



# Managing iron supply during the infection cycle of a flea borne pathogen, *Bartonella henselae*

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*Bartonella* are hemotropic bacteria responsible for emerging zoonoses. Most *Bartonella* species appear to share a natural cycle that involves an arthropod transmission, followed by exploitation of a mammalian host in which they cause long-lasting intra-erythrocytic bacteremia. Persistence in erythrocytes is considered an adaptation to transmission by bloodsucking arthropