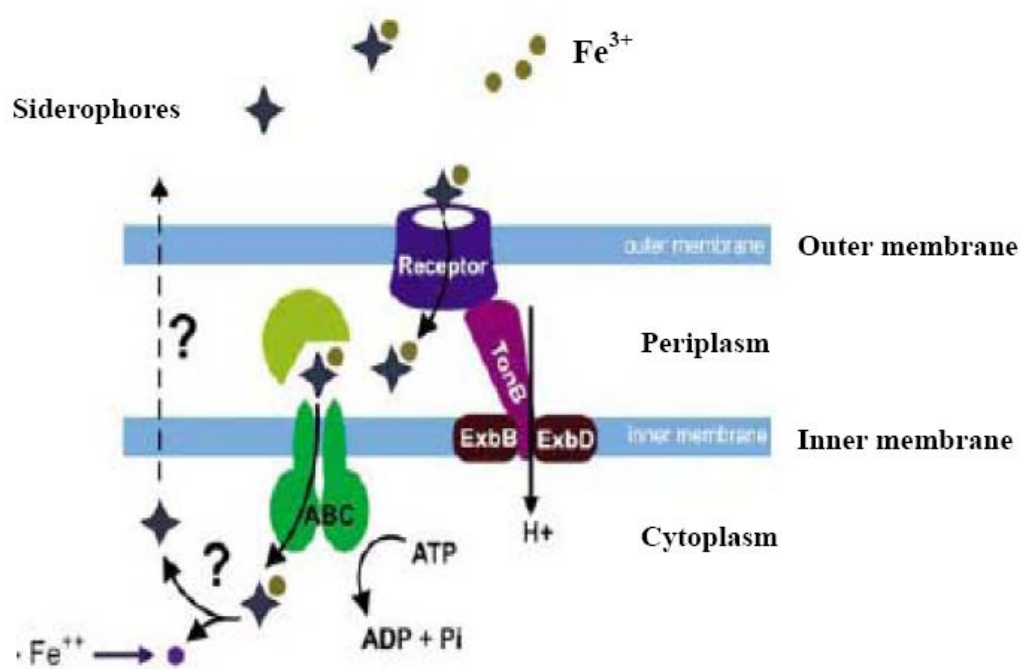


Fig 2. Organization of the siderophore-dependent Fe^{3+} uptake system in Gram-negative bacteria. Adapted from (Wandersman and Delepelaire, 2004).

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 (Letoffe et al., 1994a). Later, hemophores were identified in *P. aeruginosa* (Letoffe 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 ($K_d \sim 10^{-11}$ 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 α -helices on one face and 7 β -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).

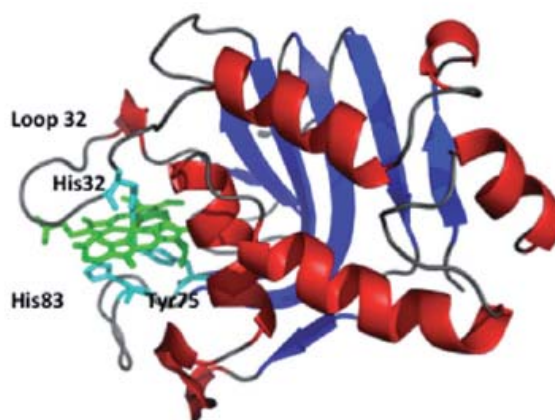


Fig 3. Crystal structure of holo-HasA from *S. marcescens*. Heme is ligated by tyrosine 75 and histidine 32. Histidine 83 stabilizes Tyr-75 and strengthens the Tyr-75 iron coordination bond. Adapted from (Arnoux et al., 1999).

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).

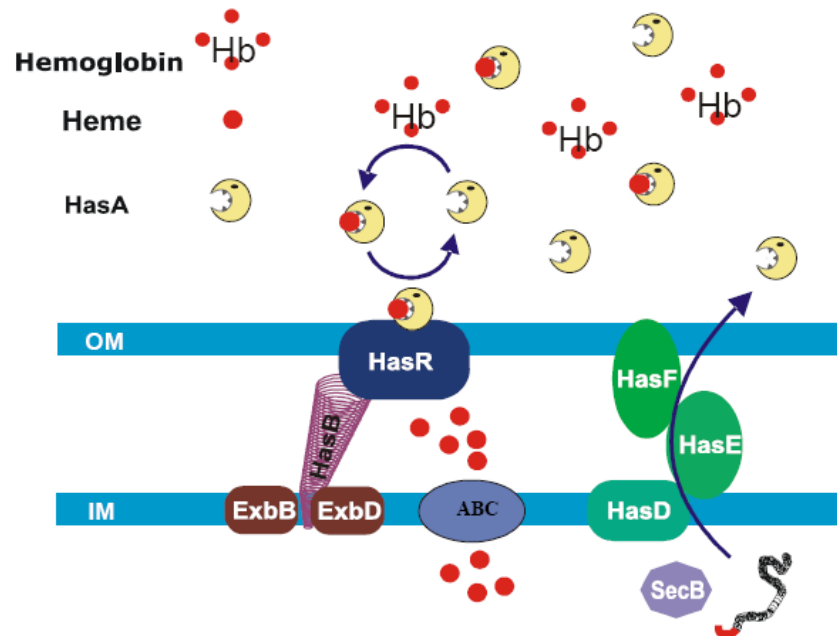


Fig 4. Organization of the *has* operon of *S. marcescens*. HasDEF and SecB allow secretion of the HasA hemophore into the extracellular medium, where it binds heme and returns it to the HasR receptor. Adapted from (Wandersman and Delepelaire, 2004).

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 *hxC*, *hxB*, and *hxA* 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 HuxA is released into the extracellular medium (Fournier et al., 2011). HuxA is secreted by a signal-peptide-dependent pathway requiring one helper protein, HxB (Cope et al., 1995). HxB was hypothesized as being involved in release of soluble HuxA from the cell surface (Cope et al., 1995). Unlike HasA, there is no heme capture by HuxA, but heme is released into the medium when HuxA interacts with heme-hemopexin (Wandersman and Delepelaire, 2012). In reconstituted *E. coli* strain C600 Δ *hemA*, expression of HxC alone enabled use of free heme or hemoglobin as a heme source (Fournier et al., 2011). However, co-expression of HuxA, HxB and HxC in C600 Δ *hemA* is required for heme acquisition from heme-loaded hemopexin (Fournier et al., 2011). It was shown that when HuxA interacts with holo-hemopexin, heme is released from hemopexin and immediately binds its cognate receptor HxC, 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 plus 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).

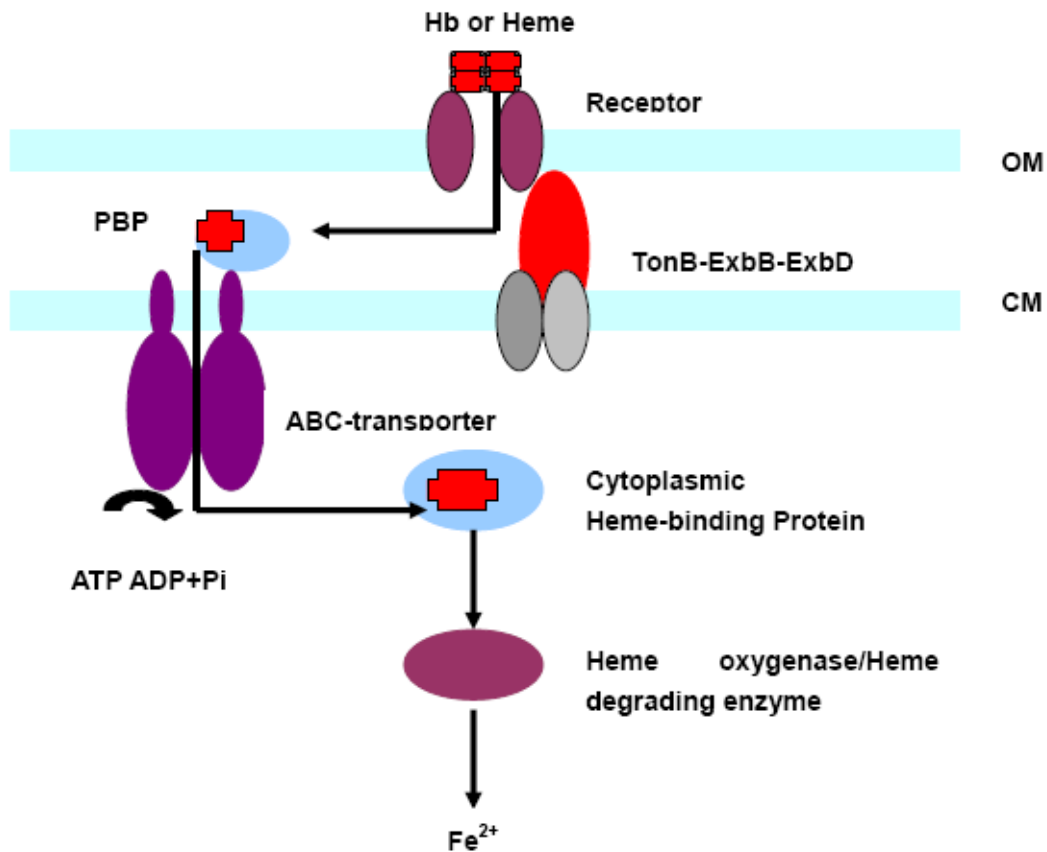


Fig 5. Organization of the direct heme uptake system in Gram-negative bacteria.

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).

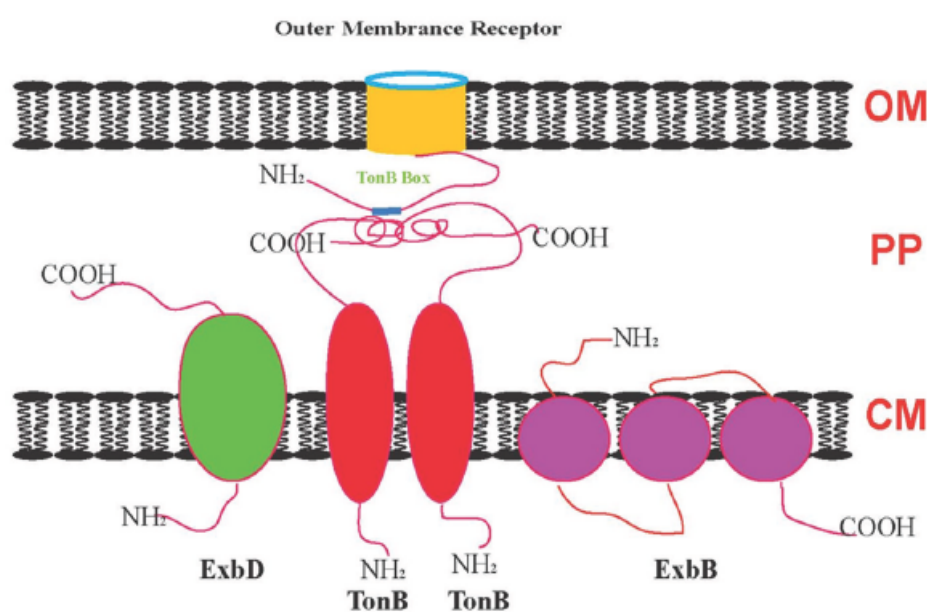


Fig. 6 The structure of the complex TonB-ExbB-ExbD. OM, outer membrane; PP, periplasmic space; CM, cytoplasmic membrane. Adapted from (Tong and Guo, 2009).

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 $\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow$

$\text{Fe(III)} + \text{OH}^- + \text{OH}^\cdot$), 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 Fe^{2+} and forms a Fur-Fe^{2+} complex which binds the Fur box, a 19 bp consensus sequence

(GATAATGATAATCATTATC). 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).

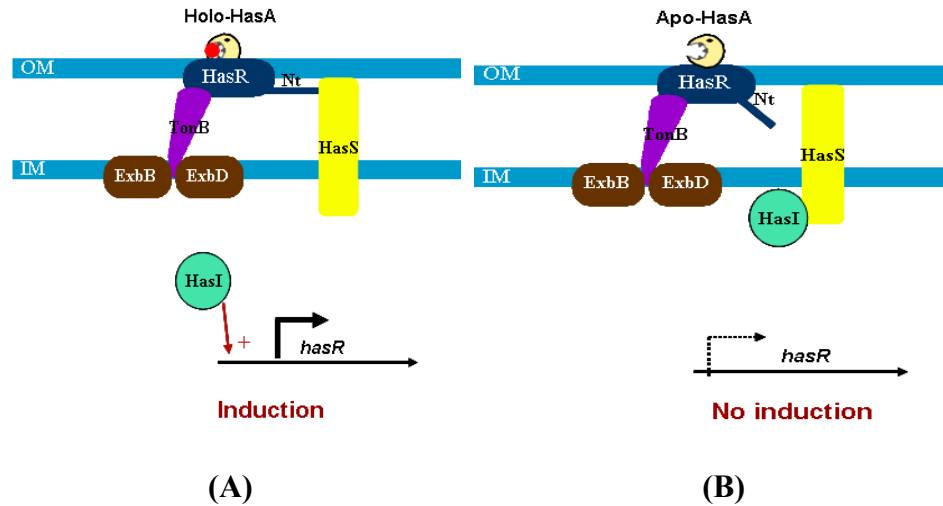


Fig. 7 The *has* operon signaling cascade. Adapted from (Cescau et al., 2007)

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*.

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

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).

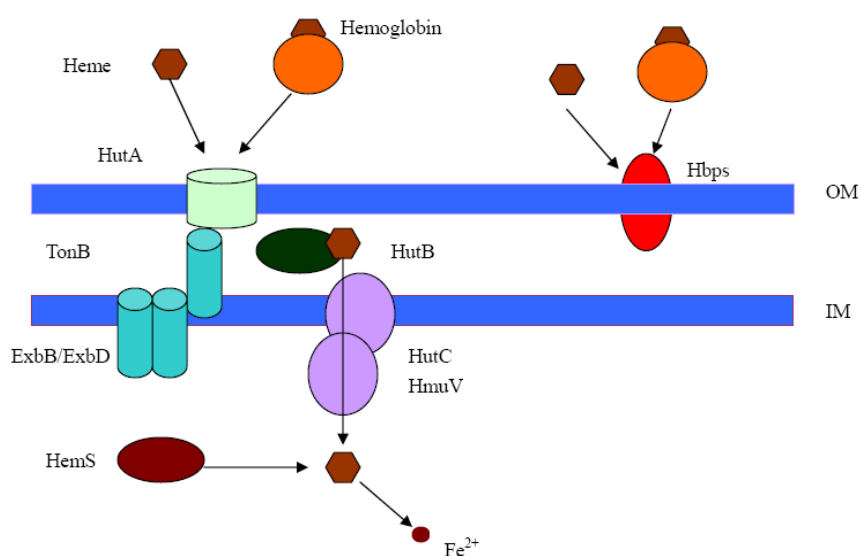


Fig. 8 Proposed organization of the heme acquisition system of *Bartonella*. The heme uptake system consists of HutA, a TonB-dependent heme receptor, HutB, HutC and HmuV (ABC transport system), and HemS (heme trafficking or degradation protein). Adapted from (Minnick and Battisti, 2009).

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. birtlessii* 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 *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 α -proteobacteria (Rudolph et al., 2006). Homologues of Irr were found in all *Rhizobia* (Rudolph et al., 2006). Irr was first identified in *B. japonicum* as a transcriptional repressor of *hemB* which encodes for heme biosynthetic enzyme δ -aminolevulinic acid dehydratase (Hamza et al., 1998). Irr has been described as a regulator of iron- and heme-associated genes in *B. japonicum* (Hamza et al., 2000), *R. leguminosarum* (Singleton et al., 2010) and *B. abortus* (Martinez et al., 2005). *B. japonicum* Irr expression is under the transcriptional control of Fur. It was shown that *B. japonicum* Fur binds to the *irr* gene promoter with high affinity, although without a consensus Fur box (Friedman and O'Brian, 2003). However, *R. leguminosarum* *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 *hut* locus of *B. quintana* (Parrow et al., 2009). These data suggested that Irr plays an important role as a heme-responsive transcriptional regulator in *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

PAPER ONE

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* (Hirotzu 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.

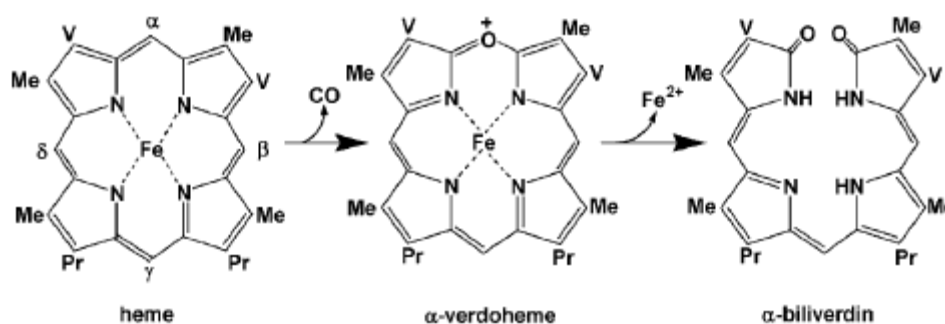


Fig. 1 Heme degradation, as catalyzed by the heme oxygenase reaction. Adapted from (Wilks and Burkhard, 2007).

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 anthracis* (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).

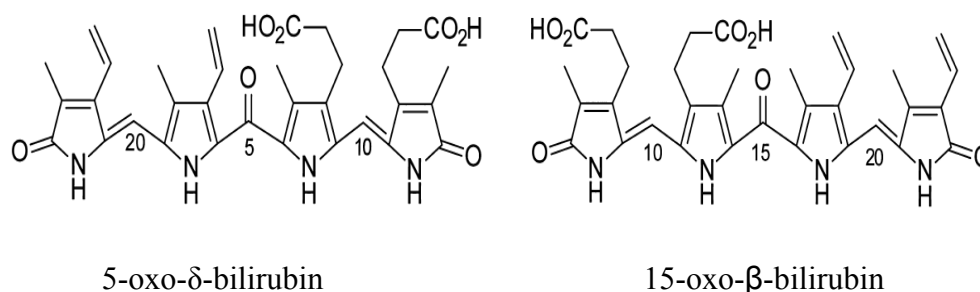


Fig 2. Linear representation of heme degradation products produced by the IsdG family. Adapted from (Reniere et al., 2010).

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.

Protein	Organism	Function	Reference
ShuS	<i>Shigella dysenteriae</i>	Heme trafficking	(Wilks, 2001)
HemS	<i>Yersinia enterocolitica</i>	Heme trafficking	(Schneider et al., 2006)
HmuS	<i>Yersinia pestis</i>	Heme trafficking	(Thompson et al., 1999)
HutZ	<i>Vibrio cholerae</i>	Heme trafficking or storage	(Wyckoff et al., 2004)
PhuS	<i>Pseudomonas aeruginosa</i>	Heme trafficking	(Lansky et al., 2006)
PigA	<i>Pseudomonas aeruginosa</i>	Heme oxygenase	(Ratliff et al., 2001)
BphO	<i>Pseudomonas aeruginosa</i>	Heme oxygenase	(Wegele et al., 2004)
HmuO	<i>Corynebacterium diptheriae</i>	Heme oxygenase	(Wilks and Schmitt, 1998)
HemO	<i>Neisseria meningitidis</i>	Heme oxygenase	(Zhu et al., 2000b)
ChuS	<i>Escherichia coli</i> O157:H7	Heme oxygenase	(Suits et al., 2005)
HugZ	<i>Helicobacter pylori</i>	Heme oxygenase	(Guo et al., 2008)
IsdG	<i>Staphylococcus aureus</i>	Heme-degrading enzyme	(Skaar et al., 2004)
IsdG	<i>Staphylococcus lugdunensis</i>	Heme-degrading enzyme	(Haley et al., 2011)
IsdI	<i>Staphylococcus aureus</i>	Heme-degrading enzyme	(Skaar et al., 2004)
IsdG	<i>Bacillus anthracis</i>	Heme-degrading monooxygenase	(Skaar et al., 2006)
HmuQ	<i>Bradyrhizobium japonicum</i>	Heme-degrading enzyme	(Puri and O'Brian, 2006)
HmuD	<i>Bradyrhizobium japonicum</i>	Heme-degrading enzyme	(Puri and O'Brian, 2006)
MhuD	<i>Mycobacterium tuberculosis</i>	Heme-degrading enzyme	(Chim et al., 2010)

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

Bartonellae are hemotropic bacteria, agents of emerging zoonoses. These bacteria are heme auxotroph *Alphaproteobacteria* 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 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 Fe^{3+} uptake. Similarly to other bacteria which use heme as an iron source, *Bartonellae* must transport this compound into the cytoplasm and degrade it to allow the release of iron from the tetrapyrrole 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, ascorbate or NADPH-cytochrome P450 reductase. Knocking down the expression of HemS in *B. henselae* reduces its ability to face H_2O_2 induced oxidative stress.

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Introduction

Bartonella species are now well established as human pathogens responsible for several emerging zoonoses [1]. *Bartonella bacilliformis* (*B. bacilliformis*), *Bartonella quintana* (*B. quintana*) and *Bartonella henselae* (*B. henselae*) are the most medically important species although several others 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 syndromes [4]. Most *Bartonella* species appear to share a similar natural cycle that involves arthropod transmission, then exploitation of a mammalian host. Each *Bartonella* species appears to be highly adapted to one or few reservoir hosts in which *Bartonella* causes a long lasting intra-erythrocytic bacteraemia 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 intraerythrocytic bacteraemia is considered to represent a unique adaptation to the mode of transmission by blood sucking arthropod vectors. Flagella [5], a deformin activity [6], and a locus containing *ialA* and *ialB* genes [7] were shown to be important for erythrocytes invasion by *B. bacilliformis*. Also, the importance of the Trw T4SS for erythrocytes invasion was demonstrated for *B. tribocorum* [8] and *B. birtlesii* [9]. The erythrocytes invasion can also be proposed to be 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. quintana* 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 siderophore biosynthesis and a complete iron Fe^{3+} transport system. Only genes sharing strong homologies with all the compounds of a Fe^{2+} uptake system already characterized in *Yersinia pestis* [12], and *Photobacterium luminescens* [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. quintana* [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 cytoplasm [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 *Corynebacterium diphtheriae* [16]. Other bacterial heme oxygenases, that provoke the release of iron by degrading heme to biliverdin were also characterized in Gram negative and Gram positive bacteria such as *Pseudomonas aeruginosa*

[17], or *Clostridium tetani* [18] (for a review see: [15]). Other heme degrading mono-oxygenases were identified in Gram positive bacteria such as *Bacillus anthracis* [19] and Gram negative bacteria such as *Bradyrhizobium japonicum* [20]. Protein sequestering heme have been identified in bacteria such as *Yersinia enterocolitica* [21], *Shigella dysenteriae* [22], *Pseudomonas aeruginosa* [23]. Enzyme allowing the release of iron from protoporphyrin IX leaving ring intact was identified in *Escherichia coli* (*E. coli*) [24]. Controversial data about this heme dechelate activity were published recently [25]. In this report, we investigated the function of HemS for *B. henselae*, using functional complementation, and partial biochemical characterization. The effect of *hemS* knock down was also investigated in *B. henselae*.

Materials and Methods

Bacterial strains and plasmid

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 0.26 µm pore size Millipore filters for bacterial growth experiments. *E. coli* strains

were grown on LB medium or M63 minimal medium aerobically at 37°C [26]. M63 medium was supplemented with 0.4% glycerol (Gly) as carbon source. Solid media contained 1.5% Difco agar. Soft M63 agar medium contained 0.7% Difco agar. Iron-depleted medium was obtained with the addition of Dip at a 70 µM final concentrations. Antibiotics were added to the following final concentrations (µg ml⁻¹): Ampicillin (Amp), 50; Kanamycin (Km), 50; Spectinomycin (Spc), 50; Chloramphenicol (Cmp), 20. Arabinose was added at 0.2% for induction of the P_{ara} promoter as indicated. *B. henselae* was grown on 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 [27] at 35°C under 5% CO₂ atmosphere.

Use of heme as iron source: *E. coli* assay

Tested strains were grown at 37°C for 18 hours in M63 medium without iron, with 0.4% glycerol as carbon source, and in the presence of 0.2% arabinose. Culture 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 M63 plates containing 0.4% Glycerol, 0.2% arabinose, and 70 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 one and two day incubation at 37°C. All experiments were performed in triplicate.

Table 1. Strains and plasmids used in this study.

<i>E. coli</i> strains	Genotype	Source or reference
XL1-blue	F ⁻ supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac ⁺ F' proAB ⁺ lacZ ⁺ lacZΔM15 Tn10 (Tet ^R)	Laboratory collection
JP313	araD139 relA rpsL 150 thi hsd5301 (lacU 139) deo7 ptsF25 Δara 174	Laboratory collection
MG1655 ttdA::Cmp	F ⁻ ttdA::Cmp Cmp ^R	[31]
FB8.27	F ⁻ , Δlac X74, entF::TnphoA'S, Tet ^R	[37]
FB8.27 (pAM238::hasR)	FB8.27 pAM238::hasR Tet ^R , Spc ^R	[24]
FB8.27 efeB::Kan yfeX::Cmp (pAM238::hasR)	FB8.27 efeB::Kan yfeX::Cmp, pAM::hasR, Tet ^R , Spc ^R , Kan ^R , Cmp ^R	[24]
XL1-blue (pBAD24::hemS _{hs})	XL1-blue, pBAD24::hemS _{hs} Amp ^R	This study
JP313 (pBAD24)	JP313 pBAD24 Amp ^R	This study
JP313 (pBAD24::hemS _{hs})	JP313 pBAD24::hemS _{hs} Amp ^R	This study
FB8.27 (pAM238::hasR), (pBAD24)	FB8.27 pAM238::hasR pBAD24 Tet ^R , Spc ^R , Amp ^R	This study
FB8.27 efeB::Kan yfeX::Cmp (pAM238::hasR), (pBAD24)	FB8.27 efeB::Kan yfeX, pAM::hasR, pBAD24, Tet ^R , Spc ^R , Kan ^R , Amp ^R , Cmp ^R	This study
FB8.27 efeB::Kan yfeX::Cmp (pAM238::hasR), (pBAD24::hemS _{hs})	FB8.27 efeB::Kan yfeX::Cmp, pAM::hasR, pBAD24::hemS _{hs} , Tet ^R , Spc ^R , Kan ^R , Amp ^R , Cmp ^R	This study
<i>Bartonella</i> strains	Genotype	Source or reference
<i>B. henselae</i>	<i>B. henselae</i> Houston-1, ATCC 49882 ^T	Laboratory collection
<i>B. henselae</i> (pNS2Trc)	<i>B. henselae</i> Houston-1(pNS2Trc) Kan ^R	This study
<i>B. henselae</i> (pNS2Trc::hemS _{hs})	<i>B. henselae</i> Houston-1, pNS2Trc::hemS _{hs} Kan ^R	This study
Plasmids		
pKOBEGA	λred functions, Amp ^R	[49]
pBAD24	pBR322 araC, arabinose-inducible promoter, Amp ^R	[50]
pNS2Trc	Kan ^R	[41]
pAM238::hasR	pAM238::hasR Spc ^R	[51]
pBAD24::hemS _{hs}	pBAD24 carrying <i>B. henselae</i> hemS _{hs} , Amp ^R	This study
pNS2Trc::hemS _{hs}	pNS2Trc carrying antisense hemS from <i>B. henselae</i> .	This study

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Effect of the *hemS* knockdown in *B. henselae*

To evaluate the effect of *hemS* knock down 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* (pNS2Trc::*hemS*_{AS}) were collected after 5 days growth on CBA plates and suspended in Schneider's medium. The OD₆₀₀ of the bacterial suspension was adjusted to 0.05. 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* (pNS2Trc::*hemS*_{AS}) were grown on CBA plates during 5 days at 35°C under 5% CO₂ atmosphere. Bacteria collected from one plate were 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 (T₀). For challenge assay, bacteria were incubated 30 minutes in PBS buffer in the presence of 1 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 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)×100%. All experiments were performed in triplicate.

Genetic techniques

E. coli cells were transformed by the calcium chloride method [28]. Pl lysates and transductions were performed as previously described by Miller [26]. *Bartonella* cells were transformed by electroporation as previously described [29].

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

A non polar mutation that deletes the entire *yfeX* gene was created by allelic exchange as previously described [30]. Briefly, plasmid pKOBEGA (an ampicillin-resistant derivative of pKOBEG) (see Table 1) was introduced into the target strain, and electrocompetent cells were prepared at 30°C after induction of the λ red system carried by pKOBEGA with 0.2% arabinose. A three-step PCR procedure was used to produce a PCR product in which the *cat* gene from pHP45Ω (9) is flanked by 500-bp homology arms corresponding to DNA regions located upstream and downstream from the *yfeX* start and stop codons, respectively. The following primers were used: for the left 500-bp *yfeX* homology arm, AmtAmtyfeX and AmtAvlyfeX and for the right 500-bp *yfeX* homology arm, AvlAmtyfeX and AvlAvlyfeX. The *cat* gene cassette (0.9 kb) was amplified from strain *E. coli* MG1655 *tetA*:Cm [31] using the primers Cat5 and Cat3. The PCR product resulting from the three-step procedure was introduced into *E. coli* XL1 blue/pKOBEGA using electroporation, and chloramphenicol-resistant deletion mutants produced by allelic exchange were selected at 37°C (to eliminate the thermosensitive plasmid pKOBEGA). Correct chromosomal insertion was checked by PCR amplification using the cat primers Cat5 and Cat3 in combination with AvlAvlyfeX and AmtAmtyfeX, respectively. The *yfeX*:Cm^r mutation was then introduced in strain FB8.27 *eflB*:*Kan* using P1 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 QIAprep Spin Miniprep kit (Qiagen). Restriction endonuclease digestions, and ligation were carried out according to the manufacture's recommendation. 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 entire coding region of *hemS* was amplified by PCR from the *B. henselae* chromosomal DNA using primers hemShamont, containing NheI restriction site and allowing addition of a His₆ - tag at the N-terminus part of the protein, and primer hemShaval containing a KpnI restriction site (Table 2). The 1070 bp PCR product was purified, digested by NheI and KpnI, and ligated with plasmid pBAD24, digested with NheI and KpnI, to give plasmid pBAD24::*hemS*_{AS}. Ligation product was introduced in *E. coli* strain XL1 blue, using calcium chloride method. Transformants were screened using PCR method with hemShamont and hemShaval primers. Six PCR positive clones were then sequenced.

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

The entire coding region of *hemS* was amplified by PCR from the *B. henselae* chromosomal DNA using primers hemSantisensamt, containing a BamHI restriction site, and hemSantisensavl, containing an XbaI restriction site (Table 2). The 1054 bp PCR product was purified, digested with BamHI and XbaI and then ligated with plasmids pNS2Trc digested with BamHI and XbaI to give pNS2Trc::*hemS*_{AS}. Ligation mixtures were introduced in *E. coli* strain XL1 blue using calcium chloride method. Transformants were screened, using PCR method with hemSantisensamt, and hemSantisensavl primers. Six PCR positive clones were then sequenced.

Expression and Purification of HemS His-tagged protein

Strain JP313 (pBAD24::*hemS*_{AS}) 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. Arabinose 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, 2.5 mM MgSO₄, 10 mM imidazole, 0.05% Triton). 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 30 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 DTT

Table 2. Primers used in this study.

Primer	Gene	Organism	Sequence
hemShamont	<i>hemS</i>	<i>B. henselae</i>	5'TTTTGGGCTAGCAGGAGGAATTCACCATGCATCATCACCATCACCATTATATACAGCCGAAAT 3'
hemShaval	<i>hemS</i>	<i>B. henselae</i>	5'ATCCCGGGTACCATGGTCTAAGCGACTGCTACTGCGTGGCTTTGAGGC 3'
hemSantisensavl	Antisens <i>hemS</i>	<i>B. henselae</i>	5' CCCCTCTAGATGTCATATACAGCCGAAAT 3'
hemSantisensamt	Antisens <i>hemS</i>	<i>B. henselae</i>	5' CCCGGATCCCTAAGCGACTGCTACTGCGTG3'
AmtAmtyfeX	Upstream of <i>yfeX</i>	XL1-blue	5' ATTTGGCGTTAATCTGGCTGCTG3'
AmtAvlyfeX	Upstream of <i>yfeX</i>	XL1-blue	5'GCGGATGAATGGCAGAAATTTGTTCTCTGAAAATAATAATGC3'
Cat5	Cmp cartridge	MG1655 <i>ttdA-Cmp</i>	5' AATTTCGCCATTATCCGC3'
Cat3	Cmp cartridge	MG1655 <i>ttdA-Cmp</i>	5' TTGATCGGCACGTAAGAGGT3'
AvlAmtyfeX	Downstream of <i>yfeX</i>	XL1-blue	5'ACCTCTTACGTGCCGATCAATTACTTCTGCTTTAACGCCGCATAC3'
AvlAvlyfeX	Downstream of <i>yfeX</i>	XL1-blue	5' TTTAAACCGCAACAAATTTGCCGCC3'

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was added. And samples were not boiled before electrophoresis. 4 µg of HemS protein and 5 µg of BSA were separated on 12% SDS-PAGE. One gel was stained with coomassie brilliant blue R. Another gel was transferred to nitrocellulose by the general methods of Towbin *et al.* [33]. Heme-binding blot was done according the protocol of Carroll *et al.* [34]. Briefly, the resulting blots were rinsed with TBST buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20) three times for 10 min and subsequently probed for 1.5 h with TBS containing heme (10^{-6} M) at room temperature. The nitrocellulose was washed three times for 10 min with TBST at room temperature. Heme was visualized by it intrinsic peroxidase activity using enhanced chemiluminescence (ECL) reagents (Amersham pharmacia, Piscataway, NJ). Heme binding protein bands were visualized by exposing the blot to autoradiographic film (LabsScientific, Livingston, NJ).

Absorption spectroscopy

Heme binding assays also were carried out using absorption spectroscopy method. HemS and heme were respectively diluted to 10 µM, and 200 µM in Tris-HCl 50 mM pH 8.0. Aliquots of heme (raising heme concentration from 1 µM to 20 µM final concentration) were successively added into the cuvette containing 100 µl of 10 µM HemS. Absorbance spectra from 300 nm to 700 nm were recorded 5 mins after each heme addition on a nanodrop 2000 spectrophotometer. Experiments were performed in triplicate.

Reaction of HemS-heme complex with NADPH-cytochrome P450 reductase

The reaction was performed according to Zhu *et al.* [35] and Skaar *et al.* [19]. Human cytochrome P450 oxidoreductase (Sigma-Aldrich) was added to the HemS-heme complex (10 µM) at a ratio of reductase to HemS equal to 0.3:1 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 µM 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.

Reaction of HemS-heme complex with ascorbate

Ascorbic acid-dependent degradation of heme was monitored spectrophotometrically as previously described [35]. HemS-heme complex (10 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

200 µl of an emulsion containing purified HemS (10 µg), ISA 61 VG adjuvant (Seppic Paris France) (120 µl) and completed with NaCl 0.9% were inoculated twice (with one month interval) by subcutaneous route in C57B6 mice (Charles River) (Ethic committee Anses/ENVA/UPEC agreement n°:14/06/2011-1). Two weeks after the second inoculation, 200 µl blood samples were collected each 3 week, using retro orbital bleeding method. Blood samples were centrifuged twice (3600 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 8000 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 [36], 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. henselae* were performed as follows: *B. henselae* (pNS2Trc) and *B. henselae* (pNS2Trc:*hemS*_{AS}) were harvested after 5 days of growth on CBA plates. Proteins contained in 20 µg of each sample were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-C Extra, GE Healthcare) according to Towbin *et al.* [33]. Nonspecific binding sites were blocked with 5% skim milk in TBS-Tween 20(0.05%). The immunoblot was probed with polyclonal mice sera raised against recombinant HemS (1:200), followed by a 1:1,000 dilution of a rabbit anti-mice 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 BC Assay protein Quantitation kit (interchim)

Statistical analysis

Data was expressed by mean \pm standard errors of the means. The statistical analysis was performed using GraphPad Prism 5 software for Windows. Statistical significance of the data was ascertained 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 HmuS 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/HmuS from *Bartonella* shared 35% to 42% identity with some of these polypeptides (Fig. 1). Analysis of the identical regions did not allow predicting that HemS/HmuS from *Bartonella* were heme degrading enzyme 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 6 \times His-tag at its N-terminus, and cloned in plasmid pBAD24. The recombinant plasmid pBAD24::hemS_{his} was introduced in *E. coli* strain FB8.27 *eflB::Km yfeX::Cmp* (pAM::hasR) to check for complementation ability. This *E. coli* strain, similarly with strain FB8.27 (pAM::hasR), which cannot grow on an iron depleted medium since it is impaired in enterobactin biosynthesis [37]. When heme is added on iron depleted medium, strain FB8.27 (pAM::hasR) can grow, due to the presence of the HasR heme transporter from *Serratia marcescens* contained in plasmid pAM::hasR [38]. Heme is transported through the outer membrane by HasR, and the deferrocyclization activity of EfeB and YfeX allows the release of iron required for growth [24]. In *E. coli* strain FB8.27 *eflB::Km yfeX::Cmp* (pAM::hasR), deferrocyclization activity is absent and consequently, heme dependent growth was abolished. When hemS from *B. henselae*, was expressed in strain FB8.27 *eflB::Km yfeX::Cmp* (pAM::hasR) (pBAD24::hemS_{his}) it restored the heme dependent growth on 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_{his} 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_{his}) and JP313 (pBAD24). A supplementary visible band of 40 KDa was observed only for the strain containing the recombinant plasmid pBAD24::hemS_{his} grown in the presence of arabinose (data not shown). In strain JP313 (pBAD24::hemS_{his}), grown in the presence of 0.2% arabinose, His-tagged HemS was expressed as a soluble protein. The protein was purified by Ni-agarose affinity chromatography, yielding a distinct protein (95% pure) migrating at \sim 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 μ g) and BSA (5 μ g) 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 added at 10^{-6} M concentration (Fig. 3B). In contrast, in our condition assay, BSA was unable to bind heme (Fig. 3B). This latter result underlines the specificity of the HemS heme binding. Also, a crude extract obtained from *E. coli* strain JP313 (pBAD24::hemS_{his}), expressed a protein of about 40 KDa that can bind heme. In contrast such a protein was not observed in a crude extract obtained from strain JP313 pBAD24 (data not shown). Taken together, these above 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 μ M 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 μ M heme, showing a 1:1 stoichiometry of heme to HemS (Fig. 4B).

HemS can degrade heme *in vitro*

Various monoheme-protein complexes like heme oxygenases [39], and other biochemically uncharacterized heme degrading enzymes [40], [20] were shown to degrade heme *in vitro* in the presence of electron donors 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. 5A, the disappearance of the Soret band was nearly complete 5 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 spectral were recorded. The Soret band decreased after each addition of NADPH, and disappears after addition of 100 μ M 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). And 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 seen 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 *Bartonella*. According to the conclusions provided by analysis of the genome content of *Bartonella*, we

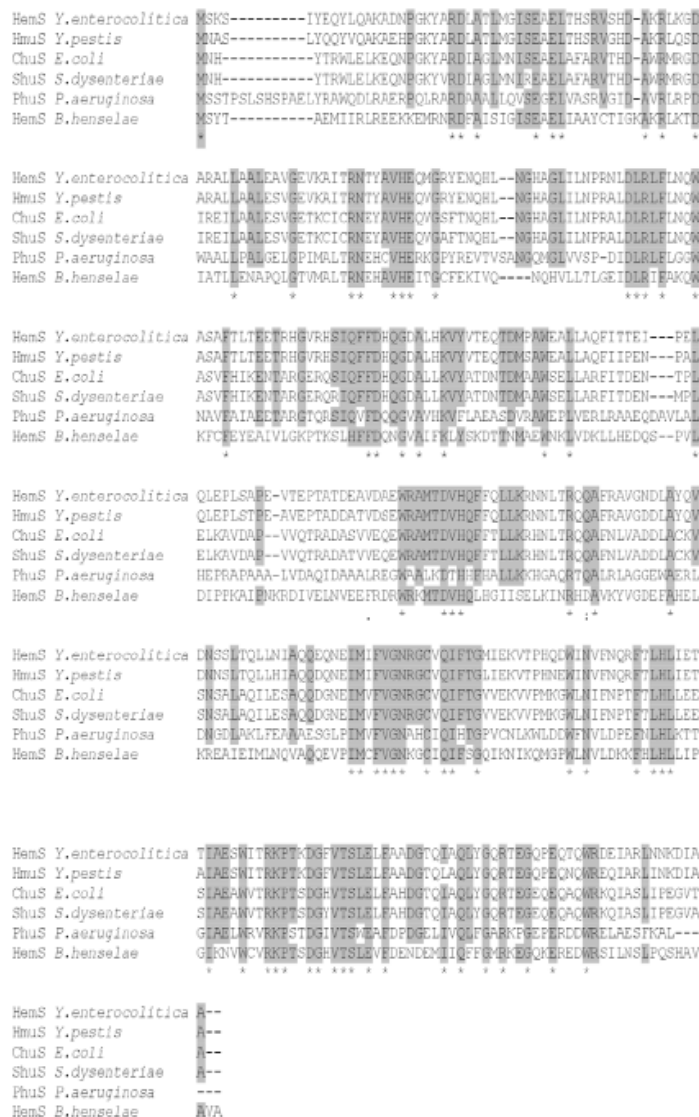


Figure 1. HemS and heme degrading or trafficking enzymes sequence alignment. ClustalW alignment of HemS from *B. henselae*, PhuS from *Pseudomonas aeruginosa* [52], ChuS from *E. coli* [46], ShuS from *Shigella dysenteriae* [43], HmuS from *Yersinia enterocolitica* [21], HmuS from *Yersinia pestis* [47]. This alignment was generated by Clustal W. Amino acids conserved in five or more polypeptides are highlighted in grey. Amino acids conserved in all protein are indicated with a star.
doi:10.1371/journal.pone.0037630.g001

hypothesized that HemS is required for growth since its activity provides iron source when bacteria are grown in aerobic condition. Preliminary unsuccessful assays to disrupt *hemS* strengthened this hypothesis (data not shown). Lacking appropriate genetic tools, it is presently not possible to generate mutant for genes presumed to be essential for *B. henselae*. However it is possible to analyse the effect of the decrease of an essential gene product in

this genus via knocking down gene expression. This method was used successfully to investigate the function of genes in *B. henselae* [41], [42]. We cloned *hemS* of *B. henselae* into a vector that allowed high level expression, pNS2Trc [41]. The gene was oriented in the reverse orientation such that the antisense strand was transcribed. Plasmids pNS2Trc and pNS2Trc:*hemS*_{AS} were introduced into *B. henselae* by electroporation. We first checked if the level of HemS

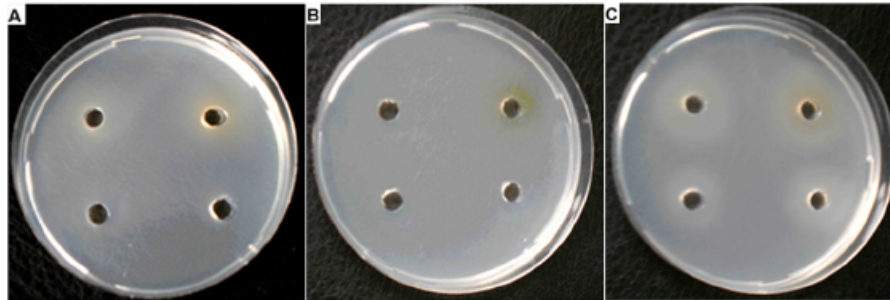


Figure 2. Functional complementation of the *E. coli* mutants impaired in iron release from heme. *E. coli* strains FB8.27 pAM:hasR (pBAD24) (A), FB8.27 *efeB::Kan yfeX::Cmp* (pAM:hasR) (pBAD24) (B) and FB8.27 *efeB::Kan yfeX::Cmp* (pAM:hasR) (pBAD24::*hemS_{4S}*) (C) were tested for the use of heme as an iron source on iron depleted medium M63 (Gly 0.4%, Ara 0.2%, Dip 70 μ M, Spc, Amp). Growth around the wells containing 1 μ M, 5 μ M, 10 μ M, or 50 μ M Hb were performed as described in "Materials and Methods". Growth around the wells was assessed by visible 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

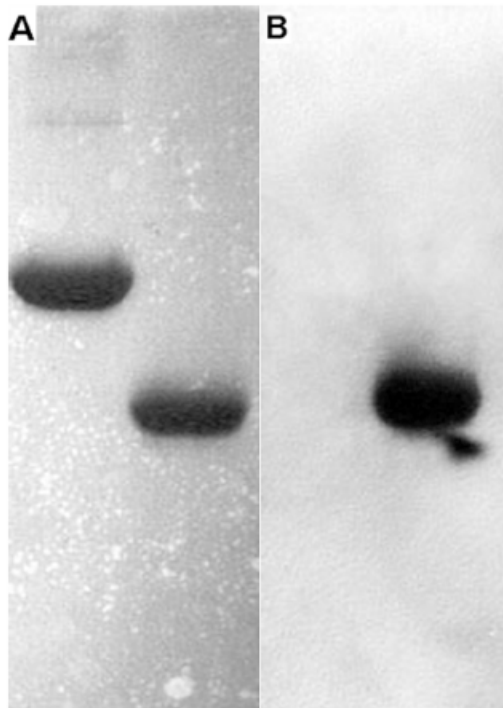


Figure 3. HemS heme blotting. After SDS gel electrophoresis, one gel was stained with coomassie brilliant blue R. Another gel was transferred to a nitrocellulose filter to do heme blotting and detected by ECL. (A) Coomassie blue staining: Line 1, 5 μ g BSA; Line 2, 4 μ g HemS; (B) Heme binding: Line 1, 5 μ g BSA; Line 2, 4 μ g 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. henselae* (pNS2Trc:*hemS_{4S}*) as compared with strain *B. henselae* (pNS2Trc) using western blot experiment performed with anti HemS mice antibodies. As seen in Fig. 6B, the level of HemS contained in strain *B. henselae* (pNS2Trc:*hemS_{4S}*) was lower than in strain *B. henselae* (pNS2Trc). Strains *B. henselae* (pNS2Trc) and *B. henselae* (pNS2Trc:*hemS_{4S}*) were then tested for growth on both on CBA plates and in Schneider's medium. Our results showed that the knock down of HemS in strain *B. henselae* (pNS2Trc:*hemS_{4S}*) did not significantly decrease its growth ability. Thus, the level of HemS in *B. henselae* (pNS2Trc:*hemS_{4S}*) is sufficient to support a normal growth on CBA plates and in Schneider'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 ShuS that was evidenced to promote heme utilization in *Shigella dysenteriae*. In this bacteria, *shuS* disruption did not increase sensitivity to hydrogen peroxide [43]. *Shigella dysenteriae* genome (<http://www.ncbi.nlm.nih.gov/genome/?term=Shigella%20dysenteriae%20>) contains genes encoding for KatG, AhpC, and AhpF Alkyl Hydroperoxide Reductase, that are involved in hydrogen peroxide degradation [44]. Also, OxyR that regulates the response to H₂O₂ 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 catalases, AhpC, and AhpF Alkyl Hydroperoxide Reductase, Dps and OxyR are absent in *Bartonella* genomes (<http://www.ncbi.nlm.nih.gov/protein?term=zwf%20Bartonella%20henselae>). Nevertheless, a previous report showed that *Bartonella bacilliformis* was able to sustain successfully a 30 minutes exposure to 1 mM H₂O₂ [45]. This latter result strongly suggested that cellular activities allow this bacterium to face oxidative stress generated by exposure to H₂O₂. 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. henselae* to face a 30 minutes exposure to 1 mM and 10 mM hydrogen peroxide.

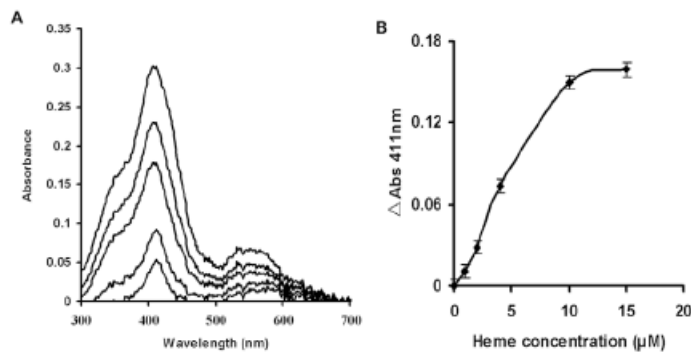


Figure 4. Binding of heme to HemS. (A): Increasing amounts of heme (1 μM–20 μM final concentration) were added to HemS (10 μM) as described in “Materials and Methods” and the spectrum (300 nm–700 nm) was recorded after 5 min for each addition. The Soret band at 411 nm increases with each addition of heme as demonstrated by absorbance peak increases at 411 nm. (B): Absorbance at 411 nm was measured for each sample and plotted versus heme concentration. Experiments were performed in triplicate and a single representative experiment is presented. doi:10.1371/journal.pone.0037630.g004

After exposure to 1 mM H_2O_2 , the survival was about 50% for both strains. After exposure to 10 mM H_2O_2 , the survival was about 20% for the control strain *B. henselae* (pNS2Trc). With strain *B. henselae* (pNS2Trc::hemS_{Δ8}), the survival was decreased by three fold (Fig. 7). This result showed that lowering HemS level in *B. henselae* enhanced its sensitivity to H_2O_2 .

Discussion

In this report, we investigated the function of HemS in *B. henselae*. Homologs of this protein are present in all the *Bartonella* genome that have been sequenced. This underlines the importance of its function for these *Alphaproteobacteria*. Predicting an important role for HemS in *Bartonella* is mainly driven by the fact that these bacteria use heme as iron source. The use of heme as

iron source requires its transportation through the outer membrane, the periplasm, and the inner membrane. The release of iron from heme inside the cytoplasm requires an enzymatic 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 protoporphyrin ring degradation [15]. Heme oxygenase first characterized in *Corynebacterium diphtheriae* [39], releases heme by degrading heme to forming α -biliverdin, CO and free iron. Others 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

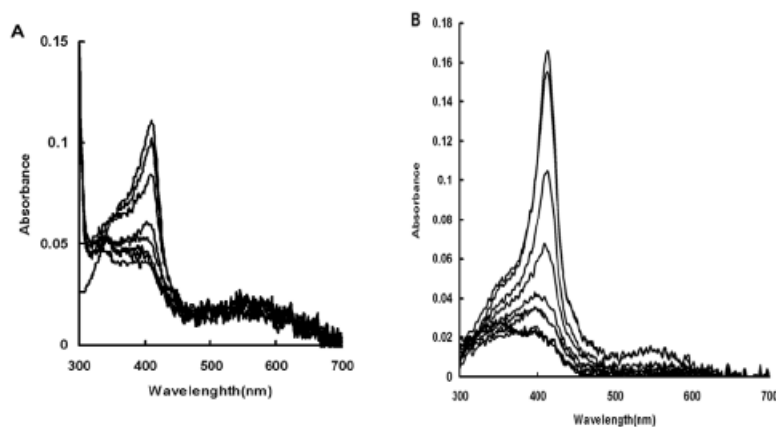


Figure 5. HemS dependent degradation of heme. (A): 10 mM final concentration of ascorbate was added to the HemS-heme complex (10 μM). The spectral changes from 300–700 nm were recorded every 1 min. (B): Cytochrome P450 reductase was added to 10 μM HemS-heme complex with a 0.3:1 molar ratio and heme degradation 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. doi:10.1371/journal.pone.0037630.g005

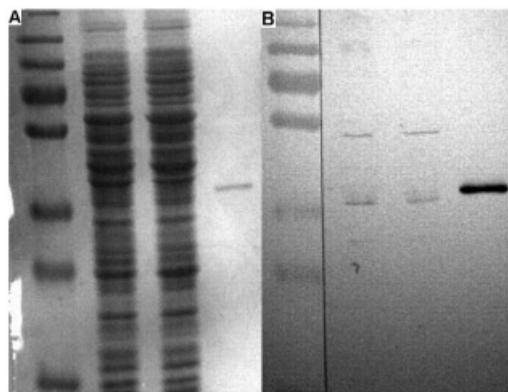


Figure 6. Detection of HemS expression level in *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::hemSAS by immunoblotting. 20 μ g samples of *B. henselae* pNS2Trc (1) and *B. henselae* pNS2Trc::hemSAS (2), 20 ng sample of purified his-tagged HemS (3) were loaded on SDS-PAGE. After electrophoresis, one gel was stained with comassie brilliant blue R (A). Another gel was transferred to a nitrocellulose filter to do immune blotting as described in "Materials and Methods" (B). Measurement of HemS band intensity using Image J software gave the following results: *B. henselae* pNS2Trc mean gray value: 24, integrated density: 3218; *B. henselae* pNS2Trc::hemSAS: mean gray value: 14, integrated density: 2075.
doi:10.1371/journal.pone.0037630.g006

[15]. Finally, a deferrochelation 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 tetrapyrrole 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], ChuS 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 *Shigella dysenteriae* [43]. For HemS from *B. henselae*, both complementation abilities and biochemical assays show that HemS can degrade heme. Similarly with IsdG from *Bacillus anthracis*, purified HemS from *B. henselae* can degrade heme in the presence of a reducing agent. During the heme degradation in the presence of NADPH-cytochrome P450 reductase, the Soret band of the heme-HemS complex only decreased in intensity. This was not the case for HmuO from *Corynebacterium diphtheriae* since the Soret band wavelength of the heme-HmuO complex also vary during the heme degradation in the presence of NADPH-cytochrome P450 reductase [39]. Heme degradation enzymes of the IsdG family were characterized in *Alphaproteobacteria* like *Bradyrhizobium japonicum* [20] and *Brucella melitensis* [20]. The heme degradation in *Bradyrhizobium japonicum* produces biliverdin [20]. In *Brucella melitensis* the product of heme degradation by BmeII0706 was not characterized [20]. Similarly with BmeII0706 from *Brucella melitensis* [20], HemS from *B. henselae* and *B. birtlesii* (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 HO activity from peroxide-coupled oxidation. HemS knock down only provoked a slight slowdown growing effect when bacteria were grown on blood

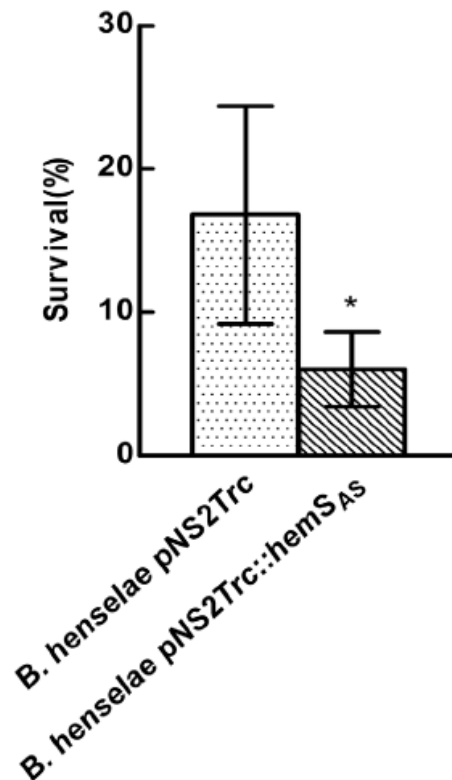


Figure 7. HemS knockdown decreases *B. henselae* ability to face exposure to H_2O_2 . *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::hemSAS were challenged with 10 mM H_2O_2 as described in "Materials and Methods". Experiments were performed in triplicate and a single representative experiment is presented.
doi:10.1371/journal.pone.0037630.g007

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 *B. henselae* to sustain exposure to hydrogen peroxide. This result suggests that HemS could be an actor of the pathway used by *Bartonella* to face oxidative stress. Since classical pathways used to face oxidative stress characterized in *E. coli* are not present in *Bartonella*, we hypothesized that *Bartonella* develop alternatives strategies to face oxidative stress encountered in erythrocytes or macrophages and during vectorisation by the cat flea *Ctenocephalides felis*. Inside erythrocytes, and in the flea gut, Hb and heme concentration are high [48]. Thus, HemS may be proposed to have a dual role for *Bartonella*, since its heme degrading activity allows iron supplying and the control of heme homeostasis.

Supporting Information

Figure S1 Purification of His-tagged HemS. Purification of the His-tagged HemS protein was achieved by Ni-agarose purification followed by gel filtration. Purified protein (5 μ g) was

run on 12% sodium SDS-PAGE and stained with Coomassie blue. Line MW: molecular weight markers. Line 1: purified HemS. (TIF)

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Author Contributions

Conceived and designed the experiments: FB. Performed the experiments: MFL. FB. Analyzed the data: MFL. FB. Contributed reagents/materials/analysis tools: FB HJB. Wrote the paper: MFL. FB HJB.

References

- Breitschwerdt EB, Maggi RG, Chomel BB, Lappin MR (2010) Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. *J Vet Emerg Crit Care (San Antonio)* 20: 8–30.
- Gupilli L (2010) Bartonellosis. *Vet Microbiol* 140: 347–359.
- Edouard S, Raoult D (2010) *Bartonella henselae*, an ubiquitous agent of proteiform zoonotic disease. *Med Mal Infect* 40: 319–330.
- Klotz SA, Janas V, Elliott SP (2011) Cat-scratch Disease. *Am Fam Physician* 83: 152–155.
- Scherer DC, DeBuron-Connors I, Minnick MF (1993) Characterization of *Bartonella bacilliformis* flagella and effect of anti-flagellin antibodies on invasion of human erythrocytes. *Infect Immun* 61: 4962–4971.
- Derrick SC, Ihler GM (2001) Deformin, a substance found in *Bartonella bacilliformis* culture supernatants, is a small, hydrophobic molecule with an affinity for albumin. *Blood Cells Mol Dis* 27: 1013–1019.
- Mitchell SJ, Minnick MF (1995) Characterization of a two-gene locus from *Bartonella bacilliformis* associated with the ability to invade human erythrocytes. *Infect Immun* 63: 1552–1562.
- Seubert A, Hiestand R, de la Cruz F, Dehio C (2003) A bacterial conjugation machinery recruited for pathogenesis. *Mol Microbiol* 49: 1253–1266.
- Vaysier-Tausant M, Le Rhun D, Deng HK, Biville F, Cescau S, et al. (2010) The Trv type IV secretion system of *Bartonella* mediates host-specific adhesion to erythrocytes. *PLoS Pathog* 6: e1000946.
- Sander A, Kretzer S, Brett W, Oberle K, Berens W (2000) Hemin-dependent growth and hemin binding of *Bartonella henselae*. *FEMS Microbiol Lett* 189: 55–59.
- Almark CM, Frank AC, Karlberg EO, Legault BA, Ardell DH, et al. (2004) The louse-borne human pathogen *Bartonella quintana* is a genomic derivative of the zoonotic agent *Bartonella henselae*. *Proc Natl Acad Sci U S A* 101: 9716–9721.
- Perry RD, Mier I, Fetherston JD (2007) Roles of the Yfe and Fec transporters of *Yersinia pestis* in iron uptake and intracellular growth. *Biomaterials* 20: 699–703.
- Watson RJ, Milichap P, Joyce SA, Reynolds S, Clarke DJ (2010) The role of iron uptake in pathogenicity and symbiosis in *Photobacterium luminescens* TT01. *BMC Microbiol* 10: 177.
- Parrow NL, Abbott J, Lockwood AR, Battisti JM, Minnick MF (2009) Function, regulation, and transcriptional organization of the hemin utilization locus of *Bartonella quintana*. *Infect Immun* 77: 307–316.
- Anzaldi LI, Skaar EP (2010) Overcoming the heme paradox: heme toxicity and tolerance in bacterial pathogens. *Infect Immun* 78: 4977–4989.
- Schmitt MP (1997) Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. *J Bacteriol* 179: 838–845.
- Ratiff M, Zhu W, Deshmukh R, Wilks A, Stojiljkovic I (2001) Homologues of neisserial heme oxygenase in gram-negative bacteria: degradation of heme by the product of the *hmoA* gene of *Pseudomonas aeruginosa*. *J Bacteriol* 183: 6394–6403.
- Bruggemann H, Bauer R, Raffesin S, Gottschalk G (2004) Characterization of a heme oxygenase of *Clostridium tetani* and its possible role in oxygen tolerance. *Arch Microbiol* 182: 259–263.
- Skaar EP, Gaspar AH, Schneewind O (2006) *Bacillus anthracis* IsdG, a heme-degrading monooxygenase. *J Bacteriol* 188: 1071–1080.
- Puri S, O'Brian MR (2006) The *hmuQ* and *hmuD* genes from *Bradyrhizobium japonicum* encode heme-degrading enzymes. *J Bacteriol* 188: 6476–6482.
- Stojiljkovic I, Hantke K (1994) Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersinia enterocolitica*. *Mol Microbiol* 13: 719–732.
- Wyckoff EE, Schmitt M, Wilks A, Payne SM (2004) HuzZ is required for efficient heme utilization in *Vibrio cholerae*. *J Bacteriol* 186: 4142–4151.
- Lansky IB, Lukat-Rodgen GS, Block D, Rodgers KR, Ratiff M, et al. (2006) The cytoplasmic heme-binding protein (PhuS) from the heme uptake system of *Pseudomonas aeruginosa* is an intracellular heme-trafficking protein to the delta-region-selective heme oxygenase. *J Biol Chem* 281: 13652–13662.
- Létoffé S, Heuck G, Deleplaire P, Lange N, Wandersman C (2009) Bacteria capture iron from heme by keeping tetrapyrrole skeleton intact. *Proc Natl Acad Sci U S A* 106: 11719–11724.
- Dailey HA, Seper AN, Daugherty I, Thames D, Gerdes S, et al. The *Escherichia coli* protein YfeX functions as a porphyrinogen oxidase, not a heme dechelatease. *MBio* 2: e00248-00211.
- Müller JH (1972) Experiments in molecular genetics. Cold Spring Harbor laboratory Press, Cold Spring Harbor, N.Y.
- Ries T, Dietrich F, Schmidt KV, Kaiser PO, Schwarz H, et al. (2008) Analysis of a novel insect cell culture medium-based growth medium for *Bartonella* species. *Appl Environ Microbiol* 74: 5224–5227.
- Maniatis T, Fritsch EF, Sambrook J (1989) Molecular Cloning. A Laboratory Manual; Harbor CS, editor. New York: Cold Spring Harbor Laboratory Press.
- Ries T, Anderson B, Fackelmayer A, Autenneth IB, Kempf VA (2003) Rapid and efficient transposon mutagenesis of *Bartonella henselae* by transposome technology. *Gene* 313: 103–109.
- Derbise A, Lesic B, Dacheux D, Ghigo JM, Camiel E (2003) A rapid and simple method for inactivating chromosomal genes in *Yersinia*. *FEMS Immunol Med Microbiol* 38: 113–116.
- Oshima T, Biville F (2006) Functional identification of YgiP as a positive regulator of the *adaA-tdkA-yge* operon. *Microbiology* 152: 2129–2135.
- Vargas C, McEwan AG, Downie JA (1993) Detection of c-type cytochromes using enhanced chemiluminescence. *Anal Biochem* 209: 323–326.
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76: 4350–4354.
- Carroll JA, Coleman SA, Smitherman LS, Minnick MF (2000) Hemin-binding surface protein from *Bartonella quintana*. *Infect Immun* 68: 6750–6757.
- Zhu W, Wilks A, Stojiljkovic I (2000) Degradation of heme in gram-negative bacteria: the product of the *hmoO* gene of *Neisseria* is a heme oxygenase. *J Bacteriol* 182: 6783–6790.
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
- Perronne-Piquemal M, Danchin A, Biville F (1999) Pyrophosphate increases the efficiency of enterobactin-dependent iron uptake in *Escherichia coli*. *Biochimie* 81: 245–253.
- Létoffé S, Deleplaire P, Wandersman C (2006) The housekeeping dipeptide permease is the *Escherichia coli* heme transporter and functions with two optional peptide binding proteins. *Proc Natl Acad Sci U S A* 103: 12891–12896.
- Wilks A, Schmitt MP (1998) Expression and characterization of a heme oxygenase (Hmo O) from *Corynebacterium diphtheriae*. Iron acquisition requires oxidative cleavage of the heme macrocycle. *J Biol Chem* 273: 837–841.
- Skaar EP, Gaspar AH, Schneewind O (2004) IsdG and IsdI, heme-degrading enzymes in the cytoplasm of *Staphylococcus aureus*. *J Biol Chem* 279: 436–443.
- Gillaspie D, Perkins I, Lanen K, McCord A, Pangonis S, et al. (2009) Plasmid-based system for high-level gene expression and antisense gene knockdown in *Bartonella henselae*. *Appl Environ Microbiol* 75: 5434–5436.
- Liu MF, Cescau S, Mechold U, Wang J, Cohen D, et al. (2012) Identification of a new nanoRNase in *Bartonella*. *Microbiology* 158: 886–895.
- Wyckoff EE, Lopreato GF, Tipton KA, Payne SM (2005) *Shigella dysenteriae* ShuS promotes utilization of heme as an iron source and protects against heme toxicity. *J Bacteriol* 187: 5658–5664.
- Imlay JA (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* 77: 755–776.
- Coleman SA, Minnick MF (2003) Differential expression of the invasion-associated locus B (*ialB*) gene of *Bartonella bacilliformis* in response to environmental cues. *Microb Pathog* 34: 179–186.
- Suiz MD, Pal GP, Nakazu K, Matte A, Cygler M, et al. (2005) Identification of an *Escherichia coli* O157:H7 heme oxygenase with tandem functional repeats. *Proc Natl Acad Sci U S A* 102: 16955–16960.
- Thompson JM, Jones HA, Perry RD (1999) Molecular characterization of the hemin uptake locus (*hmu*) from *Yersinia pestis* and analysis of *hmu* mutants for hemin and hemoprotein utilization. *Infect Immun* 67: 3879–3892.
- Silver GM, Gaines PJ, Hunter SW, Maddux JD, Thomas RE, et al. (2002) Identification, characterization, and cloning of an immunoglobulin degrading enzyme in the cat flea, *Cimex phellus felis*. *Arch Insect Biochem Physiol* 51: 136–150.
- Chaveronche MK, Ghigo JM, d'Enfert C (2000) A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res* 28: E97.
- Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose *PBAD* promoter. *J Bacteriol* 177: 4121–4130.

51. Letoffe S, Debarbieux L, Izadi N, Delepelaire P, Wandersman C (2003) Ligand delivery by haem carrier proteins: the binding of *Serratia marcescens* haemophore to its outer membrane receptor is mediated by two distinct peptide regions. *Mol Microbiol* 50: 77–89.
52. Kaur AP, Lansky IB, Wilks A (2009) The role of the cytoplasmic heme-binding protein (PhuS) of *Pseudomonas aeruginosa* in intracellular heme trafficking and iron homeostasis. *J Biol Chem* 284: 56–66.

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 melitensis* (Puri

and O'Brian, 2006). However, HemS of *Bartonella* do not have any similarity with the heme-degrading protein of *B. melitensis* 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. meningitidis* 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).

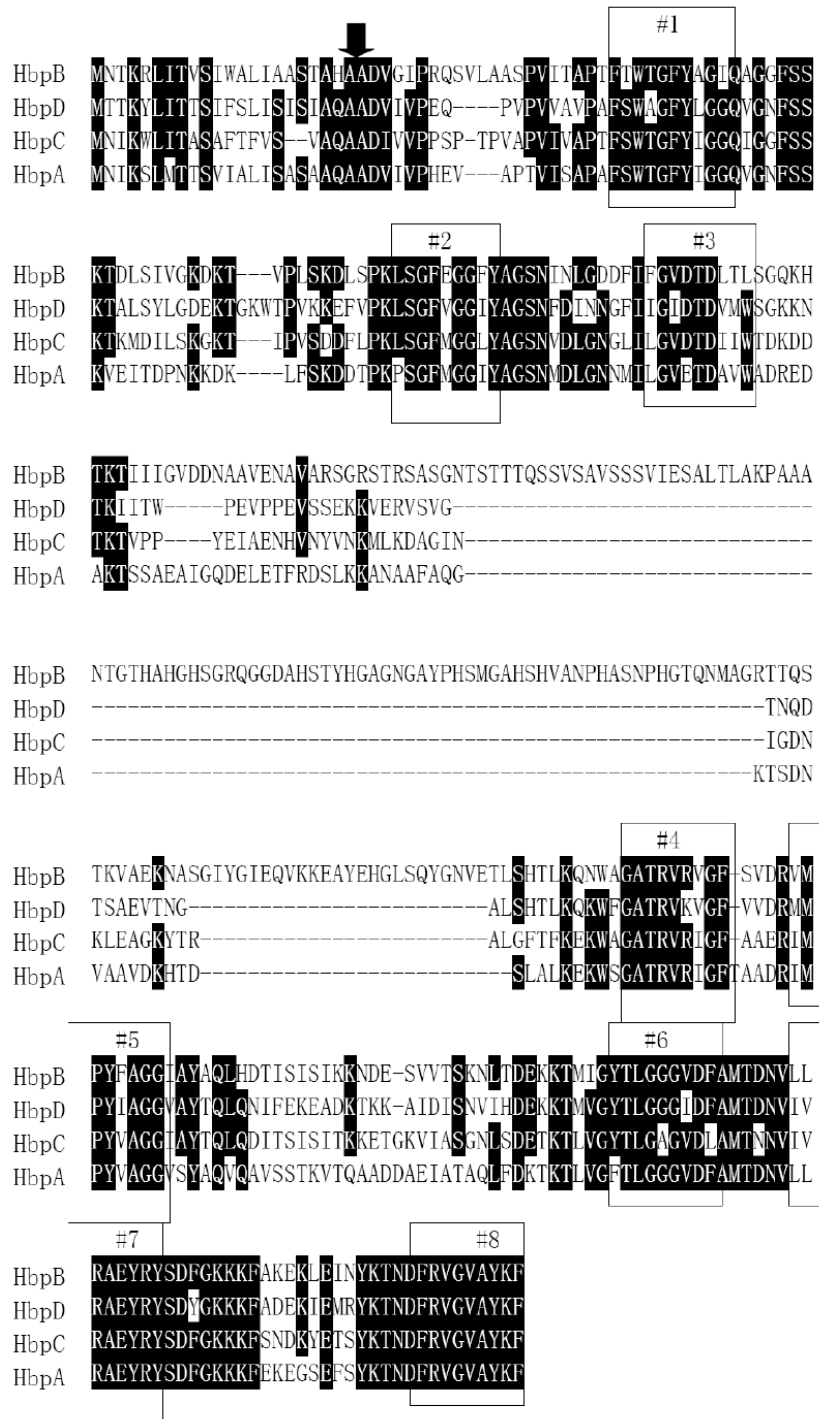


Fig 1. Multiple sequence alignment of the predicted proteins encoded by the *hbp* gene family of *B. henselae*. Identical residuals are shaded in black. Predicted β -strand transmembrane domains are boxed and numbered. Secretory signal sequence cleavage sites are indicated by an arrowhead.

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).

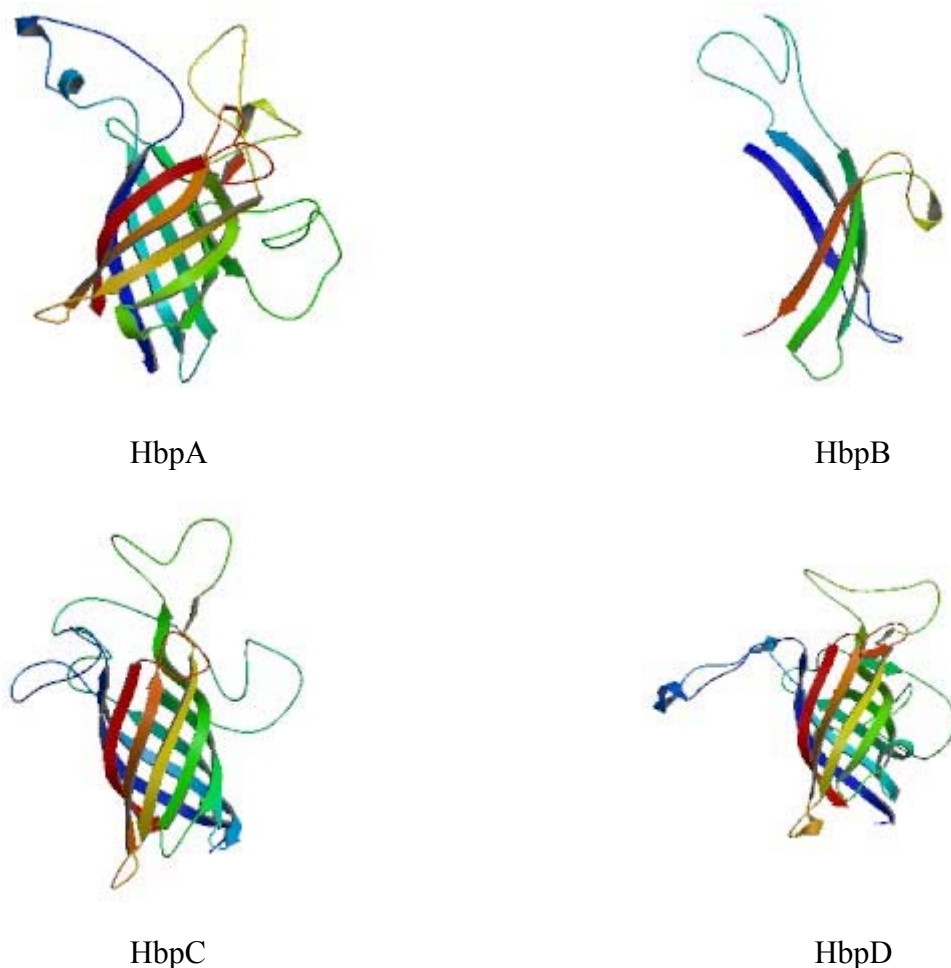


Fig 2. Three-dimensional models of *B. henselae* HbpA (region: 42aa-279aa), HbpB (region: 278aa-391aa), HbpC (region: 42aa-276aa) and HbpD (region: 41aa-274aa) were predicted by the ModBase Model.

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 μ M 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 *hbpA*, *hbpD* and *hbpE* that were overexpressed under conditions mimicking the mammal environment (low heme concentrations and 37°C) (Battisti et al., 2006) (Table 1). Transcription of *hbpA*, *hbpD* and *hbpE* was increased at low heme concentrations (0.05 mM) at 37°C, whereas transcription of *hbpB* and *hbpC* remained relatively unchanged (Battisti et al., 2006). The authors proposed that *hbpA*, *hbpD* and *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 *hbpB* and *hbpC* was upregulated during infection of endothelial cells, while *hbpA* and *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 *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% O₂, low heme) conditions using qRT-PCR. Under “bloodstream-like” (5% O₂, low heme) conditions, *irr* and *fur* expression was slightly increased, whereas *rirA* expression slightly decreased (Table 1). Under “louse-like” (30°C, high heme) conditions, *irr* expression decreased (Battisti et al., 2007). Transcription of *fur* and *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).

	30°C Compared to 37°C (0.15 mM heme)	5% Compared to 21% O ₂ (37°C, 0.15 mMheme)	0.05 mM Compared to 0.15 mM heme	5 mM Compared to 0.15 mM heme
<i>hbpA</i>	No difference	Decreased	Elevated	No difference
<i>hbpB</i>	No difference	Decreased	No difference	Elevated
<i>hbpC</i>	Significantly elevated	Decreased	No difference	Elevated
<i>hbpD</i>	No difference	Decreased	Elevated	No difference
<i>hbpE</i>	No difference	Decreased	Elevated	No difference
Regulator				
<i>irr</i>	Significantly reduced	Slightly elevated	Slightly elevated	Slightly reduced
<i>fur</i>	No difference	Slightly elevated	Slightly elevated	Slightly elevated
<i>rirA</i>	No difference	Slightly reduced	Slightly reduced	Slightly elevated

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). HbpB 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, HbpD 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*

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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 *Bartonellae* 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 *Bartonellae* species to obtain heme, an absolute requirement for growth [5]. Not all of the already sequenced *Bartonellae* 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 *Photobacterium luminescens* [8] are present in *Bartonellae* genomes. Moreover, *Bartonellae* genomes encode for a complete heme transport system shown to be active in *Bartonella quintana* [9].

Analysis of *Bartonellae* 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 sulfoxide reduction (MsrA and MsrB)[12] and oxidative stress response regulation (SoxR, OxyR) [13] [14], are not contained in *Bartonellae* genomes. Based on the above information relating to *Bartonellae* genome contents, it can be hypothesized that *Bartonellae* 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 H₂O₂ for 30 min [15]. Moreover, the lifestyle of *Bartonellae* suggests that these bacteria must face oxidative stress after the blood meal of the arthropod vector [16]. *Bartonellae* 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, *Bartonellae* 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 mimick the gut arthropod environment (high heme concentration and low temperature, high O₂ 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]. HbpC 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 ($\mu\text{g ml}^{-1}$): 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 P_{ara} 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% CO_2 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 1.98×10^8 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 OD₆₀₀ of 0.05 with overnight culture and grown at 37°C. Expression was induced at an OD₆₀₀ 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 OD₆₀₀ =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::*hbps*_{AS} 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 OD₆₀₀ 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% CO₂. OD₆₀₀ 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% CO₂. All experiments were performed in triplicate.

H₂O₂ challenge. *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::*hbps*_{AS} were grown on CBA plates for five days at 35°C under a 5% CO₂ atmosphere. Bacteria collected from one plate were suspended and washed twice in PBS buffer. The cell suspension was then diluted to OD₆₀₀ 0.5. Before H₂O₂ challenge, several dilutions of the tested cell suspension were spread on CBA plates (T₀). For the challenge assay, bacteria were incubated 30 min in PBS buffer in the presence of 1 mM and 10 mM H₂O₂ at 35°C under a 5% CO₂ atmosphere. After exposure to H₂O₂, bacteria were washed twice in PBS buffer and several dilutions plated onto CBA plates (T₁). After 15-day incubation at 35°C under a 5% CO₂ atmosphere, colonies were counted. Survival rate was expressed by (T₁/ T₀) 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 decomplexed 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 (T_I). The invasion rate was expressed as $(T_I/T_0) \times 100\%$. 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::*hbps*_{AS} 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 (T_{S24} or T_{S48}). The survival rate was expressed as

$(T_{S24}/T_I \text{ or } T_{S48}/T_I) \times 100\%$. 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::hbps_{AS}-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 1.98×10^8 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 *hbpA*, 881 bp for *hbpC* and 875 bp for *hbpD*) were purified and digested with NheI and KpnI. The *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 CaCl₂-competent *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.

Construction of the vector for decreasing the amount of Hbp in *B. henselae*. The entire coding region of *hbpA*, *hbpB*, *hbpC* and *hbpD* was amplified by PCR from the *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 *hbpA*, 1,182 bp for *hbpB*, 837 bp for *hbpC* and 831 bp for *hbpD*) was purified, digested with BamHI and XbaI and then ligated with plasmid pNS2Trc digested with BamHI and XbaI. Ligation mixtures were introduced into CaCl₂-competent *E. coli* strain XL1 Blue cells. Transformants were screened by PCR with corresponding primers. Six PCR-positive clones were then sequenced.

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 <https://www.genoscope.cns.fr/agc/microscope/home/index.php> 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 OD₆₀₀ of 0.05 with the overnight culture and grown at 37°C. Expression was induced at an OD₆₀₀ 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 μ l of an emulsion containing purified HbpB (10 μ g) and ISA 61 VG adjuvant (Seppic) (120 μ l), 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 μ l 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::*hbp*_{his} 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^{-6} M 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. birtlessii* 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

(Supplement table 2). 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 μ M in the presence of 80 μ M 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 μ M 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 μ M 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::*hbpA*_{AS} using mouse antibody directed against HbpB. As seen in Fig. 3, the level of HbpA was lower in strain *B. henselae* pNS2Trc::*hbpA*_{AS} 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::*hbpA*_{AS}, pNS2Trc::*hbpB*_{AS}, pNS2Trc::*hbpC*_{AS} or pNS2Trc::*hbpD*_{AS} were much smaller than those formed by *B. henselae* pNS2Trc (Table 4). The *B. henselae* strain containing pNS2Trc::*hbps*_{AS} 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::*hbpC_{AS}*. There existed slightly decreased survival capacity for *B. henselae* pNS2Trc::*hbpA_{AS}*, *B. henselae* pNS2Trc::*hbpB_{AS}* and *B. henselae* pNS2Trc::*hbpD_{AS}* (data not shown). After exposure to 10 mM H₂O₂, survival was about 25 % for control strain *B. henselae* pNS2Trc. With strain *B. henselae* pNS2Trc::*hbpA_{AS}*, *B. henselae* pNS2Trc::*hbpB_{AS}* and *B. henselae* pNS2Trc::*hbpC_{AS}*, 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_2O_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_2O_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_2O_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

feces [63]. 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 *Bartonellae* 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 H₂O₂ 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.

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References

1. Edouard S, Raoult D (2010) [*Bartonella henselae*, an ubiquitous agent of proteiform zoonotic disease]. *Med Mal Infect* 40: 319-330.
2. Klotz SA, Ianas V, Elliott SP (2011) Cat-scratch Disease. *Am Fam Physician* 83: 152-155.
3. Harms A, Dehio C (2012) Intruders below the Radar: Molecular Pathogenesis of *Bartonella* spp. *Clin Microbiol Rev* 25: 42-78.
4. Mandle T, Einsele H, Schaller M, Neumann D, Vogel W, et al. (2005) Infection of human CD34+ progenitor cells with *Bartonella henselae* results in intraerythrocytic presence of *B. henselae*. *Blood* 106: 1215-1222.
5. Sander A, Kretzer S, Brecht W, Oberle K, Bereswill S (2000) Hemin-dependent growth and hemin binding of *Bartonella henselae*. *FEMS Microbiol Lett* 189: 55-59.
6. Alsmark CM, Frank AC, Karlberg EO, Legault BA, Ardell DH, et al. (2004) The louse-borne human pathogen *Bartonella quintana* is a genomic derivative of the zoonotic agent *Bartonella henselae*. *Proc Natl Acad Sci U S A* 101: 9716-9721.
7. Perry RD, Mier I, Jr., Fetherston JD (2007) Roles of the Yfe and Feo transporters of *Yersinia pestis* in iron uptake and intracellular growth. *Biometals* 20: 699-703.
8. Watson RJ, Millichap P, Joyce SA, Reynolds S, Clarke DJ (2010) The role of iron uptake in pathogenicity and symbiosis in *Photobacterium luminescens* TT01. *BMC Microbiol* 10: 177.
9. Parrow NL, Abbott J, Lockwood AR, Battisti JM, Minnick MF (2009) Function, regulation, and transcriptional organization of the hemin utilization locus of *Bartonella quintana*. *Infect Immun* 77: 307-316.
10. Storz G, Imlay JA (1999) Oxidative stress. *Curr Opin Microbiol* 2: 188-194.
11. Schellhorn HE (1995) Regulation of hydroperoxidase (catalase) expression in *Escherichia coli*. *FEMS Microbiol Lett* 131: 113-119.
12. Weissbach H, Resnick L, Brot N (2005) Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage. *Biochim Biophys Acta* 1703: 203-212.
13. Amabile-Cuevas CF, Demple B (1991) Molecular characterization of the soxRS genes of *Escherichia coli*: two genes control a superoxide stress regulon. *Nucleic Acids Res* 19: 4479-4484.
14. Storz G, Tartaglia LA, Ames BN (1990) The OxyR regulon. *Antonie Van Leeuwenhoek* 58: 157-161.
15. Coleman SA, Minnick MF (2003) Differential expression of the invasion-associated locus B (*ialB*) gene of *Bartonella bacilliformis* in response to environmental cues. *Microb Pathog* 34: 179-186.
16. Graca-Souza AV, Maya-Monteiro C, Paiva-Silva GO, Braz GR, Paes MC, et al. (2006) Adaptations against heme toxicity in blood-feeding arthropods. *Insect Biochem Mol Biol* 36: 322-335.

17. Minnick MF, Sappington KN, Smitherman LS, Andersson SG, Karlberg O, et al. (2003) Five-member gene family of *Bartonella quintana*. *Infect Immun* 71: 814-821.
18. Wandersman C, Delepelaire P (2004) Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol* 58: 611-647.
19. Battisti JM, Sappington KN, Smitherman LS, Parrow NL, Minnick MF (2006) Environmental signals generate a differential and coordinated expression of the heme receptor gene family of *Bartonella quintana*. *Infect Immun* 74: 3251-3261.
20. Roden JA, Wells DH, Chomel BB, Kasten RW, Koehler JE (2012) Hemin binding protein C is found in outer membrane vesicles and protects *Bartonella henselae* against toxic concentrations of hemin. *Infect Immun*.
21. Zimmermann R, Kempf VA, Schiltz E, Oberle K, Sander A (2003) Hemin binding, functional expression, and complementation analysis of Pap 31 from *Bartonella henselae*. *J Bacteriol* 185: 1739-1744.
22. Carroll JA, Coleman SA, Smitherman LS, Minnick MF (2000) Hemin-binding surface protein from *Bartonella quintana*. *Infect Immun* 68: 6750-6757.
23. Simon D, Rest RF (1992) *Escherichia coli* expressing a *Neisseria gonorrhoeae* opacity-associated outer membrane protein invade human cervical and endometrial epithelial cell lines. *Proc Natl Acad Sci U S A* 89: 5512-5516.
24. Dabo SM, Confer AW, Anderson BE, Gupta S (2006) *Bartonella henselae* Pap31, an extracellular matrix adhesin, binds the fibronectin repeat III13 module. *Infect Immun* 74: 2513-2521.
25. Dabo SM, Confer AW, Saliki JT, Anderson BE (2006) Binding of *Bartonella henselae* to extracellular molecules: identification of potential adhesins. *Microb Pathog* 41: 10-20.
26. Saenz HL, Engel P, Stoeckli MC, Lanz C, Raddatz G, et al. (2007) Genomic analysis of *Bartonella* identifies type IV secretion systems as host adaptability factors. *Nat Genet* 39: 1469-1476.
27. Kyme PA, Haas A, Schaller M, Peschel A, Iredell J, et al. (2005) Unusual trafficking pattern of *Bartonella henselae* -containing vacuoles in macrophages and endothelial cells. *Cell Microbiol* 7: 1019-1034.
28. Miller JH (1972) *Experiments in molecular genetics.*: Cold Spring Harbor laboratory Press, Cold Spring Harbor, N.Y.
29. Riess T, Dietrich F, Schmidt KV, Kaiser PO, Schwarz H, et al. (2008) Analysis of a novel insect cell culture medium-based growth medium for *Bartonella* species. *Appl Environ Microbiol* 74: 5224-5227.
30. Chang CC, Chen YJ, Tseng CS, Lai WL, Hsu KY, et al. (2011) A comparative study of the interaction of *Bartonella henselae* strains with human endothelial cells. *Vet Microbiol* 149: 147-156.
31. Mehock JR, Greene CE, Gherardini FC, Hahn TW, Krause DC (1998) *Bartonella henselae* invasion of feline erythrocytes in vitro. *Infect Immun* 66: 3462-3466.
32. Kempf VA, Volkmann B, Schaller M, Sander CA, Alitalo K, et al. (2001) Evidence of a leading role for VEGF in *Bartonella henselae*-induced

- endothelial cell proliferations. *Cell Microbiol* 3: 623-632.
33. Maniatis T, Fritsch EF, Sambrook J (1989) *Molecular Cloning. A Laboratory Manual*; Harbor CS, editor. New York: Cold Spring Harbor Laboratory Press.
 34. Riess T, Anderson B, Fackelmayer A, Autenrieth IB, Kempf VA (2003) Rapid and efficient transposon mutagenesis of *Bartonella henselae* by transposome technology. *Gene* 313: 103-109.
 35. Reif KE, Stout RW, Henry GC, Foil LD, Macaluso KR (2008) Prevalence and infection load dynamics of *Rickettsia felis* in actively feeding cat fleas. *PLoS One* 3: e2805.
 36. Vargas C, McEwan AG, Downie JA (1993) Detection of c-type cytochromes using enhanced chemiluminescence. *Anal Biochem* 209: 323-326.
 37. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76: 4350-4354.
 38. Feissner R, Xiang Y, Kranz RG (2003) Chemiluminescent-based methods to detect subpicomole levels of c-type cytochromes. *Anal Biochem* 315: 90-94.
 39. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
 40. Stugard CE, Daskaleros PA, Payne SM (1989) A 101-kilodalton heme-binding protein associated with congo red binding and virulence of *Shigella flexneri* and enteroinvasive *Escherichia coli* strains. *Infect Immun* 57: 3534-3539.
 41. Hare JM, McDonough KA (1999) High-frequency RecA-dependent and -independent mechanisms of Congo red binding mutations in *Yersinia pestis*. *J Bacteriol* 181: 4896-4904.
 42. Olczak T, Sroka A, Potempa J, Olczak M (2008) *Porphyromonas gingivalis* HmuY and HmuR: further characterization of a novel mechanism of heme utilization. *Arch Microbiol* 189: 197-210.
 43. Nijssen E, Cescau S, Vayssier-Taussat M, Wang Jd, Biville F (2009) Identification of mechanisms involved in iron and heme uptake in *Bartonella birtlesii*: In silico and in vivo approaches. *Clinical Microbiology and Infection*.
 44. Perrotte-Piquemal M, Danchin A, Biville F (1999) Pyrophosphate increases the efficiency of enterobactin-dependent iron uptake in *Escherichia coli*. *Biochimie* 81: 245-253.
 45. Letoffe S, Delepelaire P, Wandersman C (2006) The housekeeping dipeptide permease is the *Escherichia coli* heme transporter and functions with two optional peptide binding proteins. *Proc Natl Acad Sci U S A* 103: 12891-12896.
 46. Letoffe S, Heuck G, Delepelaire P, Lange N, Wandersman C (2009) Bacteria capture iron from heme by keeping tetrapyrrole skeleton intact. *Proc Natl Acad Sci U S A* 106: 11719-11724.
 47. Liu M, Boulouis H-J, Biville F (2012) Heme Degrading Protein HemS Is Involved in Oxidative Stress Response of *Bartonella henselae*. *PLoS One* 7: e37630.
 48. Benevides-Matos N, Biville F (2010) The Hem and Has haem uptake systems in

- Serratia marcescens*. Microbiology 156: 1749-1757.
49. Gillaspie D, Perkins I, Larsen K, McCord A, Pangonis S, et al. (2009) Plasmid-based system for high-level gene expression and antisense gene knockdown in *Bartonella henselae*. Appl Environ Microbiol 75: 5434-5436.
 50. Liu MF, Cescau S, Mechold U, Wang J, Cohen D, et al. (2012) Identification of a novel nanoRNase in *Bartonella*. Microbiology 158: 886-895.
 51. Chenoweth MR, Greene CE, Krause DC, Gherardini FC (2004) Predominant outer membrane antigens of *Bartonella henselae*. Infect Immun 72: 3097-3105.
 52. Li DM, Liu QY, Zhao F, Hu Y, Xiao D, et al. (2011) Proteomic and bioinformatic analysis of outer membrane proteins of the protobacterium *Bartonella henselae* (Bartonellaceae). Genet Mol Res 10: 1789-1818.
 53. Saisongkorh W, Kowalczywska M, Azza S, Decloquement P, Rolain JM, et al. Identification of candidate proteins for the diagnosis of *Bartonella henselae* infections using an immunoproteomic approach. FEMS Microbiol Lett 310: 158-167.
 54. Eberhardt C, Engelmann S, Kusch H, Albrecht D, Hecker M, et al. (2009) Proteomic analysis of the bacterial pathogen *Bartonella henselae* and identification of immunogenic proteins for serodiagnosis. Proteomics 9: 1967-1981.
 55. Boonjakuakul JK, Gerns HL, Chen YT, Hicks LD, Minnick MF, et al. (2007) Proteomic and immunoblot analyses of *Bartonella quintana* total membrane proteins identify antigens recognized by sera from infected patients. Infect Immun 75: 2548-2561.
 56. Rhomberg TA, Karlberg O, Mini T, Zimny-Arndt U, Wickenberg U, et al. (2004) Proteomic analysis of the sarcosine-insoluble outer membrane fraction of the bacterial pathogen *Bartonella henselae*. Proteomics 4: 3021-3033.
 57. Minnick MF, Battisti JM (2009) Pestilence, persistence and pathogenicity: infection strategies of *Bartonella*. Future Microbiol 4: 743-758.
 58. Bogdan C, Rollinghoff M, Diefenbach A (2000) Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 12: 64-76.
 59. Sahni SK, Rydkina E (2009) Host-cell interactions with pathogenic *Rickettsia* species. Future Microbiol 4: 323-339.
 60. Kempf VA, Schaller M, Behrendt S, Volkmann B, Aepfelbacher M, et al. (2000) Interaction of *Bartonella henselae* with endothelial cells results in rapid bacterial rRNA synthesis and replication. Cell Microbiol 2: 431-441.
 61. Koehler JE, Glaser CA, Tappero JW (1994) *Rochalimaea henselae* infection. A new zoonosis with the domestic cat as reservoir. JAMA 271: 531-535.
 62. Chomel BB, Kasten RW, Floyd-Hawkins K, Chi B, Yamamoto K, et al. (1996) Experimental transmission of *Bartonella henselae* by the cat flea. J Clin Microbiol 34: 1952-1956.
 63. Chomel BB, Boulouis HJ, Breitschwerdt EB, Kasten RW, Vayssier-Taussat M, et al. (2009) Ecological fitness and strategies of adaptation of *Bartonella* species to their hosts and vectors. Vet Res 40: 29.

64. Ghigo JM, Letoffe S, Wandersman C (1997) A new type of hemophore-dependent heme acquisition system of *Serratia marcescens* reconstituted in *Escherichia coli*. *J Bacteriol* 179: 3572-3579.
65. Rydkina E, Turpin LC, Sahni SK *Rickettsia rickettsii* infection of human macrovascular and microvascular endothelial cells reveals activation of both common and cell type-specific host response mechanisms. *Infect Immun* 78: 2599-2606.
66. Zhou W, Russell CW, Johnson KL, Mortensen RD, Erickson DL Gene expression analysis of *Xenopsylla cheopis* (Siphonaptera: Pulicidae) suggests a role for reactive oxygen species in response to *Yersinia pestis* infection. *J Med Entomol* 49: 364-370.
67. Zhou W, Russell CW, Johnson KL, Mortensen RD, Erickson DL (2012) Gene expression analysis of *Xenopsylla cheopis* (Siphonaptera: Pulicidae) suggests a role for reactive oxygen species in response to *Yersinia pestis* infection. *J Med Entomol* 49: 364-370.
68. Oliveira MF, Silva JR, Dansa-Petretski M, de Souza W, Lins U, et al. (1999) Haem detoxification by an insect. *Nature* 400: 517-518.
69. Paes MC, Oliveira MB, Oliveira PL (2001) Hydrogen peroxide detoxification in the midgut of the blood-sucking insect, *Rhodnius prolixus*. *Arch Insect Biochem Physiol* 48: 63-71.
70. Molina-Cruz A, DeJong RJ, Charles B, Gupta L, Kumar S, et al. (2008) Reactive oxygen species modulate *Anopheles gambiae* immunity against bacteria and *Plasmodium*. *J Biol Chem* 283: 3217-3223.
71. Ryter SW, Tyrrell RM (2000) The heme synthesis and degradation pathways: role in oxidant sensitivity. Heme oxygenase has both pro- and antioxidant properties. *Free Radic Biol Med* 28: 289-309.
72. Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, et al. (2002) Pro-oxidant and cytotoxic effects of circulating heme. *Blood* 100: 879-887.
73. Smalley JW, Birss AJ, Silver J (2000) The periodontal pathogen *Porphyromonas gingivalis* harnesses the chemistry of the mu-oxo bishaem of iron protoporphyrin IX to protect against hydrogen peroxide. *FEMS Microbiol Lett* 183: 159-164.
74. Dehio C (2005) *Bartonella*-host-cell interactions and vascular tumour formation. *Nat Rev Microbiol* 3: 621-631.
75. Weel JF, Hopman CT, van Putten JP (1991) In situ expression and localization of *Neisseria gonorrhoeae* opacity proteins in infected epithelial cells: apparent role of Opa proteins in cellular invasion. *J Exp Med* 173: 1395-1405.
76. Benevides-Matos N, Wandersman C, Biville F (2008) HasB, the *Serratia marcescens* TonB paralog, is specific to HasR. *J Bacteriol* 190: 21-27.

Table 1. Strains and plasmids used in this study

<i>E. coli</i> strains	Genotype	Source or reference
XL1-Blue	F ⁻ supE44 hdsR17 recA1 endA1 gyrA46 thi relA1 lac ⁻ F ['] proAB ⁻ lacI ^q lacZAM15 Tn10 (Tet ^R)	Laboratory collection
XL1-Blue pBAD24	XL1-Blue carrying pBAD24, Amp ^R	This study
XL1-Blue pBAD24:: <i>hbpA</i>	XL1-Blue carrying pBAD24:: <i>hbpA</i> , Amp ^R	This study
XL1-Blue pBAD24:: <i>hbpB</i>	XL1-Blue carrying pBAD24:: <i>hbpB</i> , Amp ^R	This study
XL1-Blue pBAD24:: <i>hbpC</i>	XL1-Blue carrying pBAD24:: <i>hbpC</i> , Amp ^R	This study
XL1-Blue pBAD24:: <i>hbpD</i>	XL1-Blue carrying pBAD24:: <i>hbpD</i> , Amp ^R	This study
FB8.27	F ⁻ , Δ lac X74, <i>entF</i> :: <i>TnphoA</i> '5, Tet ^R	[44]
FB8.27 pAM239:: <i>hemR</i> , pBAD24	FB8.27 pAM239:: <i>hemR</i> , pBAD24, Tet ^R , Spc ^R , Amp ^R	This study
FB8.27 pAM239:: <i>hemR</i> , pBAD24:: <i>hbpA</i>	FB8.27 pAM239:: <i>hemR</i> , pBAD24:: <i>hbpA</i> , Tet ^R , Spc ^R , Amp ^R	This study
FB8.27 pAM239:: <i>hemR</i> , pBAD24:: <i>hbpB</i>	FB8.27 pAM239:: <i>hemR</i> , pBAD24:: <i>hbpB</i> , Tet ^R , Spc ^R , Amp ^R	This study
FB8.27 pAM239:: <i>hemR</i> , pBAD24:: <i>hbpC</i>	FB8.27 pAM239:: <i>hemR</i> , pBAD24:: <i>hbpC</i> , Tet ^R , Spc ^R , Amp ^R	This study
FB8.27 pAM239:: <i>hemR</i> , pBAD24:: <i>hbpD</i>	FB8.27 pAM239:: <i>hemR</i> , pBAD24:: <i>hbpD</i> , Tet ^R , Spc ^R , Amp ^R	This study
<i>Bartonella</i> strains	Genotype	Source or reference
<i>B. henselae</i> Houston-1	Houston-1, ATCC 49882 ^T	Laboratory collection
<i>B. henselae</i> pNS2Trc	<i>B. henselae</i> carrying pNS2Trc, Amp ^R	This study
<i>B. henselae</i> pNS2Trc:: <i>hbpA</i> _{AS}	<i>B. henselae</i> carrying pNS2Trc:: <i>hbpA</i> _{AS} , Amp ^R	This study
<i>B. henselae</i> pNS2Trc:: <i>hbpB</i> _{AS}	<i>B. henselae</i> carrying pNS2Trc:: <i>hbpB</i> _{AS} , Amp ^R	This study
<i>B. henselae</i> pNS2Trc:: <i>hbpC</i> _{AS}	<i>B. henselae</i> carrying pNS2Trc:: <i>hbpC</i> _{AS} , Amp ^R	This study
<i>B. henselae</i> pNS2Trc:: <i>hbpD</i> _{AS}	<i>B. henselae</i> carrying pNS2Trc:: <i>hbpD</i> _{AS} , Amp ^R	This study
Plasmids		
pBAD24	pBR322 araC, arabinose-inducible promoter, Amp ^R	Laboratory collection
pAM239:: <i>hemR</i>	pAM239 carrying <i>hemR</i> from <i>Serratia marcescens</i>	[76]
pBAD24:: <i>hbpA</i>	pBAD24 carrying <i>hbpA</i> from <i>B. henselae</i> , Amp ^R	This study
pBAD24:: <i>hbpB</i>	pBAD24 carrying <i>hbpB</i> from <i>B. henselae</i> , Amp ^R	This study
pBAD24:: <i>hbpC</i>	pBAD24 carrying <i>hbpC</i> from <i>B. henselae</i> , Amp ^R	This study
pBAD24:: <i>hbpD</i>	pBAD24 carrying <i>hbpD</i> from <i>B. henselae</i> , Amp ^R	This study
pNS2Trc	Km ^R	This study
pNS2Trc:: <i>hbpA</i> _{AS}	pNS2Trc carrying anti-sense <i>hbpA</i> , km ^R	This study
pNS2Trc:: <i>hbpB</i> _{AS}	pNS2Trc carrying anti-sense <i>hbpB</i> , km ^R	This study
pNS2Trc:: <i>hbpC</i> _{AS}	pNS2Trc carrying anti-sense <i>hbpC</i> , km ^R	This study
pNS2Trc:: <i>hbpD</i> _{AS}	pNS2Trc carrying anti-sense <i>hbpD</i> , km ^R	This study

Table 2. Primers used in this study

Primer	Gene	Organism	Sequence
HbpABhamont	<i>hbpA</i>	<i>B. henselae</i>	5' CTAGCTAGCAGGAGGAATTCACCATGAATATAAAAATCTTTAATGA 3'
HbpABhaval	<i>hbpA</i>	<i>B. henselae</i>	5' CGGGGTACCTCAGTGGTGGTGGTGGTGGTGAATTTGTACGCTACACCAACACGG 3'
HbpBBhamont	<i>hbpB</i>	<i>B. henselae</i>	5' CTAGCTAGCAGGAGGAATTCACCATGAATACGAAACGTTTAATAACAG 3'
HbpBBhaval	<i>hbpB</i>	<i>B. henselae</i>	5' ATCCCCGAAGCTTATGGTTTAGTGGTGGTGGTGGTGGTGAATTTGTAAGCGACAC 3'
HbpCBhamont	<i>hbpC</i>	<i>B. henselae</i>	5' CTAGCTAGCAGGAGGAATTCACCATGAAATCGCGTGTTCAAATAT3'
HbpCBhaval	<i>hbpC</i>	<i>B. henselae</i>	5' CGGGGTACCTCAGTGGTGGTGGTGGTGGTGAATTTATAAGCGACACCAACACGG 3'
HbpDBhamont	<i>hbpD</i>	<i>B. henselae</i>	5' CTAGCTAGCAGGAGGAATTCACCATGACTACAAAATATTTAATCACAA 3'
HbpDBhaval	<i>hbpD</i>	<i>B. henselae</i>	5' CGGGGTACCTCAGTGGTGGTGGTGGTGGTGAAACTTGTACGCTACACCAACACGA 3'
hbpAantisensamt	Antisens <i>hbpA</i>	<i>B. henselae</i>	5' CCCGGATCCTTAGAATTTGTACGCTACACC3'
hbpAantisensavl	Antisens <i>hbpA</i>	<i>B. henselae</i>	5' CCCTCTAGAATGAATATAAAAATCTTTAATG 3'
hbpBantisensamt	Antisens <i>hbpB</i>	<i>B. henselae</i>	5' CCCGGATCCTTAGAATTTGTAAGCGACACC3'
hbpBantisensavl	Antisens <i>hbpB</i>	<i>B. henselae</i>	5' CCCTCTAGAATGAATACGAAACGTTTAATAAC3'
hbpCantisensamt	Antisens <i>hbpC</i>	<i>B. henselae</i>	5' CCCGGATCCTTAAAATTTATAAGCGACACC3'
hbpCantisensavl	Antisens <i>hbpC</i>	<i>B. henselae</i>	5' CCCTCTAGAATGAATATAAAAATGGTTAATA3'
hbpDantisensamt	Antisens <i>hbpD</i>	<i>B. henselae</i>	5' CCCGGATCCTCAAAACTTGTACGCTACACCA 3'
hbpDantisensavl	Antisens <i>hbpD</i>	<i>B. henselae</i>	5' CCCTCTAGAATGACTACAAAATATTTAATCACA 3'
bh2390fo	bh2390	<i>B. henselae</i>	5'-GGTGAATGTGTGCAAAGTTTAAAG 3'
bh2390re	bh2390	<i>B. henselae</i>	5' CCAATAAACGCCAACAAAGAC 3'.
Cf18Sf	18S rDNA	<i>C. felis</i>	5' TGCTCACCGTTTGACTTGG 3'
Cf18Sr	18S rDNA	<i>C. felis</i>	5' GTTTCTCAGGCTCCCTCTCC 3'

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 μ M, Spc, Amp). Growth around the wells containing 1 μ M, 5 μ M, 10 μ M, or 50 μ M 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 \pm SD of the diameter (in cm) obtained for the three plates. NM: Not measurable

<i>E. coli</i> strain	Diameter of the halo (cm) (Dip 80 μ M)			
	50 μ M	10 μ M	5 μ M	1 μ M
FB8.27 pAM:: <i>hemR</i> (pBAD24)	1.82 \pm 0.015	NM	NM	NM
FB8.27 pAM:: <i>hemR</i> (pBAD24:: <i>hbpA</i>)	2.23 \pm 0.04	1.78 \pm 0.03	1.48 \pm 0.09	NM
FB8.27 pAM:: <i>hemR</i> (pBAD24:: <i>hbpB</i>)	2.77 \pm 0.11	2.37 \pm 0.08	2.31 \pm 0.09	1.84 \pm 0.1
FB8.27 pAM:: <i>hemR</i> (pBAD24:: <i>hbpC</i>)	2.09 \pm 0.04	1.64 \pm 0.03	1.42 \pm 0.08	NM
FB8.27 pAM:: <i>hemR</i> (pBAD24:: <i>hbpD</i>)	2.55 \pm 0.10	1.92 \pm 0.09	1.68 \pm 0.09	1.22 \pm 0.06
<i>E. coli</i> strain	Diameter of the halo (cm) (Dip 100 μ M)			
	50 μ M	10 μ M	5 μ M	1 μ M
FB8.27 pAM:: <i>hemR</i> (pBAD24)	-	-	-	-
FB8.27 pAM:: <i>hemR</i> (pBAD24:: <i>hbpA</i>)	-	-	-	-
FB8.27 pAM:: <i>hemR</i> (pBAD24:: <i>hbpB</i>)	+	+	+	+
FB8.27 pAM:: <i>hemR</i> (pBAD24:: <i>hbpC</i>)	-	-	-	-
FB8.27 pAM:: <i>hemR</i> (pBAD24:: <i>hbpD</i>)	+	-	-	-

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

Strain	Colony size (mm)	
	Day 6	Day 10
<i>B. henselae</i> (pNS2Trc)	0.58±0.12	1.00 ± 0.149
<i>B. henselae</i> (pNS2Trc:: <i>hbpA_{AS}</i>)	NM	0.50 ± 0.105 (p<0.0001)
<i>B. henselae</i> (pNS2Trc:: <i>hbpB_{AS}</i>)	NM	0.53 ± 0.125 (p<0.0001)
<i>B. henselae</i> (pNS2Trc:: <i>hbpC_{AS}</i>)	NM	0.51 ± 0.120 (p<0.0001)
<i>B. henselae</i> (pNS2Trc:: <i>hbpD_{AS}</i>)	NM	0.449 ± 0.072 (p<0.0001)

Table 5. Detection of *B. henselae* DNA from flea feces samples using PCR.

About 500 fleas were first feed with blood containing 500 μ l 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.

	Flea feces samples (days)									
	1	2	3	4	5	6	7	8	9	10
<i>B. henselae</i> pNS2Trc	+	+	+	+	+	+	+	+	+	+
<i>B. henselae</i> pNS2Trc:: <i>hbpA</i> _{AS}	+	+	+	+	+	+	-	-	-	-
<i>B. henselae</i> pNS2Trc:: <i>hbpB</i> _{AS}	+	+	+	+	+	+	-	-	-	-
<i>B. henselae</i> pNS2Trc:: <i>hbpC</i> _{AS}	+	+	+	+	+	+	-	-	-	-
<i>B. henselae</i> pNS2Trc:: <i>hbpD</i> _{AS}	+	+	+	+	+	+	-	-	-	-

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

R (A). Another gel was transferred to a nitrocellulose filter for performing immune blotting as described in Materials and methods (B).

Fig 4. Effect of *hbps* knockdown on growth of *B. henselae* in Schneider's medium.

Strains *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::*hbps*_{AS} 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 OD₆₀₀ of 0.05. Cultures were grown at 35°C in the presence of 5% CO₂ and OD₆₀₀ 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 H₂O₂.

B. henselae pNS2Trc and *B. henselae* pNS2Trc::*hbps*_{AS} were challenged with 10 mM H₂O₂ 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 *B. henselae* pNS2Trc)

Fig 7. Effect of Hbp knockdown on survival of *B. henselae* in endothelial cells.

Survival of *B. henselae* pNS2Trc and *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 *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).

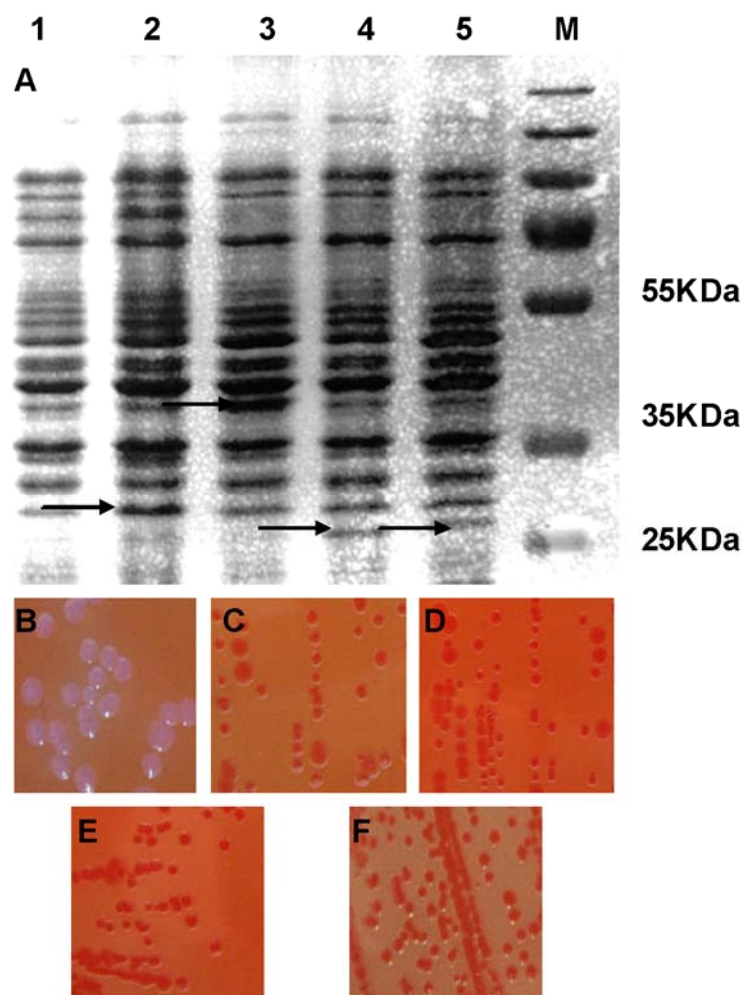


Fig 2. Detection of Hbp binding to heme.

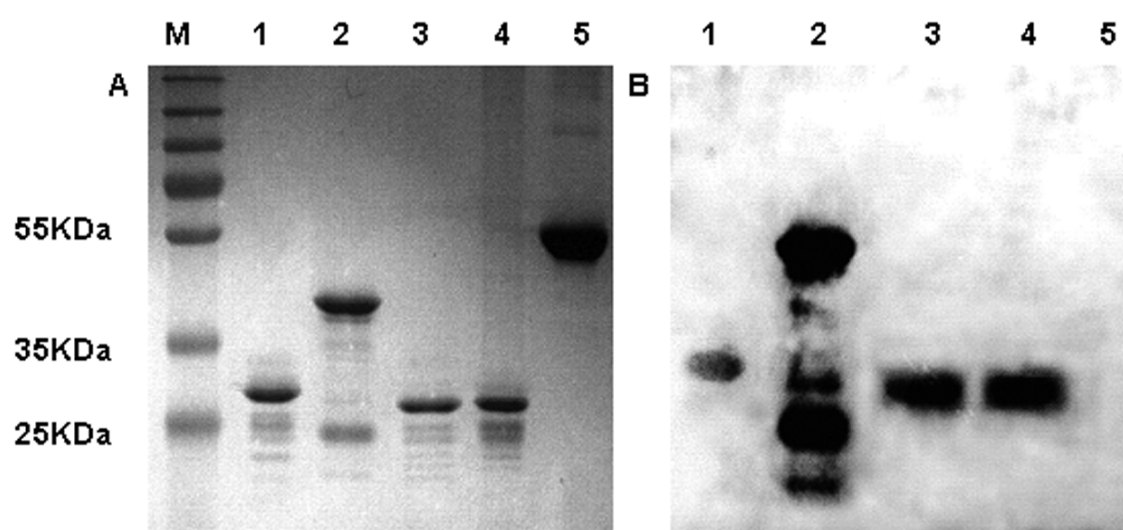


Fig 3. Detection of the HbpA expression level in *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::*hbpA*_{AS} by immunoblotting.

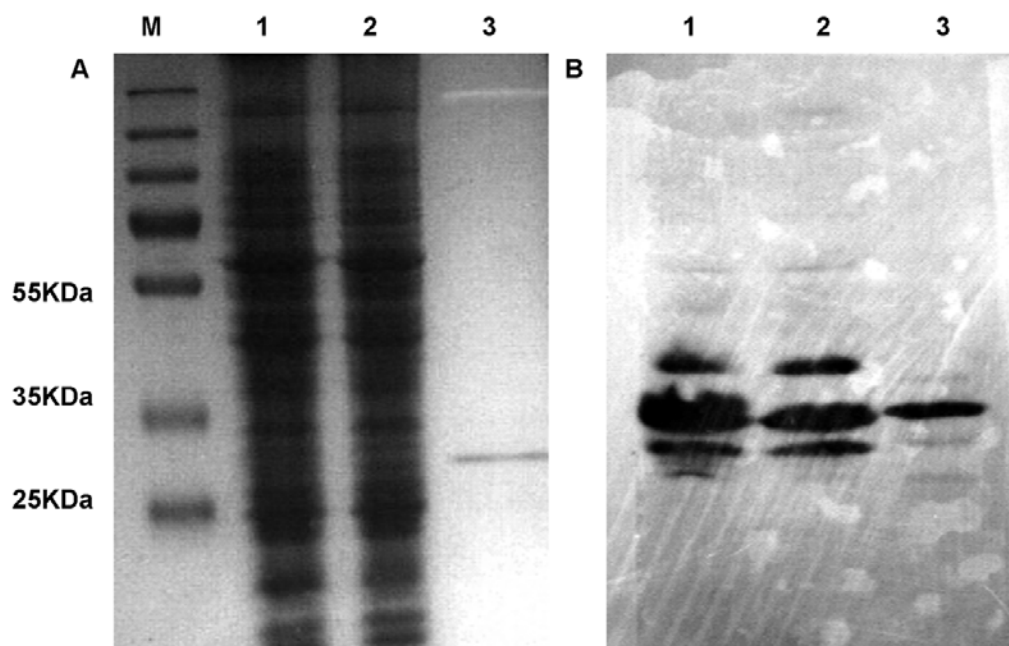


Fig 4. Effect of *hbp* knockdown on growth of *B. henselae* in Schneider's medium.

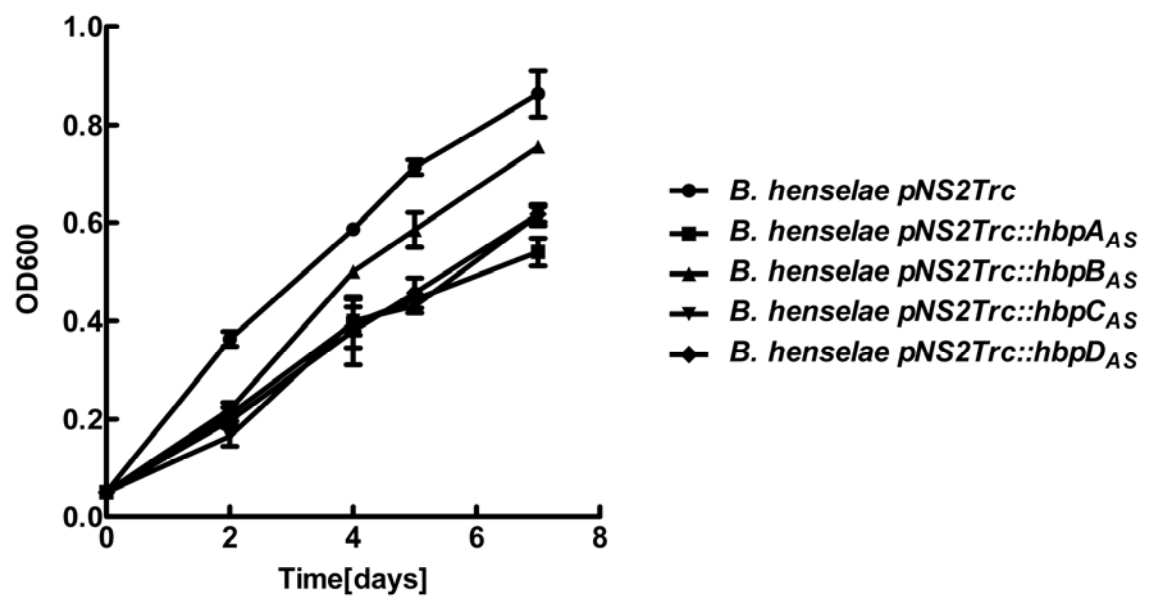


Fig 5: Hbp knockdown decreases *B. henselae* capacity to face H₂O₂ challenge.

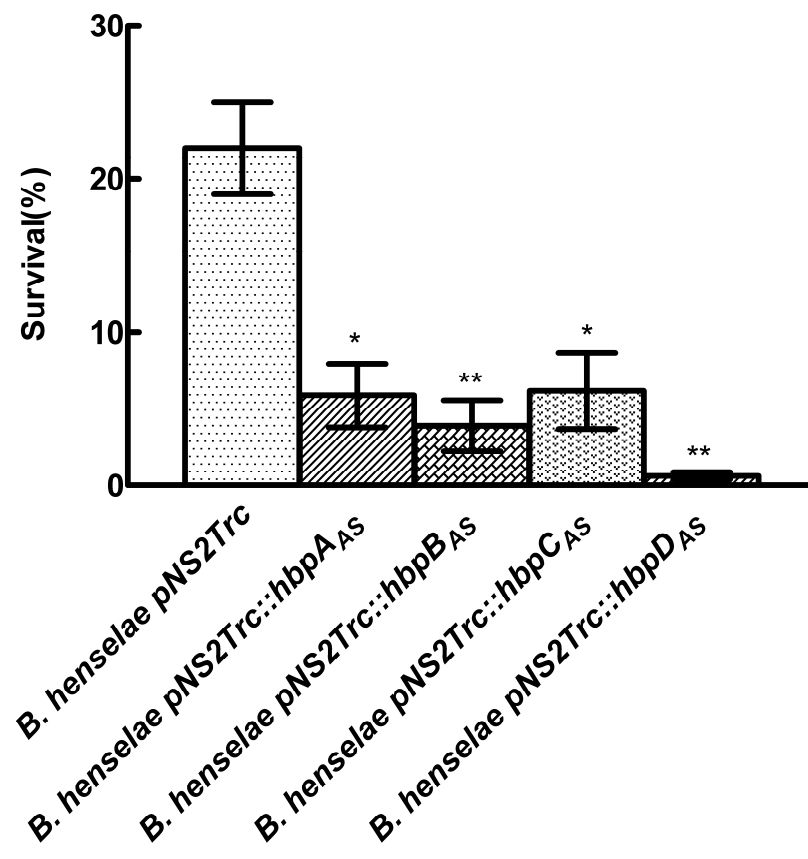


Fig 6: Effect of Hbp knockdown on endothelial cell invasion.

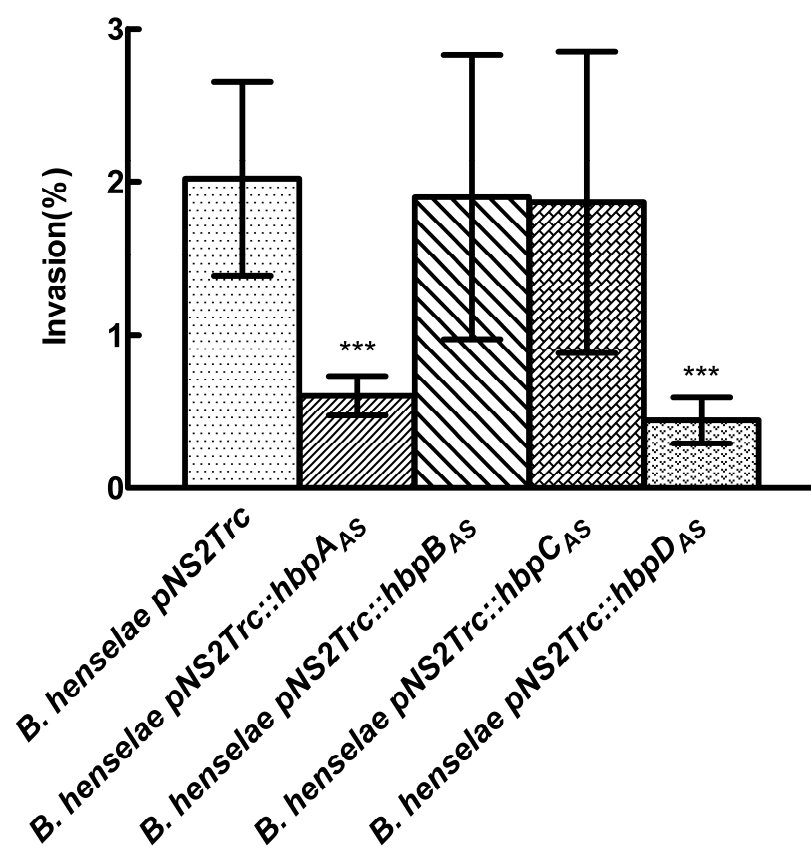
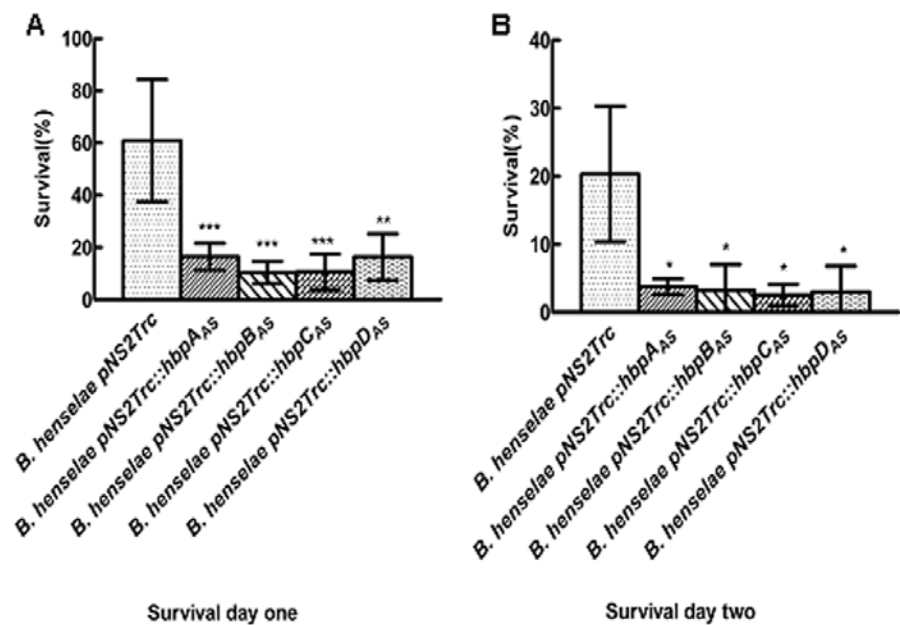


Fig 7: Effect of Hbp knockdown on survival of *B. henselae* in endothelial cells.



Supplement Table 1

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

C600 Δ *hemA* is incapable of the synthesis of Δ ala, 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), C600 Δ *hemA* 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 μ l of 10^{-4} M, 10^{-5} M or 10^{-6} M 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*). C600 Δ *hemA* pBAD24 expressing Hbps have no any growth was observed.

Wells	C600 Δ <i>hemA</i> pAM:: <i>hasR</i>	C600 Δ <i>hemA</i> pBAD24	C600 Δ <i>hemA</i> pBAD24:: <i>hbpA</i>	C600 Δ <i>hemA</i> pBAD24:: <i>hbpB</i>	C600 Δ <i>hemA</i> pBAD24:: <i>hbpC</i>	C600 Δ <i>hemA</i> pBAD24:: <i>hbpD</i>
Heme 10^{-4} M	+	-	-	-	-	-
Heme 10^{-5} M	+	-	-	-	-	-
Heme 10^{-6} M	-	-	-	-	-	-
Hb 10^{-4} M	+	-	-	-	-	-
Hb 10^{-5} M	+	-	-	-	-	-
Hb 10^{-6} M	-	-	-	-	-	-

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 μ l of 50 μ M, 10 μ M, 5 μ M, 1 μ M of filter-sterilized Hb solution. Growth was recorded around the wells after 48-hours incubation at 37°C.

<i>E. coli</i> strain	Hb			
	50 μ M	10 μ M	5 μ M	1 μ M
FB8.27 pAM:: <i>hemR</i>	+	+	-	-
FB8.27 pBAD24	-	-	-	-
FB8.27 pBAD24:: <i>hutA</i>	-	-	-	-

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 *in vivo* and heme *in vitro*. We next showed that heme binding proteins of *B. henselae* cannot transport heme when expressed in the *E. coli hemA* mutant. We then sought to determine whether heme binding proteins affect HutA-mediated heme uptake activity when co-expressed in *E. coli*. We failed to reconstitute the activity of HutA as a heme transporter in *E. coli*, although it has been claimed that HutA of *B. quintana* can transport heme when expressed in an *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 *S. marcescens* when expressed in *E. coli*. Our results clearly showed that heme binding proteins increase HemR-mediated heme transport when expressed in *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, *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 *E. coli* are not present in *Bartonellae*. The fact that *B. bacilliformis* was able to successfully endure 30 min exposure to 1 mM H₂O₂ (Coleman and Minnick, 2003) implies that *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 H₂O₂.

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 *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 *B. henselae*.

Discussion and perspectives

To accomplish their infection cycle, *Bartonellae* 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_2O_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 *Bartonellae* genomes (Table 1).

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

	Function in <i>E. coli</i>	<i>B. henselae</i>	<i>B. quintana</i>	<i>B. bacilliformis</i>
Ros degradation				
<i>sodA</i>	Manganese superoxide dismutase	+	+	+
<i>sodB</i>	Iron superoxide dismutase	+	+	+
<i>sodC</i>	Copper-zinc superoxide dismutase	-	-	-
<i>katG</i>	Hydroperoxidase I	-	-	-
<i>katE</i>	Hydroperoxidase II	-	-	-
<i>ahpC</i>	Alkyl hydroperoxide reductase	-	-	-
<i>ahpF</i>	Alkyl hydroperoxide reductase	-	-	-
<i>dps</i>	Non-specific DNA binding protein	-	-	-
<i>acrA</i>	ROS excretion	?	?	?
<i>acrB</i>	ROS excretion	+	+	+
ROS damage repair				
<i>gorA</i>	Glutathione reductase	+	+	+
<i>grxA</i>	Glutaredoxin 1	+	+	+
<i>msrA</i>	Methionine sulfoxide reductase	-	-	-
<i>msrB</i>	Methionine sulfoxide reductase	-	-	-
<i>trxB</i>	Thioredoxin reductase	+	+	+
<i>zwf</i>	Glucose-6-phosphate dehydrogenase	+	+	-
<i>fpr</i>	Ferredoxin reductase	+	+	+
<i>nfo</i>	Endonuclease IV	-	-	-
<i>xthA</i>	Exonuclease III	+	+	+
Regulators				
<i>soxR</i>	<i>sodA</i> , <i>acrA</i> , <i>acrB</i> , <i>zwf</i> , <i>fpr</i> , <i>nfo</i> , <i>xthA</i>	-	-	-
<i>oxyR</i>	<i>katG</i> , <i>ahpC</i> , <i>ahpF</i> , <i>gor</i> , <i>grxA</i>	-	-	-
<i>rpoS</i>	<i>sodC</i> , <i>katE</i> , <i>dps</i> , <i>gor</i> , <i>trxB</i> , <i>xthA</i>	-	-	-

It was thus hypothesized that *Bartonellae* 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 H₂O₂. This hypothesis was supported by the absence of a strategy for endogenous H₂O₂ detoxification, according to genome analysis (Table 1). For heme binding proteins, we showed that *B.*

henselae was more sensitive to H₂O₂ 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 H₂O₂. 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 O₂ 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 blood stream (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 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 H₂O₂ 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.

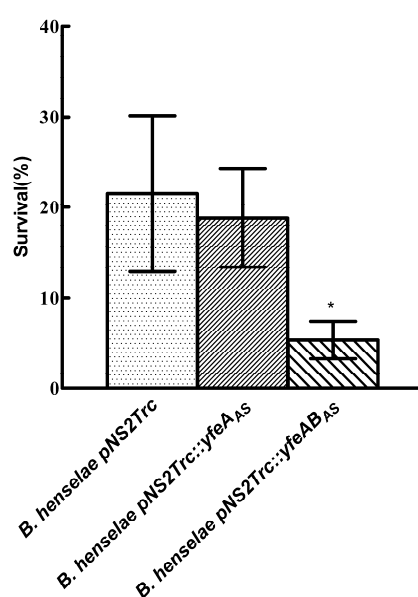


Fig. 1: YfeA and YfeAB knockdown decreases the ability of *B. henselae* to face exposure to H₂O₂.

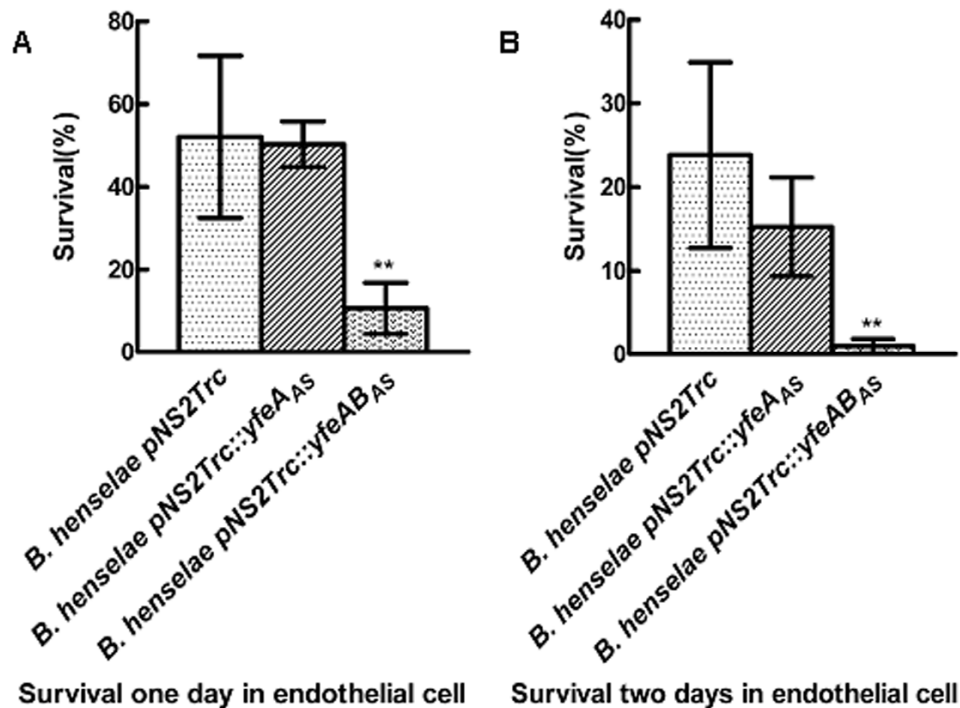


Fig 2: Effect of YfeA and YfeAB knockdown on survival of *B. henselae* in endothelial cells.

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.

	Flea feces samples (days)									
	1	2	3	4	5	6	7	8	9	10
<i>B. henselae</i> pNS2Trc	+	+	+	+	+	+	+	+	+	+
<i>B. henselae</i> pNS2Trc::yfeA _{AS}	+	+	+	+	+	+	+	+	+	+
<i>B. henselae</i> pNS2Trc::yfeAB _{AS}	+	+	+	+	+	+	-	-	-	-

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 ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (HO^{\bullet}) (Storz and Imlay, 1999). The sources of oxidative stress mainly include the following: 1) ROS, endogenously produced by aerobic metabolism (Storz and Imlay, 1999). $O_2^{\bullet -}$ 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).

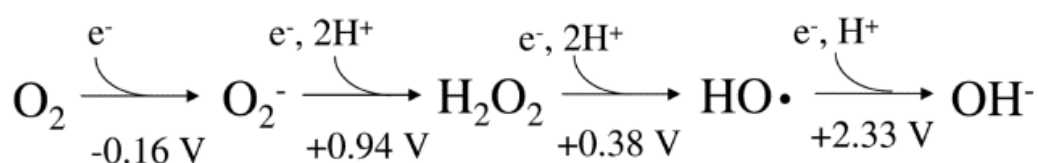


Fig. 3 The redox state of oxygen with standard reduction potential. Adapted from (Imlay, 2008).

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 ($O_2^{\bullet -}$) 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 (Orn) can degrade oligoribonucleotides of five residues and shorter in length (nanoRNA). In *Bacillus subtilis*, NrnA and NrnB, which do not show any sequence similarity to Orn, have been identified as functional analogues of Orn. Sequence comparisons did not identify *orn*, *nrnA* or *nrnB* homologues in the genomes of the *Chlamydia/Cyanobacteria* and *Alphaproteobacteria* family members. Screening a genomic library from *Bartonella birtlesii*, a member of the *Alphaproteobacteria*, for genes that can complement a conditional *orn* mutant in *E. coli*, we identified BA0969 (NmC) as a functional analogue of Orn. NmC 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 Orn from *E. coli*, NmC is inhibited by micromolar amounts of 3'-phosphoadenosine 5'-phosphate *in vitro*. NmC homologues are widely present in genomes of *Alphaproteobacteria*. Knock down of *nrnC* 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 (Lagesen *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 RNases on long RNA molecules, in particular mRNAs. 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 RNases have been identified so far (Arraiano *et al.*, 2010). In this organism, the essential RNase E initiates the degradation of RNA endonucleolytically (Condon, 2007), and other RNases exhibiting a 3' to 5' activity complete RNA degradation. RNase II produces 3- to 5mers or 4- to 6mers (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 (Orn) (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

Abbreviations: Amp, ampicillin; Atc, anhydrotetracycline; Km, kanamycin; Orn, oligoribonuclease; PAA, polyacrylamide; pAp, 3'-phosphoadenosine 5'-phosphate.

The GenBank/EMBL/DBJ accession number for the *B. birtlesii* *nrnC* locus is HE603915.

One supplementary table and three supplementary figures are available with the online version of this paper.

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'-phosphoadenosine 5'-phosphate (pAp) *in vitro* (Mechold *et al.*, 2006). Among the RNases with the ability to degrade nanoRNA are NrnA and NrnB from *Bacillus subtilis* (Fang *et al.*, 2009). Similarly to Orn from *E. coli*, these enzymes strongly prefer nanoRNA as substrate over longer RNA molecules. NrnA homologues are widely present in bacterial species that do not have a homologue of Orn. In addition to its activity on nanoRNA, NrnA can degrade pAp (Mechold *et al.*, 2007). In contrast to an earlier report on the NrnA homologue of *Streptococcus mutans* (Zhang & Biswas, 2009), bifunctionality seems to be common among NrnA homologues (Postic *et al.*, 2012). NanoRNases from *Gammaproteobacteria* and A+T-rich *Firmicutes*, in particular, have widely divergent structures while displaying the same activity (Fang *et al.*, 2009). In *Chlamydiae*, *Cyanobacteria* and *Alphaproteobacteria*, no counterparts of the enzymes of either the *Gammaproteobacteria* 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 & Battisti, 2009). The genus *Bartonella* comprises a unique group of facultative intracellular bacteria that use haemotrophism and arthropod transmission as a mammalian parasitism strategy (Chomel

et al., 2009). Among the 25 species identified to date (Guptill, 2010), 10 were shown to be human pathogens. *Bartonella* species are recognized to be responsible for emerging zoonoses (Breitschwerdt *et al.*, 2010). Genomes of *Bartonella* species sequenced to date are short, 1.4–1.9 Mb in size (Alsmark *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 *Gammaproteobacteria* 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 birtlesii*. This protein, BA0969 (now renamed NrnC), was identified by screening a genomic library of *B. birtlesii* for genes that can complement a conditional lethal *orn* mutant in *E. coli*. NrnC was characterized both *in vitro* and *in vivo* in *E. coli* and *Bartonella henselae*.

METHODS

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

Media and growth conditions. *E. coli* strains were grown on Luria-Bertani (LB) medium (Sigma) or M63 minimal medium aerobically at 37 °C (Miller, 1972). M63 medium was supplemented with 0.4% glycerol as carbon source. Solid media contained 1.5% Difco agar. When required, ampicillin (Amp) or kanamycin (Km) was added to a final concentration of 50 µg ml⁻¹. Arabinose was added to a final concentration of 0.2% for induction of the P_{ara} promoter as indicated. Anhydrotetracycline (Atc) was added to a concentration

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
<i>E. coli</i>		
XL1-blue	F ⁻ supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac ⁻ F' proAB ⁻ lacI ^q lacZΔM15 Tn10 (Tet ^R)	Laboratory collection
JP313	araD139 relA rpsL150 thi flb5301 (lacU139) deo7 ptsF25 Δara174	Laboratory collection
UM341	CF10230 with <i>orn</i> under control of P _{14et-1} , Tet ^R Km ^R	Mechold <i>et al.</i> (2007)
UM285	As CF10230 but ΔcysQ, Km ^R	Mechold <i>et al.</i> (2006)
UM341 (pUM408)	CF10230 with <i>orn</i> under control of P _{14et-1} , pUM408, Tet ^R Km ^R Amp ^R	Mechold <i>et al.</i> (2007)
<i>Bartonella</i>		
<i>B. birtlesii</i>	IBS 135 ^T , CIP 106691 ^T	Laboratory collection
<i>B. henselae</i> Houston-1	Houston-1, ATCC 49882 ^T	Laboratory collection
Plasmid		
pACYC184	Amp ^R Cmp ^R	Laboratory collection
pUM408	As pBAD18 (Guzman <i>et al.</i> , 1995), Orn with C-terminal His-tag, Amp ^R	Mechold <i>et al.</i> (2006)
pBAD24	pBR322 <i>araC</i> , arabinose-inducible promoter, Amp ^R	Guzman <i>et al.</i> (1995)
pNS2Amp	Km ^R	Gillaspie <i>et al.</i> (2009)
pNS2Trc	Km ^R	Gillaspie <i>et al.</i> (2009)
pBAD24:: <i>nrnC</i>	pBAD24 carrying <i>nrnC</i> from <i>B. birtlesii</i> , Amp ^R	This study
pBAD24:: <i>nrnC-s</i>	pBAD24 carrying <i>B. birtlesii</i> <i>nrnC</i> synthetic gene with C-terminal His-tag	This study
pBAD24:: <i>nrnC</i> B.h	pBAD24 carrying <i>nrnC</i> from <i>B. henselae</i> , Amp ^R	This study
pNS2Amp:: <i>nrnC</i> _{AS}	pNS2Amp carrying antisense <i>nrnC</i> , Km ^R	This study
pNS2Trc:: <i>nrnC</i> _{AS}	pNS2Trc carrying antisense <i>nrnC</i> , Km ^R	This study

of 250 ng ml⁻¹ for induction of the P_{L_{act-1}} promoter. *B. birtlesii* and *B. henselae* were grown on 5% defibrinated rabbit blood brain heart infusion (BHI) agar (Difco) or in Schneider's medium (Gibco) supplemented with 10% fetal calf serum (Riess *et al.*, 2008) at 35 °C under 5% CO₂ atmosphere.

Physiological characterization of *nrnC* knockdown strains. To evaluate the effect of *nrnC* knock down on growth of *Bartonella*, the test strains were grown both in Schneider's liquid medium and on BHI blood agar plates. *B. henselae* pNS2Trc and *B. henselae* pNS2Trc::*nrnC*_{AS} were collected after 5 days growth on BHI blood agar plates and suspended in Schneider's medium. The OD₆₀₀ of the bacterial suspension was adjusted to 0.05. Then, 2 ml of this suspension was poured into a 12-well plate and grown at 35 °C in the presence of 5% CO₂. OD₆₀₀ was checked on days 2, 4, 6 and 7 after inoculation. Serial dilutions of the bacterial suspension were plated on BHI blood agar plates, and colony size was evaluated after 6 and 10 days growth at 35 °C in the presence of 5% CO₂.

Genetic techniques. *E. coli* cells were transformed by the calcium chloride method (Maniatis *et al.*, 1989) or electroporation (Dower *et al.*, 1988). *Bartonella* cells were transformed by electroporation (Riess *et al.*, 2003).

DNA manipulations. *B. birtlesii* chromosomal DNA was isolated using the Wizard Genomic DNA purification kit (Promega). Small-scale plasmid DNA preparation was performed by using a QIAprep 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 PCRs, restriction reactions or agarose gels was performed using a Macherey-Nagel NucleoSpin Extract II kit.

Preparation of the *B. birtlesii* genomic library. *B. birtlesii* DNA was submitted to partial digestion by *Sau3AI*. After migration on 1% agarose gel, fragments of 2–3 kbp in length were purified from agarose and partially filled with A and G using DNA Polymerase I, Large (Klenow) Fragment (Invitrogen). Plasmid pACYC184 was digested by *Sall*, purified and partially filled with C and T using DNA Polymerase I, Large (Klenow) Fragment (Invitrogen). Vector and chromosomal DNA fragments were mixed, ligated and introduced into *E. coli* strain XL1-blue by using electroporation.

Construction of a recombinant vector expressing *NrnC* from *B. birtlesii*. The complete *B. birtlesii nrnC* gene with a C-terminal His-tag (His₆) was synthesized according to the *E. coli* codon usage (Millegen) (see Supplementary Fig. S1, available with the online version of this paper). The synthetic gene coding for the C-terminally His₆-tagged *nrnC* gene was amplified by PCR using primers ornBbamontsynt and ornBbavalsynt (Table 2). The amplified products (682 bp) were purified, digested with *NheI* and *KpnI*, and ligated with pBAD24 plasmid digested with *NheI* and *KpnI*. Ligation mixtures were

introduced into CaCl₂-competent *E. coli* strain XL1-blue cells. Transformants were screened by PCR with primers ornBbamontsynt and ornBbavalsynt. Six PCR-positive clones were then sequenced.

Construction of a recombinant vector expressing *NrnC* from *B. henselae*. The complete *B. henselae nrnC* gene (named *mdl* in the *B. henselae* genome database NC_005956) was amplified by PCR with primers ornBhamont and ornBhaval (Table 2) using *B. henselae* chromosomal DNA as template. The 658 bp PCR product was purified, digested with *NheI* and *KpnI*, and ligated with pBAD24 plasmid digested with *NheI* and *KpnI*. Ligation mixtures were introduced into CaCl₂-competent *E. coli* strain XL1-blue cells. Transformants were screened by PCR with primers ornBhamont and ornBhaval (Table 2). Six PCR-positive clones were then sequenced.

Construction of the vector to decrease the amount of *NrnC* in *B. henselae*. The entire coding region of *nrnC* (named *mdl* in the *B. henselae* genome database NC_005956) was amplified by PCR from *B. henselae* chromosomal DNA using primers ornantisensamt, containing a *BamHI* restriction site, and ornantisensavl, containing an *XbaI* restriction site (Table 2). The 627 bp PCR product was purified, digested with *BamHI* and *XbaI*, and then ligated with plasmids pNS2Amp or pNS2Trc digested with *BamHI* and *XbaI*. Ligation mixtures were introduced into CaCl₂-competent *E. coli* strain XL1-blue cells. Transformants were screened by PCR with primers ornantisensamt and ornantisensavl. For each plasmid, six PCR-positive clones were then sequenced.

Expression and purification of *NrnC* His-tagged protein. Strain JP313 (pBAD24::*nrnC-s*) was grown overnight at 37 °C in LB medium containing 50 µg Amp ml⁻¹. One litre of LB medium containing 50 µg Amp ml⁻¹ was inoculated to OD₆₀₀ 0.05 with the overnight culture and grown at 37 °C. Expression was induced at OD₆₀₀ 0.5 for 4 h by adding arabinose (0.2% final concentration). 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, 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 sonication followed by a 3 s pause) for 30 min. The suspension was then 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 dialysed twice against a buffer containing 50 mM Tris/HCl and 250 mM NaCl 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.

***NrnC* activity assays.** Activity assays on nanoRNA were performed on a custom-made RNA fluorescent-tagged 5mer (5'-Cy5-AAAAA3') as described previously (Mechold *et al.*, 2007). The optimal buffer for *NrnC* activity was 50 mM Tris/HCl, pH 8.0, containing 5 mM MgCl₂.

Table 2. Primers used in this study

Primer	Gene	Organism	Sequence (5'–3')
ornBbavalsynt	Synthesized <i>nrnC</i>	<i>B. birtlesii</i>	ATCCCCGGGTACCATGGTCGCCATGAGCTCTTATTAGTGGTGATG
ornBbamontsynt	Synthesized <i>nrnC</i>	<i>B. birtlesii</i>	TTTTGGGCTAGCAGGAGGAATTCACATGACCGAAGTTCGTGTTCATCAG
ornBhamont	Wild-type <i>nrnC</i>	<i>B. henselae</i>	TTTTGGGCTAGCAGGAGGAATTCACATGACCGAAGTTCGTGTTCATC
ornBhaval	Wild-type <i>nrnC</i>	<i>B. henselae</i>	ATCCCCGGGTACCATGGTTTATGCTATGCGCAAAAATATCAATC
ornantisensavl	Antisense <i>nrnC</i>	<i>B. henselae</i>	CCCTCTAGAATGACGGAAATCCGTGTTC
ornantisensamt	Antisense <i>nrnC</i>	<i>B. henselae</i>	CCGGATCCTTATGCTATGCGCAAAAATATC

Rnd	1	MNYQMITTDALASLCRAVRAFFAIAL	50
NrnC	1	MTEVRVHQGD-LPNL-DNYKT-NAVAID	47
Rnd	51	HLA-LID-PLGITDMSD-LEAILEDPSITKFLHAGSEDELEVFLNVFGELD	97
NrnC	48	GTADIIQIAKQGN-APNLVKLEDKAITKISHFGPDLAILAHTFCVMP	96
Rnd	98	QPLIDTQILAAPCGRPMG--WGFAFMVEEYSGVTLDKSESRITDLARFET	145
NrnC	97	DVVPTKI-ASRLTETTYTDEHGLKEICGELLNVNISKQOQSSDMAETLS	145
Rnd	146	ERQCEFAAADVWVLLPITAKLMVTEASGWLPAALDEC-RIMQMR-RQEV	193
NrnC	146	EAQIEFAASDVLYLHRL-KDIFERLKEERSHVAPACPOFLPMRAKLDL	196
Rnd	194	VA-PE-DAMRDITHAWQLTRQLACLQLADWRLKARERDLAVNFVRE	241
NrnC	195	LGNCEVDIFAHHS	
Rnd	242	EHLMSVARYMPGSLGELDSGLSSSEIRFHGKTLALVKAQTLPEEALF	291
Rnd	292	QPMNLNMDMPGKYKAPKAKSLITDVSETHKISARLLASRRQINQLNWH	341
Rnd	342	WKLKPKQNNLPDISGWRGELMAEALNLLQEQYQ	375

Fig. 1. *E. coli* RNaseD (Rnd) and *B. birtlesii* NrnC sequence alignment. This alignment was generated by CLUSTAL W. The conserved DEDD motifs are indicated by a double-headed arrow and numbered. Asp25 and Glu27 in Exol, Asp84 in Exoll, and Tyr151 and Asp155 in Exoll of NrnC are highlighted in grey. The DW-x(2)-RPL motif of Rnd is underlined and highlighted in grey. Rnd and NrnC exonuclease domains are dotted underlined.

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. birtlesii* gene in *E. coli*.

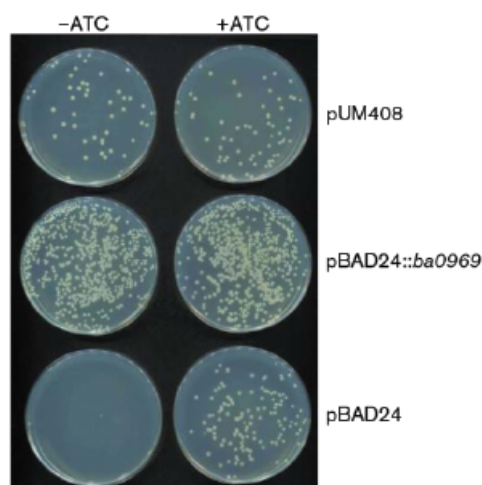


Fig. 2. Complementation of the conditional *E. coli* *om* mutant by *ba0969* from *B. birtlesii*. Strains UM341 containing pBAD24 (vector control), pUM408 (expressing *Orn* from *E. coli*) or pBAD24::*ba0969* (expressing NrnC from *B. birtlesii*) were spread on LB agar plates containing 0.2% arabinose 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.

Therefore, we decided to use a synthetic *ba0969* gene (Millegen) 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::*nrmC-s*. Expression of *ba0969-s* was able to completely rescue 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 orn*. 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::*nrmC-s* and only when expression was induced by arabinose (data not shown). Strain JP313 carrying pBAD24::*nrmC-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

the optimal conditions for nanoRNase activity of NrnC, we first measured activity on a nanoRNA 5mer (5'-Cy5-AAAAA3') 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 NrnC 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 NrnC 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 Orn-catalysed reactions (Mechold *et al.*, 2007), and pointed to a similar reaction mechanism (Fig. 3). Under

the conditions tested, the activity of NrnC measured as the disappearance of substrate 5mers was 8 nmol min⁻¹ µg⁻¹ (Fig. 3). We also tested the activity of NrnC on other RNA oligos, namely 5'-Cy5-CCCCC3', 5'-Cy5-CCC3' and 5'-Cy5-AAA3', as substrate. NrnC degraded RNA 5mer 5'-Cy5-AAAAA3' more rapidly than 5'-Cy5-CCCCC3' (Supplementary Fig. S3, available with the online version of this paper). RNA 3mer 5'-Cy5-AAA3' was also degraded more efficiently by NrnC than RNA 3mer 5'-Cy5-CCC3' (Supplementary Fig. S3). In addition, quantification of the activity on different substrates revealed that, similarly to NrnB-catalysed degradation (Fang *et al.*, 2009), RNA 5mers were a better substrate for NrnC than RNA 3mers.

NanoRNase activity of NrnC 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 Orn (Mechold *et al.*, 2006). In contrast, NrnA 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*, nrmA from *B. subtilis* can restore normal growth of a *cysQ* mutant grown in the absence of cysteine (Mechold *et al.*, 2007). We thus wondered whether nrmC from *B. birtlesii* could restore the growth to an *E. coli* *cysQ* mutant (UM285). Complementation assays showed that expression of nrmC did not restore the growth of *E. coli* strain UM285 in the absence of cysteine (data not shown). We also investigated whether pAp could inhibit the degradation of 5'-Cy5-AAAAA3' by NrnC nanoRNase activity, using 5'-Cy5-AAAAA3' as substrate. As shown in Fig. 4, the presence of 50 µM pAp inhibited NrnC nanoRNase activity.

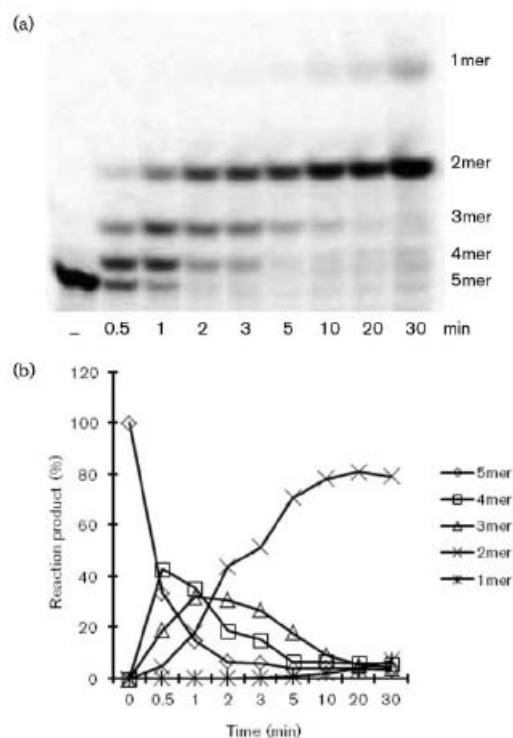


Fig. 3. NrnC-catalysed degradation of a nanoRNA 5mer (5'-Cy5-AAAAA3'). The reaction products were separated on 22% polyacrylamide gels (a). The corresponding reaction products were quantified (b). The estimated activity of NrnC, measured as the disappearance of substrate 5mers, was 8 nmol min⁻¹ µg⁻¹. The experiment was repeated three times. A representative result is presented.

Activity of NrnC on RNA 24mer

To check whether NrnC specifically degrades nanoRNA, or is active on longer RNAs as well, we tested degradation of an RNA 24mer 5'-end labelled with [³³P]ATP. The NrnC-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. birtlesii* NrnC on the 24mer was lower than that on nanoRNA, but was 4.1-fold higher than that of NrnB on 24mers and 830-fold higher than that of *B. subtilis* NrnA on 24mers (Mechold *et al.*, 2007; Fang *et al.*, 2009).

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

In *E. coli*, nanoRNase activity of Orn is required for growth (Ghosh & Deutscher, 1999). When expressed in *E. coli*, *B. birtlesii* nrmC can complement the *orn* mutation. We investigated whether NrnC activity was required for normal growth in *Bartonella*. Due to the lack of genetic tools, it is presently not possible to generate mutants in essential genes of *Bartonella*. However, it is possible to analyse 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.*,

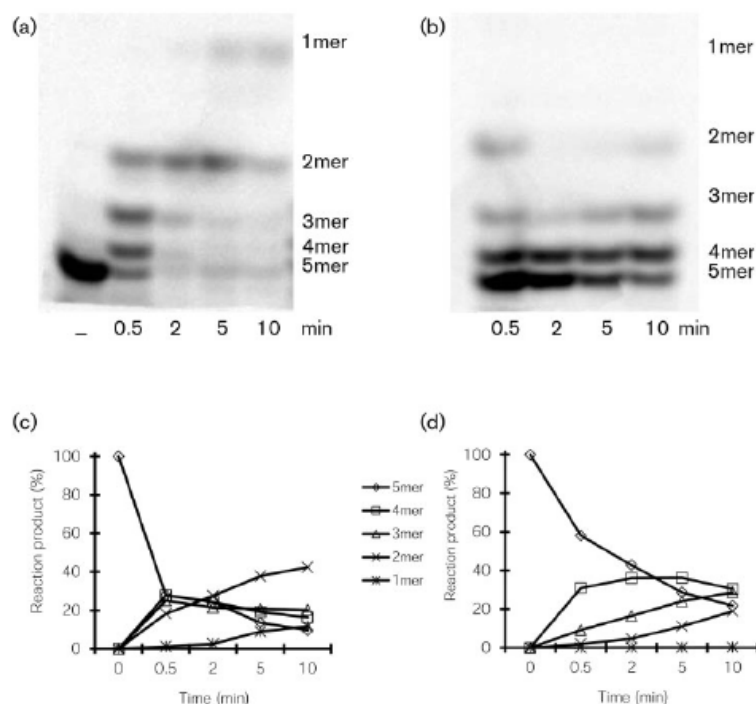


Fig. 4. NrnC activity on nanoRNA is inhibited by pAp. NrnC activity on 5mer A (5'-Cy5-AAAAA3') was measured in the absence of pAp (a, c) or in the presence of 50 μM pAp (b, d). (a, b) Separation of the corresponding reaction products on 22 % polyacrylamide gels. (c, d) Quantification of reaction products and intermediates of degradation. The experiments were repeated three times. Representative results are presented.

2009). 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 *nmC* in *B. henselae*. The primary sequence of NrnC from *B. henselae* is 90 % identical to that of NrnC from *B. birtlesii*. To test whether NrnC from *B. henselae* has nanoRNase activity *in vivo* similar to NrnC from *B. birtlesii*, we cloned *nmC* from *B. henselae* into pBAD24. The plasmid expressing *B. henselae nmC*, pBAD24::*nmCB.h*, was then introduced into *E. coli* strain UM341 to test for complementation of the lack of Orn. Our experiments demonstrated that expression of NrnC from *B. henselae* can rescue the growth defect of strain UM341 in the absence of Atc (data not shown). We cloned *nmC* of *B. henselae* into a vector that allowed low-level expression, pNS2Amp, and a vector that allowed high-level expression, pNS2Trc (Gillaspie *et al.*, 2009). The gene was in the reverse orientation such that the antisense strand was transcribed. Plasmids pNS2Amp, pNS2Trc, pNS2Amp::*nmC_{AS}* and pNS2Trc::*nmC_{AS}* 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::*nmC_{AS}* 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::*nmC_{AS}* grew more slowly in Schneider's medium (Fig. 6). The doubling time of the strain carrying pNS2Trc::*nmC_{AS}* (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::*nmC_{AS}*) were much smaller (0.65 ± 0.1 mm) than those formed by *B. henselae* (pNS2Trc) (0.56 ± 0.09 and 1.15 ± 0.13 mm after 6 and 10 days, respectively). These results show that a decrease in the amount of NrnC slowed the growth of *Bartonella*, and demonstrate the importance of NrnC activity for the physiology of this alphaproteobacterium.

Phylogenetic distribution of NrnC

Searching for NrnC homologues in bacterial genomes sequenced to date showed that this protein is well conserved

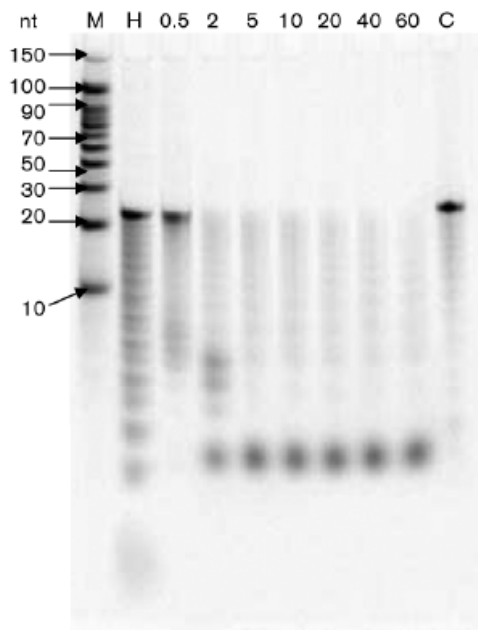


Fig. 5. Activity of NrnC on an RNA 24mer. The degradation of 5' [γ - 32 P]ATP-labelled RNA 24mers (5'-CACCACCACCACCACC-ACCACACA-3') by NrnC. Samples were taken after 0.5, 2, 5, 10, 20, 40 and 60 min and analysed as described in Methods. The negative control was performed in the absence of NrnC. M, decade marker; H, alkaline hydrolysis control; C, negative control. The experiment was repeated three times; a representative result is presented.

in all *Bartonella*. *B. birtlesii* NrnC was 87–91 % identical to the NrnC homologues found in the six *Bartonella* genomes present in the databases (<http://www.ncbi.nlm.nih.gov/>). In addition, we found NrnC homologues in the genomes of many other *Alphaproteobacteria* with identities ranging from 77 % for *Brucella melitensis* to 43 % for *Candidatus pelagibacter* (Supplementary Table S1, available with the online version of this paper). Other NrnC homologues that fulfilled our criterion of varying less than 20 % in size were found in *Cyanobacteria*, and were between 42 and 50 % identical to NrnC (Supplementary Table S1). Genomes of some *Spirochaetes* also encode NrnC homologues that are between 44 and 48 % identical to NrnC (Supplementary Table S1). NrnC homologues were absent in *Betaproteobacteria* and *Gammaproteobacteria* genomes that harbour homologues of Orn (Mechold *et al.*, 2007). Also, NrnC homologues were absent in *Chlorobi/Bacteroidetes* and *Firmicutes* that have NrnA homologues. Thus, NrnC represents a third class of nanoRNase that is mainly present in *Alphaproteobacteria* and *Cyanobacteria*. According to its size, NrnC is closer to Orn from *E. coli* (181 aa) (Ghosh &

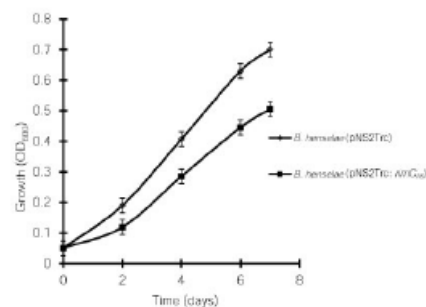


Fig. 6. Effect of *nrmC* knock down on growth of *B. henselae*. Strains *B. henselae* (pNS2Trc) and *B. henselae* (pNS2Trc::*nrmC* Δ) were cultured in Schneider's medium, collected after 5 days growth on BHI blood agar plates and suspended in PBS buffer. The bacterial suspension was used to inoculate Schneider's medium at an OD₆₀₀ of 0.05. Cultures were grown with shaking at 35 °C in the presence of 5 % CO₂ and OD₆₀₀ was measured on days 2, 4, 6 and 7 after inoculation. All experiments were repeated three times.

Deutscher, 1999) than to NrnA (313 aa) (Mechold *et al.*, 2007) and NmB (399 aa) from *B. subtilis* (Fang *et al.*, 2009). Similarity between NrnC and its functional analogues *E. coli* Orn and *B. subtilis* NmA and NmB 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-PP, 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 oligoribonucleotide 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 oligoribonucleotide degradation are undoubtedly assumed by functional analogues. We demonstrate here that this is the case for Orn, an RNase exoribonuclease that is essential for cell growth in *E. coli*. Screening for *B. birtlesii* genes that could complement a conditional lethal mutant of *E. coli* *om*, we identified a gene *ba0969* that encodes a polypeptide that has 30 % identity to the catalytic domain of *E. coli* RNase D. However, the *B. birtlesii* 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

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. birtlesii* clearly showed that this protein can degrade nanoRNA in the presence of magnesium or manganese. In both cases, the specific activity of NrnC is higher than those measured for *B. subtilis* NrnA and NrnB (Mechold *et al.*, 2007).

The nanoRNase activity of NrnC 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, NrnC 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 2- to 5mers, we noted the general tendency of decreased reaction rate with increasing chain length (Datta & Niyogi, 1975). The activity of NrnA from *B. subtilis* on 24mer oligos was shown to be insignificant (Mechold *et al.*, 2007). Despite the fact that NrnB from *B. subtilis* is more active on RNA 24mers compared with NmA, this activity is nevertheless three orders of magnitude lower than the activity on nanoRNA (Fang *et al.*, 2009). For NrnC, the activity on RNA 24mers ($8.3 \text{ pmol min}^{-1} \mu\text{g}^{-1}$) is 1000 times lower than the activity on nanoRNA ($8 \text{ nmol min}^{-1} \mu\text{g}^{-1}$). Hence all these enzymes strongly prefer nanoRNA over longer RNA substrates. Similar to Orn activity (Mechold *et al.*, 2006), the nanoRNase activity of NrnC is strongly inhibited by pAp. In contrast, the nanoRNase activity of NrnA is not sensitive to pAp at low concentrations. However, pAp is a substrate for NrnA and as such pAp can compete with nanoRNA for degradation by NrnA. In agreement with this *in vitro* activity, NrnA can restore the growth of an *E. coli* strain impaired in pAp degradation due to the lack of a functional CysQ and thus enables this strain to grow in the absence of cysteine (Mechold *et al.*, 2007). NrnC from *B. birtlesii* cannot restore growth of the *E. coli* *cysQ* mutant, and hence pAp is not a substrate for this enzyme.

NrnC is present and well conserved in the genomes of *Bartonella* species. In agreement with this observation, we show that the NrnC homologue from *B. henselae* could equally complement an *E. coli* *orn* mutant, pointing to conserved function of NrnC 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 NmA and NrnB, and it seems that other RNases can substitute for their function (Fang *et al.*, 2009). Comparison of growth rates of *B. henselae* carrying pNS2Trc::nmC_{AS} (doubling time 12 h), and the corresponding vector control (doubling time 7 h) shows that knocking down *nrcC* in *B. henselae* slows growth. This result suggests that a full nanoRNase activity of NrnC is important for optimal growth at least in this *Bartonella* species and that other RNases cannot take over this function. Hence, similar

to *E. coli*, in *Bartonella* only one enzyme, here NrnC, 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 NrnC. Members of the genera *Alphaproteobacteria* and *Cyanobacteria* do not contain Orn, or NrnA or NmB homologues. We propose that NrnC homologues fulfil the function of degrading nanoRNA in these organisms. Thus, NrnC 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 faeces.

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REFERENCES

- Alsmark, C. M., Frank, A. C., Karlberg, E. O., Legault, B. A., Ardeli, D. H., Canbäck, B., Eriksson, A. S., Näslund, A. K., Handley, S. A. & other authors (2004). The louse-borne human pathogen *Bartonella quintana* is a genomic derivative of the zoonotic agent *Bartonella henselae*. *Proc Natl Acad Sci U S A* 101, 9716–9721.
- Arraiano, C. M., Andrade, J. M., Domingues, S., Guinote, I. B., Malecki, M., Matos, R. G., Moreira, R. N., Pobre, V., Reis, F. P. & other authors (2010). The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev* 34, 883–923.
- Breitschwerdt, E. B., Maggi, R. G., Chomel, B. B. & Lappin, M. R. (2010). Bartonellosis: an emerging infectious disease of zoonotic importance to animals and human beings. *J Vet Emerg Crit Care (San Antonio)* 20, 8–30.
- Cheng, Z. F. & Deutscher, M. P. (2002). Purification and characterization of the *Escherichia coli* exoribonuclease RNase R. Comparison with RNase II. *J Biol Chem* 277, 21624–21629.
- Chomel, B. B., Boulouis, H. J., Breitschwerdt, E. B., Kasten, R. W., Vayssier-Taussat, M., Birtles, R. J., Koehler, J. E. & Dehio, C. (2009). Ecological fitness and strategies of adaptation of *Bartonella* species to their hosts and vectors. *Vet Res* 40, 29.

- Condon, C. (2007). Maturation and degradation of RNA in bacteria. *Curr Opin Microbiol* 10, 271–278.
- Datta, A. K. & Niyogi, K. (1975). A novel oligoribonuclease of *Escherichia coli*. II. Mechanism of action. *J Biol Chem* 250, 7313–7319.
- Dower, W. J., Miller, J. F. & Ragsdale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* 16, 6127–6145.
- Fang, M., Zeisberg, W. M., Condon, C., Ogryzko, V., Danchin, A. & Mechold, U. (2009). Degradation of nanoRNA is performed by multiple redundant RNases in *Bacillus subtilis*. *Nucleic Acids Res* 37, 5114–5125.
- Ghosh, S. & Deutscher, M. P. (1999). Oligoribonuclease is an essential component of the mRNA decay pathway. *Proc Natl Acad Sci U S A* 96, 4372–4377.
- Gillaspie, D., Perkins, I., Larsen, K., McCord, A., Pangonis, S., Sweger, D., Selem, N., Sriranganathan, N. & Anderson, B. E. (2009). Plasmid-based system for high-level gene expression and antisense gene knockdown in *Bartonella henselae*. *Appl Environ Microbiol* 75, 5434–5436.
- Goldman, S. R., Sharp, J. S., Vvedenskaya, I. O., Livny, J., Dove, S. L. & Nickels, B. E. (2011). NanoRNAs prime transcription initiation *in vivo*. *Mol Cell* 42, 817–825.
- Guerra, H. (2007). The Brucellae and their success as pathogens. *Crit Rev Microbiol* 33, 325–331.
- Guptill, L. (2010). Bartonellosis. *Vet Microbiol* 140, 347–359.
- Guzman, L. M., Belin, D., Carson, M. J. & Beckwith, J. (1995). Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177, 4121–4130.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lagesen, K., Ussery, D. W. & Wassenaar, T. M. (2010). Genome update: the 1000th genome – a cautionary tale. *Microbiology* 156, 603–608.
- Mechold, U., Ogryzko, V., Ngo, S. & Danchin, A. (2006). Oligoribonuclease is a common downstream target of lithium-induced pAp accumulation in *Escherichia coli* and human cells. *Nucleic Acids Res* 34, 2364–2373.
- Mechold, U., Fang, G., Ngo, S., Ogryzko, V. & Danchin, A. (2007). YtqI from *Bacillus subtilis* has both oligoribonuclease and pAp-phosphatase activity. *Nucleic Acids Res* 35, 4552–4561.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Minnick, M. F. & Battisti, J. M. (2009). Pestilence, persistence and pathogenicity: infection strategies of *Bartonella*. *Future Microbiol* 4, 743–758.
- Mueller, K. & González, J. E. (2011). Complex regulation of symbiotic functions is coordinated by MucR and quorum sensing in *Sinorhizobium meliloti*. *J Bacteriol* 193, 485–496.
- Nickels, B. E. & Dove, S. L. (2011). NanoRNAs: a class of small RNAs that can prime transcription initiation in bacteria. *J Mol Biol* 412, 772–781.
- Niyogi, S. K. & Datta, A. K. (1975). A novel oligoribonuclease of *Escherichia coli*. I. Isolation and properties. *J Biol Chem* 250, 7307–7312.
- Postic, G., Danchin, A. & Mechold, U. (2012). Characterization of RnA homologs from *Mycobacterium tuberculosis* and *Mycoplasma pneumoniae*. *RNA* 18, 155–165.
- Riess, T., Anderson, B., Fackelmayer, A., Autenrieth, I. B. & Kempf, V. A. J. (2003). Rapid and efficient transposon mutagenesis of *Bartonella henselae* by transposome technology. *Gene* 313, 103–109.
- Riess, T., Dietrich, F., Schmidt, K. V., Kaiser, P. O., Schwarz, H., Schäfer, A. & Kempf, V. A. (2008). Analysis of a novel insect cell culture medium-based growth medium for *Bartonella* species. *Appl Environ Microbiol* 74, 5224–5227.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Stacey, G., Libault, M., Brechenmacher, L., Wan, J. & May, G. D. (2006). Genetics and functional genomics of legume nodulation. *Curr Opin Plant Biol* 9, 110–121.
- Vincent, H. A. & Deutscher, M. P. (2006). Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem* 281, 29769–29775.
- Yu, D. & Deutscher, M. P. (1995). Oligoribonuclease is distinct from the other known exoribonucleases of *Escherichia coli*. *J Bacteriol* 177, 4137–4139.
- Zhang, J. & Biswas, I. (2009). 3'-Phosphoadenosine-5'-phosphate phosphatase activity is required for superoxide stress tolerance in *Streptococcus mutans*. *J Bacteriol* 191, 4330–4340.
- Zhang, X., Zhu, L. & Deutscher, M. P. (1998). Oligoribonuclease is encoded by a highly conserved gene in the 3'-5' exonuclease superfamily. *J Bacteriol* 180, 2779–2781.
- Zuo, Y. & Deutscher, M. P. (2001). Exoribonuclease superfamilies: structural analysis and phylogenetic distribution. *Nucleic Acids Res* 29, 1017–1026.
- Zuo, Y., Wang, Y. & Malhotra, A. (2005). Crystal structure of *Escherichia coli* RNase D, an exoribonuclease involved in structured RNA processing. *Structure* 13, 973–984.

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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.

References

- Abdul-Tehrani, H., Hudson, A.J., Chang, Y.S., Timms, A.R., Hawkins, C., Williams, J.M., Harrison, P.M., Guest, J.R., Andrews, S.C., 1999, Ferritin mutants of *Escherichia coli* are iron deficient and growth impaired, and fur mutants are iron deficient. *J Bacteriol* 181, 1415-1428.
- Adal, K.A., Cockerell, C.J., Petri, W.A., Jr., 1994, Cat scratch disease, bacillary angiomatosis, and other infections due to *Rochalimaea*. *N Engl J Med* 330, 1509-1515.
- Almiron, M., Link, A.J., Furlong, D., Kolter, R., 1992, A novel DNA-binding protein with regulatory and protective roles in starved *Escherichia coli*. *Genes Dev* 6, 2646-2654.
- Alsmark, C.M., Frank, A.C., Karlberg, E.O., Legault, B.A., Ardell, D.H., Canback, B., Eriksson, A.S., Naslund, A.K., Handley, S.A., Huvet, M., La Scola, B., Holmberg, M., Andersson, S.G., 2004, The louse-borne human pathogen *Bartonella quintana* is a genomic derivative of the zoonotic agent *Bartonella henselae*. *Proc Natl Acad Sci U S A* 101, 9716-9721.
- Anderson, B.E., Neuman, M.A., 1997, *Bartonella* spp. as emerging human pathogens. *Clin Microbiol Rev* 10, 203-219.
- Andrews, S.C., Harrison, P.M., Guest, J.R., 1989, Cloning, sequencing, and mapping of the bacterioferritin gene (bfr) of *Escherichia coli* K-12. *J Bacteriol* 171, 3940-3947.
- Andrews, S.C., Robinson, A.K., Rodriguez-Quinones, F., 2003, Bacterial iron homeostasis. *FEMS Microbiol Rev* 27, 215-237.
- Anjem, A., Varghese, S., Imlay, J.A., 2009, Manganese import is a key element of the OxyR response to hydrogen peroxide in *Escherichia coli*. *Mol Microbiol* 72, 844-858.
- Anzaldi, L.L., Skaar, E.P., 2010, Overcoming the heme paradox: heme toxicity and tolerance in bacterial pathogens. *Infect Immun* 78, 4977-4989.
- Appleby, C.A., Bradbury, J.H., Morris, R.J., Wittenberg, B.A., Wittenberg, J.B., Wright, P.E., 1983, Leghemoglobin. Kinetic, nuclear magnetic resonance, and optical studies of pH dependence of oxygen and carbon monoxide binding. *J Biol Chem* 258, 2254-2259.
- Arnoux, P., Haser, R., Izadi, N., Lecroisey, A., Delepierre, M., Wandersman, C., Czjzek, M., 1999, The crystal structure of HasA, a hemophore secreted by *Serratia marcescens*. *Nat Struct Biol* 6, 516-520.
- Baichoo, N., Helmann, J.D., 2002, Recognition of DNA by Fur: a reinterpretation of the Fur box consensus sequence. *J Bacteriol* 184, 5826-5832.
- Baker, H.M., Anderson, B.F., Baker, E.N., 2003, Dealing with iron: common structural principles in proteins that transport iron and heme. *Proc Natl Acad Sci U S A* 100, 3579-3583.
- Battisti, J.M., Minnick, M.F., 1999, Development of a system for genetic manipulation of *Bartonella bacilliformis*. *Appl Environ Microbiol* 65, 3441-3448.

- Battisti, J.M., Sappington, K.N., Smitherman, L.S., Parrow, N.L., Minnick, M.F., 2006, Environmental signals generate a differential and coordinated expression of the heme receptor gene family of *Bartonella quintana*. *Infect Immun* 74, 3251-3261.
- Battisti, J.M., Smitherman, L.S., Sappington, K.N., Parrow, N.L., Raghavan, R., Minnick, M.F., 2007, Transcriptional regulation of the heme binding protein gene family of *Bartonella quintana* is accomplished by a novel promoter element and iron response regulator. *Infect Immun* 75, 4373-4385.
- Battistini, T., 1931, Laverrière péruvienne: sa transmission par le Phlébotome. *Revue Sud-Américaine de Médecine et de Chirurgie* 2, 719-724.
- Benevides-Matos, N., Biville, F., 2010, The Hem and Has haem uptake systems in *Serratia marcescens*. *Microbiology* 156, 1749-1757.
- Benson, L.A., Kar, S., McLaughlin, G., Ihler, G.M., 1986, Entry of *Bartonella bacilliformis* into erythrocytes. *Infect Immun* 54, 347-353.
- Bereswill, S., Hinkelmann, S., Kist, M., Sander, A., 1999, Molecular analysis of riboflavin synthesis genes in *Bartonella henselae* and use of the *ribC* gene for differentiation of *Bartonella* species by PCR. *J Clin Microbiol* 37, 3159-3166.
- Billeter, S.A., Levy, M.G., Chomel, B.B., Breitschwerdt, E.B., 2008, Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. *Med Vet Entomol* 22, 1-15.
- Birtles, R.J., Harrison, T.G., Saunders, N.A., Molyneux, D.H., 1995, Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. *Int J Syst Bacteriol* 45, 1-8.
- Biville, F., 2008, Bacterial iron uptake. *Iron Metabolism and Disease* 393-420.
- Biville, F., Cwerman, H., Letoffe, S., Rossi, M.S., Drouet, V., Ghigo, J.M., Wandersman, C., 2004, Haemophore-mediated signalling in *Serratia marcescens*: a new mode of regulation for an extra cytoplasmic function (ECF) sigma factor involved in haem acquisition. *Mol Microbiol* 53, 1267-1277.
- Bonilla, D.L., Kabeya, H., Henn, J., Kramer, V.L., Kosoy, M.Y., 2009, *Bartonella quintana* in body lice and head lice from homeless persons, San Francisco, California, USA. *Emerg Infect Dis* 15, 912-915.
- Boonjakuakul, J.K., Gerns, H.L., Chen, Y.T., Hicks, L.D., Minnick, M.F., Dixon, S.E., Hall, S.C., Koehler, J.E., 2007, Proteomic and immunoblot analyses of *Bartonella quintana* total membrane proteins identify antigens recognized by sera from infected patients. *Infect Immun* 75, 2548-2561.
- Boulouis, H.J., Barrat, F., Bermond, D., Bernex, F., Thibault, D., Heller, R., Fontaine, J.J., Piemont, Y., Chomel, B.B., 2001, Kinetics of *Bartonella birtlesii* infection in experimentally infected mice and pathogenic effect on reproductive functions. *Infect Immun* 69, 5313-5317.
- Bown, K.J., Bennet, M., Begon, M., 2004, Flea-borne *Bartonella grahamii* and *Bartonella taylorii* in bank voles. *Emerg Infect Dis* 10, 684-687.
- Bradbeer, C., 1993, The proton motive force drives the outer membrane transport of

- cobalamin in *Escherichia coli*. *J Bacteriol* 175, 3146-3150.
- Braun, V., Braun, M., 2002, Iron transport and signaling in *Escherichia coli*. *FEBS Lett* 529, 78-85.
- Braun, V., Gaisser, S., Herrmann, C., Kampfenkel, K., Killmann, H., Traub, I., 1996, Energy-coupled transport across the outer membrane of *Escherichia coli*: ExbB binds ExbD and TonB in vitro, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. *J Bacteriol* 178, 2836-2845.
- Braun, V., Mahren, S., Ogieman, M., 2003, Regulation of the Fecl-type ECF sigma factor by transmembrane signalling. *Curr Opin Microbiol* 6, 173-180.
- Breitschwerdt, E.B., Maggi, R.G., Sigmon, B., Nicholson, W.L., 2007, Isolation of *Bartonella quintana* from a woman and a cat following putative bite transmission. *J Clin Microbiol* 45, 270-272.
- Brickman, T.J., McIntosh, M.A., 1992, Overexpression and purification of ferric enterobactin esterase from *Escherichia coli*. Demonstration of enzymatic hydrolysis of enterobactin and its iron complex. *J Biol Chem* 267, 12350-12355.
- Brouqui, P., Lascola, B., Roux, V., Raoult, D., 1999, Chronic *Bartonella quintana* bacteremia in homeless patients. *N Engl J Med* 340, 184-189.
- Buckles, E.L., McGinnis Hill, E., 2000, Interaction of *Bartonella bacilliformis* with human erythrocyte membrane proteins. *Microb Pathog* 29, 165-174.
- Byam, W., Lloyd, L., 1920, Trench Fever: Its Epidemiology and Endemiology. *Proc R Soc Med* 13, 1-27.
- Caceres, A.G., 1993, [Geographic distribution of *Lutzomyia verrucarum* (Townsend, 1913) (Diptera, Psychodidae, Phlebotominae), vector of human bartonellosis in Peru]. *Rev Inst Med Trop Sao Paulo* 35, 485-490.
- Caro-Hernandez, P., Fernandez-Lago, L., de Miguel, M.J., Martin-Martin, A.I., Cloeckaert, A., Grillo, M.J., Vizcaino, N., 2007, Role of the Omp25/Omp31 family in outer membrane properties and virulence of *Brucella ovis*. *Infect Immun* 75, 4050-4061.
- Carroll, J.A., Coleman, S.A., Smitherman, L.S., Minnick, M.F., 2000, Hemin-binding surface protein from *Bartonella quintana*. *Infect Immun* 68, 6750-6757.
- Carrondo, M.A., 2003, Ferritins, iron uptake and storage from the bacterioferritin viewpoint. *EMBO J* 22, 1959-1968.
- Cartwright, J.L., Britton, P., Minnick, M.F., McLennan, A.G., 1999, The IalA invasion gene of *Bartonella bacilliformis* encodes a (de)nucleoside polyphosphate hydrolase of the MutT motif family and has homologs in other invasive bacteria. *Biochem Biophys Res Commun* 256, 474-479.
- Cascales, E., Christie, P.J., 2003, The versatile bacterial type IV secretion systems. *Nat Rev Microbiol* 1, 137-149.
- Cescau, S., Cwerman, H., Letoffe, S., Delepelaire, P., Wandersman, C., Biville, F., 2007, Heme acquisition by hemophores. *Biometals* 20, 603-613.
- Chang, C.C., Chomel, B.B., Kasten, R.W., Romano, V., Tietze, N., 2001, Molecular evidence of *Bartonella* spp. in questing adult *Ixodes pacificus* ticks in

- California. J Clin Microbiol 39, 1221-1226.
- Chenoweth, M.R., Greene, C.E., Krause, D.C., Gherardini, F.C., 2004a, Predominant outer membrane antigens of *Bartonella henselae*. Infect Immun 72, 3097-3105.
- Chenoweth, M.R., Somerville, G.A., Krause, D.C., O'Reilly, K.L., Gherardini, F.C., 2004b, Growth characteristics of *Bartonella henselae* in a novel liquid medium: primary isolation, growth-phase-dependent phage induction, and metabolic studies. Appl Environ Microbiol 70, 656-663.
- Chiang, S.M., Schellhorn, H.E., 2012, Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria. Arch Biochem Biophys.
- Chim, N., Iniguez, A., Nguyen, T.Q., Goulding, C.W., 2010, Unusual diheme conformation of the heme-degrading protein from *Mycobacterium tuberculosis*. J Mol Biol 395, 595-608.
- Chomel, B.B., Boulouis, H.J., Breitschwerdt, E.B., Kasten, R.W., Vayssier-Taussat, M., Birtles, R.J., Koehler, J.E., Dehio, C., 2009, Ecological fitness and strategies of adaptation of *Bartonella* species to their hosts and vectors. Vet Res 40, 29.
- Chomel, B.B., Kasten, R.W., Floyd-Hawkins, K., Chi, B., Yamamoto, K., Roberts-Wilson, J., Gurfield, A.N., Abbott, R.C., Pedersen, N.C., Koehler, J.E., 1996, Experimental transmission of *Bartonella henselae* by the cat flea. J Clin Microbiol 34, 1952-1956.
- Christie, P.J., 2004, Type IV secretion: the *Agrobacterium* VirB/D4 and related conjugation systems. Biochim Biophys Acta 1694, 219-234.
- Coleman, S.A., Minnick, M.F., 2001, Establishing a direct role for the *Bartonella bacilliformis* invasion-associated locus B (IalB) protein in human erythrocyte parasitism. Infect Immun 69, 4373-4381.
- Coleman, S.A., Minnick, M.F., 2003, Differential expression of the invasion-associated locus B (IalB) gene of *Bartonella bacilliformis* in response to environmental cues. Microb Pathog 34, 179-186.
- Conley, T., Slater, L., Hamilton, K., 1994, *Rochalimaea* species stimulate human endothelial cell proliferation and migration in vitro. J Lab Clin Med 124, 521-528.
- Conyers, G.B., Bessman, M.J., 1999, The gene, *ialA*, associated with the invasion of human erythrocytes by *Bartonella bacilliformis*, designates a nudix hydrolase active on dinucleoside 5'-polyphosphates. J Biol Chem 274, 1203-1206.
- Cope, L.D., Thomas, S.E., Latimer, J.L., Slaughter, C.A., Muller-Eberhard, U., Hansen, E.J., 1994, The 100 kDa haem:haemopexin-binding protein of *Haemophilus influenzae*: structure and localization. Mol Microbiol 13, 863-873.
- Cope, L.D., Yorgev, R., Muller-Eberhard, U., Hansen, E.J., 1995, A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. J Bacteriol 177, 2644-2653.
- Cornelissen, C.N., Anderson, J.E., Sparling, P.F., 1997, Energy-dependent changes in the gonococcal transferrin receptor. Mol Microbiol 26, 25-35.

- Cornelissen, C.N., Sparling, P.F., 1994, Iron piracy: acquisition of transferrin-bound iron by bacterial pathogens. *Mol Microbiol* 14, 843-850.
- Coy, M., Neilands, J.B., 1991, Structural dynamics and functional domains of the fur protein. *Biochemistry* 30, 8201-8210.
- Cwerman, H., Wandersman, C., Biville, F., 2006, Heme and a five-amino-acid hemophore region form the bipartite stimulus triggering the has signaling cascade. *J Bacteriol* 188, 3357-3364.
- Dabo, S.M., Confer, A.W., Anderson, B.E., Gupta, S., 2006a, *Bartonella henselae* Pap31, an extracellular matrix adhesin, binds the fibronectin repeat III13 module. *Infect Immun* 74, 2513-2521.
- Dabo, S.M., Confer, A.W., Saliki, J.T., Anderson, B.E., 2006b, Binding of *Bartonella henselae* to extracellular molecules: identification of potential adhesins. *Microb Pathog* 41, 10-20.
- Dailey, H.A., Septer, A.N., Daugherty, L., Thames, D., Gerdes, S., Stabb, E.V., Dunn, A.K., Dailey, T.A., Phillips, J.D., 2011, The *Escherichia coli* protein YfeX functions as a porphyrinogen oxidase, not a heme dechelataase. *MBio* 2, e00248-00211.
- de Paz, H.D., Sangari, F.J., Bolland, S., Garcia-Lobo, J.M., Dehio, C., de la Cruz, F., Llosa, M., 2005, Functional interactions between type IV secretion systems involved in DNA transfer and virulence. *Microbiology* 151, 3505-3516.
- Dehio, C., 2001, *Bartonella* interactions with endothelial cells and erythrocytes. *Trends Microbiol* 9, 279-285.
- Dehio, C., 2004, Molecular and cellular basis of *Bartonella* pathogenesis. *Annu Rev Microbiol* 58, 365-390.
- Dehio, C., 2005, *Bartonella*-host-cell interactions and vascular tumour formation. *Nat Rev Microbiol* 3, 621-631.
- Dehio, C., 2008, Infection-associated type IV secretion systems of *Bartonella* and their diverse roles in host cell interaction. *Cell Microbiol* 10, 1591-1598.
- Dehio, C., Sander, A., 1999, *Bartonella* as emerging pathogens. *Trends Microbiol* 7, 226-228.
- Dehio, C., Sauder, U., Hiestand, R., 2004, Isolation of *Bartonella schoenbuchensis* from *Lipoptena cervi*, a blood-sucking arthropod causing deer ked dermatitis. *J Clin Microbiol* 42, 5320-5323.
- Delpino, M.V., Cassataro, J., Fossati, C.A., Goldbaum, F.A., Baldi, P.C., 2006, *Brucella* outer membrane protein Omp31 is a haemin-binding protein. *Microbes Infect* 8, 1203-1208.
- Demers, D.M., Bass, J.W., Vincent, J.M., Person, D.A., Noyes, D.K., Staeger, C.M., Samlaska, C.P., Lockwood, N.H., Regnery, R.L., Anderson, B.E., 1995, Cat-scratch disease in Hawaii: etiology and seroepidemiology. *J Pediatr* 127, 23-26.
- Deng, H.K., Le Rhun, D., Le Naour, E., Bonnet, S., Vayssier-Taussat, M., 2012, Identification of *Bartonella* trw host-specific receptor on erythrocytes. *PLoS One* 7, e41447.
- Derrick, S.C., Ihler, G.M., 2001, Deformin, a substance found in *Bartonella*

- bacilliformis culture supernatants, is a small, hydrophobic molecule with an affinity for albumin. *Blood Cells Mol Dis* 27, 1013-1019.
- Dramsi, S., Cossart, P., 1998, Intracellular pathogens and the actin cytoskeleton. *Annu Rev Cell Dev Biol* 14, 137-166.
- Dziewanowska, K., Patti, J.M., Deobald, C.F., Bayles, K.W., Trumble, W.R., Bohach, G.A., 1999, Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect Immun* 67, 4673-4678.
- Eberhardt, C., Engelmann, S., Kusch, H., Albrecht, D., Hecker, M., Autenrieth, I.B., Kempf, V.A., 2009, Proteomic analysis of the bacterial pathogen *Bartonella henselae* and identification of immunogenic proteins for serodiagnosis. *Proteomics* 9, 1967-1981.
- Edelstein, P.H., Hu, B., Shinzato, T., Edelstein, M.A., Xu, W., Bessman, M.J., 2005, *Legionella pneumophila* NudA Is a Nudix hydrolase and virulence factor. *Infect Immun* 73, 6567-6576.
- Eisenhauer, H.A., Shames, S., Pawelek, P.D., Coulton, J.W., 2005, Siderophore transport through *Escherichia coli* outer membrane receptor FhuA with disulfide-tethered cork and barrel domains. *J Biol Chem* 280, 30574-30580.
- Ekins, A., Khan, A.G., Shouldice, S.R., Schryvers, A.B., 2004, Lactoferrin receptors in gram-negative bacteria: insights into the iron acquisition process. *Biometals* 17, 235-243.
- Eskow, E., Rao, R.V., Mordechai, E., 2001, Concurrent infection of the central nervous system by *Borrelia burgdorferi* and *Bartonella henselae*: evidence for a novel tick-borne disease complex. *Arch Neurol* 58, 1357-1363.
- Ezraty, B., Aussel, L., Barras, F., 2005, Methionine sulfoxide reductases in prokaryotes. *Biochim Biophys Acta* 1703, 221-229.
- Fabian, M., Solomaha, E., Olson, J.S., Maresso, A.W., 2009, Heme transfer to the bacterial cell envelope occurs via a secreted hemophore in the Gram-positive pathogen *Bacillus anthracis*. *J Biol Chem* 284, 32138-32146.
- Fang, M., Zeisberg, W.M., Condon, C., Ogryzko, V., Danchin, A., Mechold, U., 2009, Degradation of nanoRNA is performed by multiple redundant RNases in *Bacillus subtilis*. *Nucleic Acids Res* 37, 5114-5125.
- Fenollar, F., Raoult, D., 2004, Molecular genetic methods for the diagnosis of fastidious microorganisms. *APMIS* 112, 785-807.
- Ferguson, A.D., Deisenhofer, J., 2004, Metal import through microbial membranes. *Cell* 116, 15-24.
- Florin, T.A., Zaoutis, T.E., Zaoutis, L.B., 2008, Beyond cat scratch disease: widening spectrum of *Bartonella henselae* infection. *Pediatrics* 121, e1413-1425.
- Foucalt, C., Barrau, K., Brouqui, P., Raoult, D., 2002, *Bartonella quintana* Bacteremia among Homeless People. *Clinical Infectious Diseases* 35, 684-689.
- Fournier, C., Smith, A., Delepelaire, P., Haem release from haemopexin by HxuA allows *Haemophilus influenzae* to escape host nutritional immunity. *Mol Microbiol* 80, 133-148.

- Fournier, C., Smith, A., Delepelaire, P., 2011, Haem release from haemopexin by HxuA allows *Haemophilus influenzae* to escape host nutritional immunity. *Mol Microbiol* 80, 133-148.
- Frankenberg, L., Brugna, M., Hederstedt, L., 2002, *Enterococcus faecalis* heme-dependent catalase. *J Bacteriol* 184, 6351-6356.
- Friedman, Y.E., O'Brian, M.R., 2003, A novel DNA-binding site for the ferric uptake regulator (Fur) protein from *Bradyrhizobium japonicum*. *J Biol Chem* 278, 38395-38401.
- Fuhrmann, O., Arvand, M., Gohler, A., Schmid, M., Krull, M., Hippenstiel, S., Seybold, J., Dehio, C., Suttorp, N., 2001, *Bartonella henselae* induces NF-kappaB-dependent upregulation of adhesion molecules in cultured human endothelial cells: possible role of outer membrane proteins as pathogenic factors. *Infect Immun* 69, 5088-5097.
- Fuller, C.A., Yu, R., Irwin, S.W., Schryvers, A.B., 1998, Biochemical evidence for a conserved interaction between bacterial transferrin binding protein A and transferrin binding protein B. *Microb Pathog* 24, 75-87.
- Furrer, J.L., Sanders, D.N., Hook-Barnard, I.G., McIntosh, M.A., 2002, Export of the siderophore enterobactin in *Escherichia coli*: involvement of a 43 kDa membrane exporter. *Mol Microbiol* 44, 1225-1234.
- Gao, J.L., Nguyen, K.A., Hunter, N., 2010, Characterization of a hemophore-like protein from *Porphyromonas gingivalis*. *J Biol Chem* 285, 40028-40038.
- Garcia-Caceres, U., Garcia, F.U., 1991, Bartonellosis. An immunodepressive disease and the life of Daniel Alcides Carrion. *Am J Clin Pathol* 95, S58-66.
- Garcia, F.U., Wojta, J., Broadley, K.N., Davidson, J.M., Hoover, R.L., 1990, *Bartonella bacilliformis* stimulates endothelial cells in vitro and is angiogenic in vivo. *Am J Pathol* 136, 1125-1135.
- Gaywee, J., Radulovic, S., Higgins, J.A., Azad, A.F., 2002, Transcriptional analysis of *Rickettsia prowazekii* invasion gene homolog (invA) during host cell infection. *Infect Immun* 70, 6346-6354.
- Gilmore, R.D., Jr., Bellville, T.M., Sviat, S.L., Frace, M., 2005, The *Bartonella vinsonii* subsp. *arupensis* immunodominant surface antigen BrpA gene, encoding a 382-kilodalton protein composed of repetitive sequences, is a member of a multigene family conserved among bartonella species. *Infect Immun* 73, 3128-3136.
- Girard, V., Mourez, M., 2006, Adhesion mediated by autotransporters of Gram-negative bacteria: structural and functional features. *Res Microbiol* 157, 407-416.
- Gomez, J.A., Criado, M.T., Ferreiros, C.M., 1998, Cooperation between the components of the meningococcal transferrin receptor, TbpA and TbpB, in the uptake of transferrin iron by the 37-kDa ferric-binding protein (FbpA). *Res Microbiol* 149, 381-387.
- Goodner, B., Hinkle, G., Gattung, S., Miller, N., Blanchard, M., Quorllo, B., Goldman, B.S., Cao, Y., Askenazi, M., Halling, C., Mullin, L., Houmiel, K., Gordon, J., Vaudin, M., Iartchouk, O., Epp, A., Liu, F., Wollam, C., Allinger, M., Doughty,

- D., Scott, C., Lappas, C., Markelz, B., Flanagan, C., Crowell, C., Gurson, J., Lomo, C., Sear, C., Strub, G., Cielo, C., Slater, S., 2001, Genome sequence of the plant pathogen and biotechnology agent *Agrobacterium tumefaciens* C58. *Science* 294, 2323-2328.
- Graca-Souza, A.V., Maya-Monteiro, C., Paiva-Silva, G.O., Braz, G.R., Paes, M.C., Sorgine, M.H., Oliveira, M.F., Oliveira, P.L., 2006, Adaptations against heme toxicity in blood-feeding arthropods. *Insect Biochem Mol Biol* 36, 322-335.
- Gray, G.C., Johnson, A.A., Thornton, S.A., Smith, W.A., Knobloch, J., Kelley, P.W., Obregon Escudero, L., Arones Huayda, M., Wignall, F.S., 1990, An epidemic of Oroya fever in the Peruvian Andes. *Am J Trop Med Hyg* 42, 215-221.
- Guerra, H., 2007, The brucellae and their success as pathogens. *Crit Rev Microbiol* 33, 325-331.
- Guo, Y., Guo, G., Mao, X., Zhang, W., Xiao, J., Tong, W., Liu, T., Xiao, B., Liu, X., Feng, Y., Zou, Q., 2008, Functional identification of HugZ, a heme oxygenase from *Helicobacter pylori*. *BMC Microbiol* 8, 226.
- Guptill, L., 2010, Bartonellosis. *Vet Microbiol* 140, 347-359.
- Haley, K.P., Janson, E.M., Heilbronner, S., Foster, T.J., Skaar, E.P., 2011, *Staphylococcus lugdunensis* IsdG liberates iron from host heme. *J Bacteriol* 193, 4749-4757.
- Halliwell, B., Gutteridge, J.M., 1984, Free radicals, lipid peroxidation, and cell damage. *Lancet* 2, 1095.
- Halos, L., Jamal, T., Maillard, R., Girard, B., Guillot, J., Chomel, B., Vayssier-Taussat, M., Boulouis, H.J., 2004, Role of Hippoboscidae flies as potential vectors of *Bartonella* spp. infecting wild and domestic ruminants. *Appl Environ Microbiol* 70, 6302-6305.
- Hamza, I., Chauhan, S., Hassett, R., O'Brian, M.R., 1998, The bacterial irr protein is required for coordination of heme biosynthesis with iron availability. *J Biol Chem* 273, 21669-21674.
- Hamza, I., Qi, Z., King, N.D., O'Brian, M.R., 2000, Fur-independent regulation of iron metabolism by Irr in *Bradyrhizobium japonicum*. *Microbiology* 146 (Pt 3), 669-676.
- Hantke, K., 1981, Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. *Mol Gen Genet* 182, 288-292.
- Hantke, K., 2003, Is the bacterial ferrous iron transporter FeoB a living fossil? *Trends Microbiol* 11, 192-195.
- Harms, A., Dehio, C., 2012, Intruders below the Radar: Molecular Pathogenesis of *Bartonella* spp. *Clin Microbiol Rev* 25, 42-78.
- Helmann, J.D., 2002, The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* 46, 47-110.
- Hendrix, L.R., 2000, Contact-dependent hemolytic activity distinct from deforming activity of *Bartonella bacilliformis*. *FEMS Microbiol Lett* 182, 119-124.
- Herrer, A., 1953a, Carrion's disease. I. Studies on plants claimed to be reservoirs of *Bartonella bacilliformis*. *Am J Trop Med Hyg* 2, 637-643.
- Herrer, A., 1953b, Carrion's disease. II. Presence of *Bartonella bacilliformis* in the

- peripheral blood of patients with the benign form. *Am J Trop Med Hyg* 2, 645-649.
- Higgins, J.A., Radulovic, S., Jaworski, D.C., Azad, A.F., 1996, Acquisition of the cat scratch disease agent *Bartonella henselae* by cat fleas (Siphonaptera:Pulicidae). *J Med Entomol* 33, 490-495.
- Hill, E.M., Raji, A., Valenzuela, M.S., Garcia, F., Hoover, R., 1992, Adhesion to and invasion of cultured human cells by *Bartonella bacilliformis*. *Infect Immun* 60, 4051-4058.
- Hinnebusch, B.J., Perry, R.D., Schwan, T.G., 1996, Role of the *Yersinia pestis* hemin storage (hms) locus in the transmission of plague by fleas. *Science* 273, 367-370.
- Hirotsu, S., Chu, G.C., Unno, M., Lee, D.S., Yoshida, T., Park, S.Y., Shiro, Y., Ikeda-Saito, M., 2004, The crystal structures of the ferric and ferrous forms of the heme complex of HmuO, a heme oxygenase of *Corynebacterium diphtheriae*. *J Biol Chem* 279, 11937-11947.
- Horsburgh, M.J., Wharton, S.J., Cox, A.G., Ingham, E., Peacock, S., Foster, S.J., 2002, MntR modulates expression of the PerR regulon and superoxide resistance in *Staphylococcus aureus* through control of manganese uptake. *Mol Microbiol* 44, 1269-1286.
- Hunt, G., Rankin, A., 1915, Intermittent fever of obscure origin occurring among British soldiers in France. The so-called "trench fever". *Lancet*. 2, 1133-1136.
- Idei, A., Kawai, E., Akatsuka, H., Omori, K., 1999, Cloning and characterization of the *Pseudomonas fluorescens* ATP-binding cassette exporter, HasDEF, for the heme acquisition protein HasA. *J Bacteriol* 181, 7545-7551.
- Ihler, G.M., 1996, *Bartonella bacilliformis*: dangerous pathogen slowly emerging from deep background. *FEMS Microbiol Lett* 144, 1-11.
- Imlay, J.A., 2008, Cellular defenses against superoxide and hydrogen peroxide. *Annu Rev Biochem* 77, 755-776.
- Ismail, T.M., Hart, C.A., McLennan, A.G., 2003, Regulation of dinucleoside polyphosphate pools by the YgdP and ApaH hydrolases is essential for the ability of *Salmonella enterica* serovar typhimurium to invade cultured mammalian cells. *J Biol Chem* 278, 32602-32607.
- Iwaki-Egawa, S., Ihler, G.M., 1997, Comparison of the abilities of proteins from *Bartonella bacilliformis* and *Bartonella henselae* to deform red cell membranes and to bind to red cell ghost proteins. *FEMS Microbiol Lett* 157, 207-217.
- Izadi-Pruneyre, N., Huche, F., Lukat-Rodgers, G.S., Lecroisey, A., Gilli, R., Rodgers, K.R., Wandersman, C., Delepelaire, P., 2006, The heme transfer from the soluble HasA hemophore to its membrane-bound receptor HasR is driven by protein-protein interaction from a high to a lower affinity binding site. *J Biol Chem* 281, 25541-25550.
- Jendro, M.C., Weber, G., Brabant, T., Zeidler, H., Wollenhaupt, J., 1998, [Reactive arthritis after cat bit: a rare manifestation of cat scratch disease--case report and overview]. *Z Rheumatol* 57, 159-163.
- Jensen, W.A., Fall, M.Z., Rooney, J., Kordick, D.L., Breitschwerdt, E.B., 2000, Rapid

- identification and differentiation of *Bartonella* species using a single-step PCR assay. *J Clin Microbiol* 38, 1717-1722.
- Joh, D., Wann, E.R., Kreikemeyer, B., Speziale, P., Hook, M., 1999, Role of fibronectin-binding MSCRAMMs in bacterial adherence and entry into mammalian cells. *Matrix Biol* 18, 211-223.
- Johnston, A.W., Todd, J.D., Curson, A.R., Lei, S., Nikolaidou-Katsaridou, N., Gelfand, M.S., Rodionov, D.A., 2007, Living without Fur: the subtlety and complexity of iron-responsive gene regulation in the symbiotic bacterium *Rhizobium* and other alpha-proteobacteria. *Biometals* 20, 501-511.
- Jones, P.D., 1993, Cat scratch disease and *Rochalimaea henselae*. *Med J Aust* 159, 211.
- Kaiser, P.O., Linke, D., Schwarz, H., Leo, J.C., Kempf, V.A., 2012, Analysis of the BadA stalk from *Bartonella henselae* reveals domain-specific and domain-overlapping functions in the host cell infection process. *Cell Microbiol* 14, 198-209.
- Kaiser, P.O., Riess, T., O'Rourke, F., Linke, D., Kempf, V.A., 2011, *Bartonella* spp.: throwing light on uncommon human infections. *Int J Med Microbiol* 301, 7-15.
- Kaiser, P.O., Riess, T., Wagner, C.L., Linke, D., Lupas, A.N., Schwarz, H., Raddatz, G., Schafer, A., Kempf, V.A., 2008, The head of *Bartonella* adhesin A is crucial for host cell interaction of *Bartonella henselae*. *Cell Microbiol* 10, 2223-2234.
- Kammler, M., Schon, C., Hantke, K., 1993, Characterization of the ferrous iron uptake system of *Escherichia coli*. *J Bacteriol* 175, 6212-6219.
- Kehres, D.G., Maguire, M.E., 2003, Emerging themes in manganese transport, biochemistry and pathogenesis in bacteria. *FEMS Microbiol Rev* 27, 263-290.
- Kelly, P., Rolain, J.M., Maggi, R., Sontakke, S., Keene, B., Hunter, S., Lepidi, H., Breitschwerdt, K.T., Breitschwerdt, E.B., 2006, *Bartonella quintana* endocarditis in dogs. *Emerg Infect Dis* 12, 1869-1872.
- Kerkhoff, F.T., Bergmans, A.M., van Der Zee, A., Rothova, A., 1999, Demonstration of *Bartonella grahamii* DNA in ocular fluids of a patient with neuroretinitis. *J Clin Microbiol* 37, 4034-4038.
- Kirby, J.E., Nekorchuk, D.M., 2002, *Bartonella*-associated endothelial proliferation depends on inhibition of apoptosis. *Proceedings of the National Academy of Sciences of the United States of America* 99, 4656-4661.
- Koebnik, R., Locher, K.P., Van Gelder, P., 2000, Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* 37, 239-253.
- Koehler, J.E., Glaser, C.A., Tappero, J.W., 1994, *Rochalimaea henselae* infection. A new zoonosis with the domestic cat as reservoir. *JAMA* 271, 531-535.
- Koesling, J., Aebischer, T., Falch, C., Schulein, R., Dehio, C., 2001, Cutting edge: antibody-mediated cessation of hemotropic infection by the intraerythrocytic mouse pathogen *Bartonella grahamii*. *J Immunol* 167, 11-14.
- Kordick, D.L., Breitschwerdt, E.B., 1995, Intraerythrocytic presence of *Bartonella henselae*. *J Clin Microbiol* 33, 1655-1656.
- Kordick, D.L., Papich, M.G., Breitschwerdt, E.B., 1997, Efficacy of enrofloxacin or

- doxycycline for treatment of *Bartonella henselae* or *Bartonella clarridgeiae* infection in cats. *Antimicrob Agents Chemother* 41, 2448-2455.
- Koster, W., 2001, ABC transporter-mediated uptake of iron, siderophores, heme and vitamin B12. *Res Microbiol* 152, 291-301.
- Kostianovsky, M., Greco, M.A., 1994, Angiogenic process in bacillary angiomatosis. *Ultrastruct Pathol* 18, 349-355.
- Kostrzewski, J., 1950, [Epidemiology of trench fever]. *Med Dosw Mikrobiol* 2, 19-51.
- Krewulak, K.D., Vogel, H.J., 2008, Structural biology of bacterial iron uptake. *Biochim Biophys Acta* 1778, 1781-1804.
- Kruszewska, D., Tylewska-Wierzbanowska, S., 1996, Unknown species of rickettsiae isolated from *Ixodes ricinus* tick in Walcz. *Rocz Akad Med Bialymst* 41, 129-135.
- Kyme, P.A., Haas, A., Schaller, M., Peschel, A., Iredell, J., Kempf, V.A., 2005, Unusual trafficking pattern of *Bartonella henselae* -containing vacuoles in macrophages and endothelial cells. *Cell Microbiol* 7, 1019-1034.
- La Scola, B., Raoult, D., 1996, Serological cross-reactions between *Bartonella quintana*, *Bartonella henselae*, and *Coxiella burnetii*. *J Clin Microbiol* 34, 2270-2274.
- Lange, R., Hengge-Aronis, R., 1991, Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol* 5, 49-59.
- Lansky, I.B., Lukat-Rodgers, G.S., Block, D., Rodgers, K.R., Ratliff, M., Wilks, A., 2006, The cytoplasmic heme-binding protein (PhuS) from the heme uptake system of *Pseudomonas aeruginosa* is an intracellular heme-trafficking protein to the delta-regioselective heme oxygenase. *J Biol Chem* 281, 13652-13662.
- Lascelles, A.K., 1962, The absorption of serum albumin and casein from the mammary gland of the merino ewe. *Q J Exp Physiol Cogn Med Sci* 47, 48-56.
- Lee, B.C., 1992, Isolation of an outer membrane hemin-binding protein of *Haemophilus influenzae* type b. *Infect Immun* 60, 810-816.
- Lee, B.C., 1994, Isolation and characterization of the haemin-binding proteins from *Neisseria meningitidis*. *Microbiology* 140 (Pt 6), 1473-1480.
- Lee, B.C., Levesque, S., 1997, A monoclonal antibody directed against the 97-kilodalton gonococcal hemin-binding protein inhibits hemin utilization by *Neisseria gonorrhoeae*. *Infect Immun* 65, 2970-2974.
- Legrain, M., Mazarin, V., Irwin, S.W., Bouchon, B., Quentin-Millet, M.J., Jacobs, E., Schryvers, A.B., 1993, Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. *Gene* 130, 73-80.
- Letoffe, S., Deniau, C., Wolff, N., Dassa, E., Delepelaire, P., Lecroisey, A., Wandersman, C., 2001, Haemophore-mediated bacterial haem transport: evidence for a common or overlapping site for haem-free and haem-loaded haemophore on its specific outer membrane receptor. *Mol Microbiol* 41, 439-450.
- Letoffe, S., Ghigo, J.M., Wandersman, C., 1994a, Iron acquisition from heme and

- hemoglobin by a *Serratia marcescens* extracellular protein. *Proc Natl Acad Sci U S A* 91, 9876-9880.
- Letoffe, S., Ghigo, J.M., Wandersman, C., 1994b, Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. *J Bacteriol* 176, 5372-5377.
- Letoffe, S., Heuck, G., Delepelaire, P., Lange, N., Wandersman, C., 2009, Bacteria capture iron from heme by keeping tetrapyrrol skeleton intact. *Proc Natl Acad Sci U S A* 106, 11719-11724.
- Letoffe, S., Redeker, V., Wandersman, C., 1998, Isolation and characterization of an extracellular haem-binding protein from *Pseudomonas aeruginosa* that shares function and sequence similarities with the *Serratia marcescens* HasA haemophore. *Mol Microbiol* 28, 1223-1234.
- Li, D.M., Liu, Q.Y., Zhao, F., Hu, Y., Xiao, D., Gu, Y.X., Song, X.P., Zhang, J.Z., 2011, Proteomic and bioinformatic analysis of outer membrane proteins of the protobacterium *Bartonella henselae* (Bartonellaceae). *Genet Mol Res* 10, 1789-1818.
- Linke, D., Riess, T., Autenrieth, I.B., Lupas, A., Kempf, V.A., 2006, Trimeric autotransporter adhesins: variable structure, common function. *Trends Microbiol* 14, 264-270.
- Litwin, C.M., Boyko, S.A., Calderwood, S.B., 1992, Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae* fur gene. *J Bacteriol* 174, 1897-1903.
- Liu, X., Olczak, T., Guo, H.C., Dixon, D.W., Genco, C.A., 2006, Identification of amino acid residues involved in heme binding and hemoprotein utilization in the *Porphyromonas gingivalis* heme receptor HmuR. *Infect Immun* 74, 1222-1232.
- Locher, K.P., Rees, B., Koebnik, R., Mitschler, A., Moulinier, L., Rosenbusch, J.P., Moras, D., 1998, Transmembrane signaling across the ligand-gated FhuA receptor: crystal structures of free and ferrichrome-bound states reveal allosteric changes. *Cell* 95, 771-778.
- Lucey, D., Dolan, M.J., Moss, C.W., Garcia, M., Hollis, D.G., Wegner, S., Morgan, G., Almeida, R., Leong, D., Greisen, K.S., et al., 1992, Relapsing illness due to *Rochalimaea henselae* in immunocompetent hosts: implication for therapy and new epidemiological associations. *Clin Infect Dis* 14, 683-688.
- MacKichan, J.K., Gerns, H.L., Chen, Y.T., Zhang, P., Koehler, J.E., 2008, A SacB mutagenesis strategy reveals that the *Bartonella quintana* variably expressed outer membrane proteins are required for bloodstream infection of the host. *Infect Immun* 76, 788-795.
- Maggi, R.G., Duncan, A.W., Breitschwerdt, E.B., 2005, Novel chemically modified liquid medium that will support the growth of seven bartonella species. *J Clin Microbiol* 43, 2651-2655.
- Maguina, C., Guerra, H., Ventosilla, P., 2009, Bartonellosis. *Clin Dermatol* 27, 271-280.
- Manders, S.M., 1996, Bacillary angiomatosis. *Clin Dermatol* 14, 295-299.
- Mandle, T., Einsele, H., Schaller, M., Neumann, D., Vogel, W., Autenrieth, I.B.,

- Kempf, V.A., 2005, Infection of human CD34+ progenitor cells with *Bartonella henselae* results in intraerythrocytic presence of *B. henselae*. *Blood* 106, 1215-1222.
- Marignac, G., Barrat, F., Chomel, B., Vayssier-Taussat, M., Gandoin, C., Bouillin, C., Boulouis, H.J., 2010, Murine model for *Bartonella birtlesii* infection: New aspects. *Comp Immunol Microbiol Infect Dis* 33, 95-107.
- Martinez, M., Ugalde, R.A., Almiron, M., 2005, Dimeric *Brucella abortus* Irr protein controls its own expression and binds haem. *Microbiology* 151, 3427-3433.
- Matzanke, B.F., Anemuller, S., Schunemann, V., Trautwein, A.X., Hantke, K., 2004, FhuF, part of a siderophore-reductase system. *Biochemistry* 43, 1386-1392.
- Maurin, M., Eb, F., Etienne, J., Raoult, D., 1997, Serological cross-reactions between *Bartonella* and *Chlamydia* species: implications for diagnosis. *J Clin Microbiol* 35, 2283-2287.
- Maurin, M., Raoult, D., 1996, *Bartonella* (Rochalimaea) *quintana* infections. *Clin Microbiol Rev* 9, 273-292.
- Mavris, M., Saenz, H., Monteil, M., Boulouis, H.J., Dehio, C., Vayssier-Taussat, M., 2005, Characterization of Genes Involved in Long-Term Bacteremia in Mice by *Bartonella birtlesii*. *Ann N Y Acad Sci* 1063, 312-314.
- Mernaugh, G., Ihler, G.M., 1992, Deformation factor: an extracellular protein synthesized by *Bartonella bacilliformis* that deforms erythrocyte membranes. *Infect Immun* 60, 937-943.
- Messner, K.R., Imlay, J.A., 1999, The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. *J Biol Chem* 274, 10119-10128.
- Miller, V.L., Falkow, S., 1988, Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. *Infect Immun* 56, 1242-1248.
- Minnick, M.F., Battisti, J.M., 2009, Pestilence, persistence and pathogenicity: infection strategies of *Bartonella*. *Future Microbiol* 4, 743-758.
- Minnick, M.F., Sappington, K.N., Smitherman, L.S., Andersson, S.G., Karlberg, O., Carroll, J.A., 2003, Five-member gene family of *Bartonella quintana*. *Infect Immun* 71, 814-821.
- Mitchell, S.J., Minnick, M.F., 1995, Characterization of a two-gene locus from *Bartonella bacilliformis* associated with the ability to invade human erythrocytes. *Infect Immun* 63, 1552-1562.
- Mitchell, S.J., Minnick, M.F., 1997, A carboxy-terminal processing protease gene is located immediately upstream of the invasion-associated locus from *Bartonella bacilliformis*. *Microbiology* 143 (Pt 4), 1221-1233.
- Moeck, G.S., Coulton, J.W., 1998, TonB-dependent iron acquisition: mechanisms of siderophore-mediated active transport. *Mol Microbiol* 28, 675-681.
- Morera, D., Mackenzie, S.A., 2011, Is there a direct role for erythrocytes in the immune response? *Vet Res* 42, 89.
- Morick, D., Krasnov, B.R., Khokhlova, I.S., Shenbrot, G.I., Kosoy, M.Y., Harrus, S., 2010, *Bartonella* genotypes in fleas (insecta: siphonaptera) collected from rodents in the negev desert, Israel. *Appl Environ Microbiol* 76, 6864-6869.

- Myers, W.F., Cutler, L.D., Wisseman, C.L., Jr., 1969, Role of erythrocytes and serum in the nutrition of *Rickettsia quintana*. *J Bacteriol* 97, 663-666.
- Myers, W.F., Osterman, J.V., Wisseman, C.L., Jr., 1972, Nutritional studies of *Rickettsia quintana*: nature of the hematin requirement. *J Bacteriol* 109, 89-95.
- Niederhoffer, E.C., Naranjo, C.M., Bradley, K.L., Fee, J.A., 1990, Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus. *J Bacteriol* 172, 1930-1938.
- Nijssen, E., Cescau, S., Vayssier-Taussat, M., Wang, J., Biville, F., 2009, Identification of mechanisms involved in iron and haem uptake in *Bartonella birtlesii*: in silico and in vivo approaches. *Clin Microbiol Infect* 15 Suppl 2, 118-119.
- Noguchi, H., 1926, Etiology of Oroya Fever : V. The Experimental Transmission of *Bartonella Bacilliformis* by Ticks (*Dermacentor Andersoni*). *J Exp Med* 44, 729-734.
- Noguchi, H., Shannon, R.C., Tilden, E.B., Tyler, J.R., 1929, Etiology of Oroya Fever : Xiv. The Insect Vectors of Carrion's Disease. *J Exp Med* 49, 993-1008.
- O'Brian, M.R., 1996, Heme synthesis in the rhizobium-legume symbiosis: a palette for bacterial and eukaryotic pigments. *J Bacteriol* 178, 2471-2478.
- O'Callaghan, D., Cazevielle, C., Allardet-Servent, A., Boschioli, M.L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y., Ramuz, M., 1999, A homologue of the *Agrobacterium tumefaciens* *VirB* and *Bordetella pertussis* *Ptl* type IV secretion systems is essential for intracellular survival of *Brucella suis*. *Mol Microbiol* 33, 1210-1220.
- O'Rourke, F., Schmidgen, T., Kaiser, P.O., Linke, D., Kempf, V.A., 2011, Adhesins of *Bartonella* spp. *Adv Exp Med Biol* 715, 51-70.
- O'Rourke, L.G., Pitulle, C., Hegarty, B.C., Kraycirik, S., Killary, K.A., Grosenstein, P., Brown, J.W., Breitschwerdt, E.B., 2005, *Bartonella quintana* in cynomolgus monkey (*Macaca fascicularis*). *Emerg Infect Dis* 11, 1931-1934.
- Olczak, T., Siudeja, K., Olczak, M., 2006, Purification and initial characterization of a novel *Porphyromonas gingivalis* HmuY protein expressed in *Escherichia coli* and insect cells. *Protein Expr Purif* 49, 299-306.
- Olczak, T., Sroka, A., Potempa, J., Olczak, M., 2008, *Porphyromonas gingivalis* HmuY and HmuR: further characterization of a novel mechanism of heme utilization. *Arch Microbiol* 189, 197-210.
- Oliveira, M.F., Silva, J.R., Dansa-Petretski, M., de Souza, W., Lins, U., Braga, C.M., Masuda, H., Oliveira, P.L., 1999, Haem detoxification by an insect. *Nature* 400, 517-518.
- Otto, B.R., Verweij-van Vught, A.M., MacLaren, D.M., 1992, Transferrins and heme-compounds as iron sources for pathogenic bacteria. *Crit Rev Microbiol* 18, 217-233.
- Padmalayam, I., Kareem, K., Baumstark, B., Massung, R., 2000, The gene encoding the 17-kDa antigen of *Bartonella henselae* is located within a cluster of genes homologous to the *virB* virulence operon. *DNA Cell Biol* 19, 377-382.
- Park, S.Y., Kelminson, K.L., Lee, A.K., Zhang, P., Warner, R.E., Rehkopf, D.H.,

- Calderwood, S.B., Koehler, J.E., 2001, Identification, characterization, and functional analysis of a gene encoding the ferric uptake regulation protein in *Bartonella* species. *J Bacteriol* 183, 5751-5755.
- Parrow, N.L., Abbott, J., Lockwood, A.R., Battisti, J.M., Minnick, M.F., 2009, Function, regulation, and transcriptional organization of the hemin utilization locus of *Bartonella quintana*. *Infect Immun* 77, 307-316.
- Patel, R., Newell, J.O., Procop, G.W., Persing, D.H., 1999, Use of polymerase chain reaction for citrate synthase gene to diagnose *Bartonella quintana* endocarditis. *Am J Clin Pathol* 112, 36-40.
- Perry, R.D., Mier, I., Jr., Fetherston, J.D., 2007, Roles of the Yfe and Feo transporters of *Yersinia pestis* in iron uptake and intracellular growth. *Biometals* 20, 699-703.
- Pitassi, L.H., Magalhaes, R.F., Barjas-Castro, M.L., de Paula, E.V., Ferreira, M.R., Velho, P.E., 2007, *Bartonella henselae* infects human erythrocytes. *Ultrastruct Pathol* 31, 369-372.
- Postle, K., 1993, TonB protein and energy transduction between membranes. *J Bioenerg Biomembr* 25, 591-601.
- Pulliainen, A.T., Dehio, C., 2009, *Bartonella henselae*: subversion of vascular endothelial cell functions by translocated bacterial effector proteins. *Int J Biochem Cell Biol* 41, 507-510.
- Pulliainen, A.T., Dehio, C., 2012, Persistence of *Bartonella* spp. stealth pathogens: from subclinical infections to vasoproliferative tumor formation. *FEMS Microbiol Rev* 36, 563-599.
- Puri, S., O'Brian, M.R., 2006, The hmuQ and hmuD genes from *Bradyrhizobium japonicum* encode heme-degrading enzymes. *J Bacteriol* 188, 6476-6482.
- Quebatte, M., Dehio, M., Tropel, D., Basler, A., Toller, I., Raddatz, G., Engel, P., Huser, S., Schein, H., Lindroos, H.L., Andersson, S.G., Dehio, C., 2010, The BatR/BatS two-component regulatory system controls the adaptive response of *Bartonella henselae* during human endothelial cell infection. *J Bacteriol* 192, 3352-3367.
- Raivio, T.L., Silhavy, T.J., 2001, Periplasmic stress and ECF sigma factors. *Annu Rev Microbiol* 55, 591-624.
- Raoult, D., Roux, V., 1999, The body louse as a vector of reemerging human diseases. *Clin Infect Dis* 29, 888-911.
- Ratliff, M., Zhu, W., Deshmukh, R., Wilks, A., Stojiljkovic, I., 2001, Homologues of neisserial heme oxygenase in gram-negative bacteria: degradation of heme by the product of the pigA gene of *Pseudomonas aeruginosa*. *J Bacteriol* 183, 6394-6403.
- Regnery, R.L., Olson, J.G., Perkins, B.A., Bibb, W., 1992, Serological response to "Rochalimaea henselae" antigen in suspected cat-scratch disease. *Lancet* 339, 1443-1445.
- Regnery, R.L., Rooney, J.A., Johnson, A.M., Nesby, S.L., Manzewitsch, P., Beaver, K., Olson, J.G., 1996, Experimentally induced *Bartonella henselae* infections followed by challenge exposure and antimicrobial therapy in cats. *Am J Vet*

Res 57, 1714-1719.

- Reniere, M.L., Torres, V.J., Skaar, E.P., 2007, Intracellular metalloporphyrin metabolism in *Staphylococcus aureus*. *Biometals* 20, 333-345.
- Reniere, M.L., Ukpabi, G.N., Harry, S.R., Stec, D.F., Krull, R., Wright, D.W., Bachmann, B.O., Murphy, M.E., Skaar, E.P., 2010, The IsdG-family of haem oxygenases degrades haem to a novel chromophore. *Mol Microbiol* 75, 1529-1538.
- Reynafarje, C., Ramos, J., 1961, The hemolytic anemia of human bartonellosis. *Blood* 17, 562-578.
- Rhomberg, T.A., Karlberg, O., Mini, T., Zimny-Arndt, U., Wickenberg, U., Rottgen, M., Jungblut, P.R., Jenö, P., Andersson, S.G., Dehio, C., 2004, Proteomic analysis of the sarcosine-insoluble outer membrane fraction of the bacterial pathogen *Bartonella henselae*. *Proteomics* 4, 3021-3033.
- Rhomberg, T.A., Truttmann, M.C., Guye, P., Ellner, Y., Dehio, C., 2009, A translocated protein of *Bartonella henselae* interferes with endocytic uptake of individual bacteria and triggers uptake of large bacterial aggregates via the invasome. *Cell Microbiol* 11, 927-945.
- Riess, T., Andersson, S.G., Lupas, A., Schaller, M., Schafer, A., Kyme, P., Martin, J., Walzlein, J.H., Eehalt, U., Lindroos, H., Schirle, M., Nordheim, A., Autenrieth, I.B., Kempf, V.A., 2004, *Bartonella* adhesin A mediates a proangiogenic host cell response. *J Exp Med* 200, 1267-1278.
- Riess, T., Dietrich, F., Schmidt, K.V., Kaiser, P.O., Schwarz, H., Schafer, A., Kempf, V.A., 2008, Analysis of a novel insect cell culture medium-based growth medium for *Bartonella* species. *Appl Environ Microbiol* 74, 5224-5227.
- Riess, T., Raddatz, G., Linke, D., Schafer, A., Kempf, V.A., 2007, Analysis of *Bartonella* adhesin A expression reveals differences between various *B. henselae* strains. *Infect Immun* 75, 35-43.
- Robey, M., Cianciotto, N.P., 2002, *Legionella pneumophila* feoAB promotes ferrous iron uptake and intracellular infection. *Infect Immun* 70, 5659-5669.
- Roden, J.A., Wells, D.H., Chomel, B.B., Kasten, R.W., Koehler, J.E., 2012, Hemin binding protein C is found in outer membrane vesicles and protects *Bartonella henselae* against toxic concentrations of hemin. *Infect Immun*.
- Rolain, J.M., Arnoux, D., Parzy, D., Sampil, J., Raoult, D., 2003a, Experimental infection of human erythrocytes from alcoholic patients with *Bartonella quintana*. *Ann N Y Acad Sci* 990, 605-611.
- Rolain, J.M., Foucault, C., Guieu, R., La Scola, B., Brouqui, P., Raoult, D., 2002, *Bartonella quintana* in human erythrocytes. *Lancet* 360, 226-228.
- Rolain, J.M., Franc, M., Davoust, B., Raoult, D., 2003b, Molecular detection of *Bartonella quintana*, *B. koehlerae*, *B. henselae*, *B. clarridgeiae*, *Rickettsia felis*, and *Wolbachia pipientis* in cat fleas, France. *Emerg Infect Dis* 9, 338-342.
- Rossi, M.S., Fetherston, J.D., Letoffe, S., Carniel, E., Perry, R.D., Ghigo, J.M., 2001, Identification and characterization of the hemophore-dependent heme acquisition system of *Yersinia pestis*. *Infect Immun* 69, 6707-6717.
- Rudolph, G., Hennecke, H., Fischer, H.M., 2006, Beyond the Fur paradigm:

- iron-controlled gene expression in rhizobia. *FEMS Microbiol Rev* 30, 631-648.
- Rydkina, E., Sahni, A., Silverman, D.J., Sahni, S.K., 2002, *Rickettsia rickettsii* infection of cultured human endothelial cells induces heme oxygenase 1 expression. *Infect Immun* 70, 4045-4052.
- Rydkina, E., Turpin, L.C., Sahni, S.K., 2010, *Rickettsia rickettsii* infection of human macrovascular and microvascular endothelial cells reveals activation of both common and cell type-specific host response mechanisms. *Infect Immun* 78, 2599-2606.
- Rydkina, E.B., Roux, V., Gagua, E.M., Predtechenski, A.B., Tarasevich, I.V., Raoult, D., 1999, *Bartonella quintana* in body lice collected from homeless persons in Russia. *Emerg Infect Dis* 5, 176-178.
- Sabri, M., Leveille, S., Dozois, C.M., 2006, A SitABCD homologue from an avian pathogenic *Escherichia coli* strain mediates transport of iron and manganese and resistance to hydrogen peroxide. *Microbiology* 152, 745-758.
- Sadarangani, M., Pollard, A.J., Gray-Owen, S.D., 2011, Opa proteins and CEACAMs: pathways of immune engagement for pathogenic *Neisseria*. *FEMS Microbiol Rev* 35, 498-514.
- Saenz, H.L., Engel, P., Stoeckli, M.C., Lanz, C., Raddatz, G., Vayssier-Taussat, M., Birtles, R., Schuster, S.C., Dehio, C., 2007, Genomic analysis of *Bartonella* identifies type IV secretion systems as host adaptability factors. *Nat Genet* 39, 1469-1476.
- Saisongkroh, W., Kowalczywska, M., Azza, S., Decloquement, P., Rolain, J.M., Raoult, D., 2010, Identification of candidate proteins for the diagnosis of *Bartonella henselae* infections using an immunoproteomic approach. *FEMS Microbiol Lett* 310, 158-167.
- Sander, A., Kaliebe, T., Bredt, W., 1996, [*Bartonella* (*Rochalimaea*) infections: cat-scratch disease and bacillary angiomatosis]. *Dtsch Med Wochenschr* 121, 65-69.
- Sander, A., Kretzer, S., Bredt, W., Oberle, K., Bereswill, S., 2000a, Hemin-dependent growth and hemin binding of *Bartonella henselae*. *FEMS Microbiol Lett* 189, 55-59.
- Sander, A., Kretzer, S., Bredt, W., Oberle, K., Bereswill, S., 2000b, Hemin-dependent growth and hemin binding of *Bartonella henselae*. *FEMS Microbiol Lett* 189, 55-59.
- Sasaki, T., Kobayashi, M., Agui, N., 2002, Detection of *Bartonella quintana* from body lice (*Anoplura*: *Pediculidae*) infesting homeless people in Tokyo by molecular technique. *J Med Entomol* 39, 427-429.
- Scheidegger, F., Ellner, Y., Guye, P., Rhomberg, T.A., Weber, H., Augustin, H.G., Dehio, C., 2009, Distinct activities of *Bartonella henselae* type IV secretion effector proteins modulate capillary-like sprout formation. *Cell Microbiol* 11, 1088-1101.
- Scherer, D.C., DeBuron-Connors, I., Minnick, M.F., 1993, Characterization of *Bartonella bacilliformis* flagella and effect of anti-flagellin antibodies on

- invasion of human erythrocytes. *Infect Immun* 61, 4962-4971.
- Schmid, M.C., Scheidegger, F., Dehio, M., Balmelle-Devaux, N., Schulein, R., Guye, P., Chennakesava, C.S., Biedermann, B., Dehio, C., 2006, A translocated bacterial protein protects vascular endothelial cells from apoptosis. *PLoS Pathog* 2, e115.
- Schmiederer, M., Anderson, B., 2000, Cloning, sequencing, and expression of three *Bartonella henselae* genes homologous to the *Agrobacterium tumefaciens* VirB region. *DNA Cell Biol* 19, 141-147.
- Schneider, S., Sharp, K.H., Barker, P.D., Paoli, M., 2006, An induced fit conformational change underlies the binding mechanism of the heme transport proteobacteria-protein HemS. *J Biol Chem* 281, 32606-32610.
- Schroder, G., Dehio, C., 2005, Virulence-associated type IV secretion systems of *Bartonella*. *Trends Microbiol* 13, 336-342.
- Schroder, G., Schulein, R., Quebatte, M., Dehio, C., 2011, Conjugative DNA transfer into human cells by the VirB/VirD4 type IV secretion system of the bacterial pathogen *Bartonella henselae*. *Proc Natl Acad Sci U S A* 108, 14643-14648.
- Schryvers, A.B., Bonnah, R., Yu, R.H., Wong, H., Retzer, M., 1998, Bacterial lactoferrin receptors. *Adv Exp Med Biol* 443, 123-133.
- Schulein, R., Dehio, C., 2002, The VirB/VirD4 type IV secretion system of *Bartonella* is essential for establishing intraerythrocytic infection. *Mol Microbiol* 46, 1053-1067.
- Schulein, R., Guye, P., Rhomberg, T.A., Schmid, M.C., Schroder, G., Vergunst, A.C., Carena, I., Dehio, C., 2005, A bipartite signal mediates the transfer of type IV secretion substrates of *Bartonella henselae* into human cells. *Proc Natl Acad Sci U S A* 102, 856-861.
- Schulein, R., Seubert, A., Gille, C., Lanz, C., Hansmann, Y., Piemont, Y., Dehio, C., 2001, Invasion and persistent intracellular colonization of erythrocytes. A unique parasitic strategy of the emerging pathogen *Bartonella*. *J Exp Med* 193, 1077-1086.
- Schulte, B., Linke, D., Klumpp, S., Schaller, M., Riess, T., Autenrieth, I.B., Kempf, V.A., 2006, *Bartonella quintana* variably expressed outer membrane proteins mediate vascular endothelial growth factor secretion but not host cell adherence. *Infect Immun* 74, 5003-5013.
- Schultz, M.G., 1968, A history of bartonellosis (Carrion's disease). *Am J Trop Med Hyg* 17, 503-515.
- Schwartzman, W.A., Nesbit, C.A., Baron, E.J., 1993, Development and evaluation of a blood-free medium for determining growth curves and optimizing growth of *Rochalimaea henselae*. *J Clin Microbiol* 31, 1882-1885.
- Seki, N., Kasai, S., Saito, N., Komagata, O., Mihara, M., Sasaki, T., Tomita, T., Kobayashi, M., 2007, Quantitative analysis of proliferation and excretion of *Bartonella quintana* in body lice, *Pediculus humanus* L. *Am J Trop Med Hyg* 77, 562-566.
- Seubert, A., Hiestand, R., de la Cruz, F., Dehio, C., 2003, A bacterial conjugation machinery recruited for pathogenesis. *Mol Microbiol* 49, 1253-1266.

- Seubert, A., Schulein, R., Dehio, C., 2002, Bacterial persistence within erythrocytes: a unique pathogenic strategy of *Bartonella* spp. *Int J Med Microbiol* 291, 555-560.
- Singleton, C., White, G.F., Todd, J.D., Marritt, S.J., Cheesman, M.R., Johnston, A.W., Le Brun, N.E., 2010, Heme-responsive DNA binding by the global iron regulator Irr from *Rhizobium leguminosarum*. *J Biol Chem* 285, 16023-16031.
- Skaar, E.P., Gaspar, A.H., Schneewind, O., 2004, IsdG and IsdI, heme-degrading enzymes in the cytoplasm of *Staphylococcus aureus*. *J Biol Chem* 279, 436-443.
- Skaar, E.P., Gaspar, A.H., Schneewind, O., 2006, *Bacillus anthracis* IsdG, a heme-degrading monooxygenase. *J Bacteriol* 188, 1071-1080.
- Smalley, J.W., Birss, A.J., Silver, J., 2000, The periodontal pathogen *Porphyromonas gingivalis* harnesses the chemistry of the mu-oxo bis-haem of iron protoporphyrin IX to protect against hydrogen peroxide. *FEMS Microbiol Lett* 183, 159-164.
- Smalley, J.W., Silver, J., Marsh, P.J., Birss, A.J., 1998, The periodontopathogen *Porphyromonas gingivalis* binds iron protoporphyrin IX in the mu-oxo dimeric form: an oxidative buffer and possible pathogenic mechanism. *Biochem J* 331 (Pt 3), 681-685.
- Spach, D.H., Kanter, A.S., Dougherty, M.J., Larson, A.M., Coyle, M.B., Brenner, D.J., Swaminathan, B., Matar, G.M., Welch, D.F., Root, R.K., et al., 1995, *Bartonella* (*Rochalimaea*) *quintana* bacteremia in inner-city patients with chronic alcoholism [see comments]. *N Engl J Med* 332, 424-428.
- Spach, D.H., Koehler, J.E., 1998, *Bartonella*-associated infections. *Infect Dis Clin North Am* 12, 137-155.
- Stacey, G., Libault, M., Brechenmacher, L., Wan, J., May, G.D., 2006, Genetics and functional genomics of legume nodulation. *Curr Opin Plant Biol* 9, 110-121.
- Staggs, T.M., Perry, R.D., 1991, Identification and cloning of a fur regulatory gene in *Yersinia pestis*. *J Bacteriol* 173, 417-425.
- Stintzi, A., Raymond, K.N., 2000, Amonabactin-mediated iron acquisition from transferrin and lactoferrin by *Aeromonas hydrophila*: direct measurement of individual microscopic rate constants. *J Biol Inorg Chem* 5, 57-66.
- Stojiljkovic, I., Cobeljic, M., Hantke, K., 1993, *Escherichia coli* K-12 ferrous iron uptake mutants are impaired in their ability to colonize the mouse intestine. *FEMS Microbiol Lett* 108, 111-115.
- Stojiljkovic, I., Hantke, K., 1994, Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersinia enterocolitica*. *Mol Microbiol* 13, 719-732.
- Stojiljkovic, I., Hantke, K., 1995, Functional domains of the *Escherichia coli* ferric uptake regulator protein (Fur). *Mol Gen Genet* 247, 199-205.
- Storz, G., Imlay, J.A., 1999, Oxidative stress. *Curr Opin Microbiol* 2, 188-194.
- Suits, M.D., Pal, G.P., Nakatsu, K., Matte, A., Cygler, M., Jia, Z., 2005, Identification of an *Escherichia coli* O157:H7 heme oxygenase with tandem functional repeats. *Proc Natl Acad Sci U S A* 102, 16955-16960.

- Sweger, D., Resto-Ruiz, S., Johnson, D.P., Schmiederer, M., Hawke, N., Anderson, B., 2000, Conservation of the 17-kilodalton antigen gene within the genus *Bartonella*. *Clin Diagn Lab Immunol* 7, 251-257.
- Taye, A., Chen, H., Duncan, K., Zhang, Z., Hendrix, L., Gonzalez, J., Ching, W., 2005, Production of recombinant protein Pap31 and its application for the diagnosis of *Bartonella bacilliformis* infection. *Ann N Y Acad Sci* 1063, 280-285.
- Telford, S.R., 3rd, Wormser, G.P., 2010, *Bartonella* spp. transmission by ticks not established. *Emerg Infect Dis* 16, 379-384.
- Thompson, J.M., Jones, H.A., Perry, R.D., 1999, Molecular characterization of the hemin uptake locus (*hmu*) from *Yersinia pestis* and analysis of *hmu* mutants for hemin and hemoprotein utilization. *Infect Immun* 67, 3879-3892.
- Todd, J.D., Sawers, G., Johnston, A.W., 2005, Proteomic analysis reveals the wide-ranging effects of the novel, iron-responsive regulator *RirA* in *Rhizobium leguminosarum* bv. *viciae*. *Mol Genet Genomics* 273, 197-206.
- Tong, Y., Guo, M., 2009, Bacterial heme-transport proteins and their heme-coordination modes. *Arch Biochem Biophys* 481, 1-15.
- Touati, D., 2000, Iron and oxidative stress in bacteria. *Arch Biochem Biophys* 373, 1-6.
- Townsend, C.H., 1913, *A Phlebotomus* the Practically Certain Carrier of *Verruga*. *Science* 38, 194-195.
- Truttmann, M.C., Rhomberg, T.A., Dehio, C., 2011, Combined action of the type IV secretion effector proteins *BepC* and *BepF* promotes invasome formation of *Bartonella henselae* on endothelial and epithelial cells. *Cell Microbiol* 13, 284-299.
- Tsai, Y.L., Chang, C.C., Chuang, S.T., Chomel, B.B., 2011a, *Bartonella* species and their ectoparasites: selective host adaptation or strain selection between the vector and the mammalian host? *Comp Immunol Microbiol Infect Dis* 34, 299-314.
- Tsai, Y.L., Lin, C.C., Chomel, B.B., Chuang, S.T., Tsai, K.H., Wu, W.J., Huang, C.G., Yu, J.C., Sung, M.H., Kass, P.H., Chang, C.C., 2011b, *Bartonella* infection in shelter cats and dogs and their ectoparasites. *Vector Borne Zoonotic Dis* 11, 1023-1030.
- Tsaneva, I.R., Weiss, B., 1990, *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. *J Bacteriol* 172, 4197-4205.
- Tsolis, R.M., Baumber, A.J., Heffron, F., Stojiljkovic, I., 1996, Contribution of *TonB*- and *Fer*-mediated iron uptake to growth of *Salmonella typhimurium* in the mouse. *Infect Immun* 64, 4549-4556.
- Unkmeir, A., Latsch, K., Dietrich, G., Wintermeyer, E., Schinke, B., Schwender, S., Kim, K.S., Eigenthaler, M., Frosch, M., 2002, Fibronectin mediates *Opd*-dependent internalization of *Neisseria meningitidis* in human brain microvascular endothelial cells. *Mol Microbiol* 46, 933-946.
- van Putten, J.P., Duensing, T.D., Cole, R.L., 1998, Entry of *OpaA*⁺ gonococci into HEP-2 cells requires concerted action of glycosaminoglycans, fibronectin and integrin receptors. *Mol Microbiol* 29, 369-379.

- Varela, G., Fournier, R., Mooser, H., 1954, [Presence of *Rickettsia quintana* in *Pediculus humanus* from Mexico City; experimental inoculation]. *Rev Inst Salubr Enferm Trop* 14, 39-42.
- Vaughan, J.A., Azad, A.F., 1993, Patterns of erythrocyte digestion by bloodsucking insects: constraints on vector competence. *J Med Entomol* 30, 214-216.
- Vayssier-Taussat, M., Le Rhun, D., Deng, H.K., Biville, F., Cescau, S., Danchin, A., Marignac, G., Lenaour, E., Boulouis, H.J., Mavris, M., Arnaud, L., Yang, H., Wang, J., Quebatte, M., Engel, P., Saenz, H., Dehio, C., 2010, The Trw type IV secretion system of *Bartonella* mediates host-specific adhesion to erythrocytes. *PLoS Pathog* 6, e1000946.
- Velayudhan, J., Hughes, N.J., McColm, A.A., Bagshaw, J., Clayton, C.L., Andrews, S.C., Kelly, D.J., 2000, Iron acquisition and virulence in *Helicobacter pylori*: a major role for FeoB, a high-affinity ferrous iron transporter. *Mol Microbiol* 37, 274-286.
- Vigil, A., Ortega, R., Jain, A., Nakajima-Sasaki, R., Tan, X., Chomel, B.B., Kasten, R.W., Koehler, J.E., Felgner, P.L., 2010, Identification of the feline humoral immune response to *Bartonella henselae* infection by protein microarray. *PLoS One* 5, e11447.
- Vinson, J.W., 1966, In vitro cultivation of the rickettsial agent of trench fever. *Bull World Health Organ* 35, 155-164.
- Vinson, J.W., Varela, G., Molina-Pasquel, C., 1969, Trench fever. 3. Induction of clinical disease in volunteers inoculated with *Rickettsia quintana* propagated on blood agar. *Am J Trop Med Hyg* 18, 713-722.
- Vizcaino, N., Cloeckert, A., Zygmunt, M.S., Dubray, G., 1996, Cloning, nucleotide sequence, and expression of the *Brucella melitensis* omp31 gene coding for an immunogenic major outer membrane protein. *Infect Immun* 64, 3744-3751.
- Wagner, C.L., Riess, T., Linke, D., Eberhardt, C., Schafer, A., Reutter, S., Maggi, R.G., Kempf, V.A., 2008, Use of *Bartonella* adhesin A (BadA) immunoblotting in the serodiagnosis of *Bartonella henselae* infections. *Int J Med Microbiol* 298, 579-590.
- Walker, T.S., Winkler, H.H., 1981, *Bartonella bacilliformis*: colonial types and erythrocyte adherence. *Infect Immun* 31, 480-486.
- Wandersman, C., Delepelaire, P., 1990, TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proc Natl Acad Sci U S A* 87, 4776-4780.
- Wandersman, C., Delepelaire, P., 2004, Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol* 58, 611-647.
- Wandersman, C., Delepelaire, P., 2012, Haemophore functions revisited. *Mol Microbiol*.
- Watson, R.J., Millichap, P., Joyce, S.A., Reynolds, S., Clarke, D.J., 2010, The role of iron uptake in pathogenicity and symbiosis in *Photobacterium luminescens* TT01. *BMC Microbiol* 10, 177.
- Weel, J.F., Hopman, C.T., van Putten, J.P., 1991, In situ expression and localization of *Neisseria gonorrhoeae* opacity proteins in infected epithelial cells: apparent

- role of Opa proteins in cellular invasion. *J Exp Med* 173, 1395-1405.
- Wegele, R., Tasler, R., Zeng, Y., Rivera, M., Frankenberg-Dinkel, N., 2004, The heme oxygenase(s)-phytochrome system of *Pseudomonas aeruginosa*. *J Biol Chem* 279, 45791-45802.
- Wexler, M., Todd, J.D., Kolade, O., Bellini, D., Hemmings, A.M., Sawers, G., Johnston, A.W., 2003, Fur is not the global regulator of iron uptake genes in *Rhizobium leguminosarum*. *Microbiology* 149, 1357-1365.
- White, D.C., Granick, S., 1963, Hemin Biosynthesis in *Haemophilus*. *J Bacteriol* 85, 842-850.
- Wilks, A., 2001, The ShuS protein of *Shigella dysenteriae* is a heme-sequestering protein that also binds DNA. *Arch Biochem Biophys* 387, 137-142.
- Wilks, A., Burkhard, K.A., 2007, Heme and virulence: how bacterial pathogens regulate, transport and utilize heme. *Nat Prod Rep* 24, 511-522.
- Wilks, A., Schmitt, M.P., 1998, Expression and characterization of a heme oxygenase (Hmu O) from *Corynebacterium diphtheriae*. Iron acquisition requires oxidative cleavage of the heme macrocycle. *J Biol Chem* 273, 837-841.
- Wittenberg, J.B., Wittenberg, B.A., Gibson, Q.H., Trinick, M.J., Appleby, C.A., 1986, The kinetics of the reactions of *Parasponia andersonii* hemoglobin with oxygen, carbon monoxide, and nitric oxide. *J Biol Chem* 261, 13624-13631.
- Wojtowicz, H., Guevara, T., Tallant, C., Olczak, M., Sroka, A., Potempa, J., Sola, M., Olczak, T., Gomis-Ruth, F.X., 2009, Unique structure and stability of HmuY, a novel heme-binding protein of *Porphyromonas gingivalis*. *PLoS Pathog* 5, e1000419.
- Wolff, N., Deniau, C., Letoffe, S., Simenel, C., Kumar, V., Stojiljkovic, I., Wandersman, C., Delepierre, M., Lecroisey, A., 2002, Histidine pK(a) shifts and changes of tautomeric states induced by the binding of gallium-protoporphyrin IX in the hemophore HasA(SM). *Protein Sci* 11, 757-765.
- Wong, M.T., Thornton, D.C., Kennedy, R.C., Dolan, M.J., 1995, A chemically defined liquid medium that supports primary isolation of *Rochalimaea* (*Bartonella*) *henselae* from blood and tissue specimens. *J Clin Microbiol* 33, 742-744.
- Wyckoff, E.E., Lopreato, G.F., Tipton, K.A., Payne, S.M., 2005, *Shigella dysenteriae* ShuS promotes utilization of heme as an iron source and protects against heme toxicity. *J Bacteriol* 187, 5658-5664.
- Wyckoff, E.E., Schmitt, M., Wilks, A., Payne, S.M., 2004, HutZ is required for efficient heme utilization in *Vibrio cholerae*. *J Bacteriol* 186, 4142-4151.
- Xu, Y.H., Lu, Z.Y., Ihler, G.M., 1995, Purification of deformin, an extracellular protein synthesized by *Bartonella bacilliformis* which causes deformation of erythrocyte membranes. *Biochim Biophys Acta* 1234, 173-183.
- Yang, J., Sangwan, I., Lindemann, A., Hauser, F., Hennecke, H., Fischer, H.M., O'Brian, M.R., 2006, *Bradyrhizobium japonicum* senses iron through the status of haem to regulate iron homeostasis and metabolism. *Mol Microbiol* 60, 427-437.
- Yue, W.W., Grizot, S., Buchanan, S.K., 2003, Structural evidence for iron-free citrate

- and ferric citrate binding to the TonB-dependent outer membrane transporter FecA. *J Mol Biol* 332, 353-368.
- Zeaiter, Z., Fournier, P.E., Ogata, H., Raoult, D., 2002, Phylogenetic classification of *Bartonella* species by comparing groEL sequences. *Int J Syst Evol Microbiol* 52, 165-171.
- Zhang, P., Chomel, B.B., Schau, M.K., Goo, J.S., Droz, S., Kelminson, K.L., George, S.S., Lerche, N.W., Koehler, J.E., 2004, A family of variably expressed outer-membrane proteins (Vomp) mediates adhesion and autoaggregation in *Bartonella quintana*. *Proc Natl Acad Sci U S A* 101, 13630-13635.
- Zhou, W., Russell, C.W., Johnson, K.L., Mortensen, R.D., Erickson, D.L., 2012, Gene expression analysis of *Xenopsylla cheopis* (Siphonaptera: Pulicidae) suggests a role for reactive oxygen species in response to *Yersinia pestis* infection. *J Med Entomol* 49, 364-370.
- Zhu, W., Hunt, D.J., Richardson, A.R., Stojiljkovic, I., 2000a, Use of heme compounds as iron sources by pathogenic neisseriae requires the product of the hemO gene. *J Bacteriol* 182, 439-447.
- Zhu, W., Wilks, A., Stojiljkovic, I., 2000b, Degradation of heme in gram-negative bacteria: the product of the hemO gene of Neisseriae is a heme oxygenase. *J Bacteriol* 182, 6783-6790.
- Zimmermann, R., Kempf, V.A., Schiltz, E., Oberle, K., Sander, A., 2003, Hemin binding, functional expression, and complementation analysis of Pap 31 from *Bartonella henselae*. *J Bacteriol* 185, 1739-1744.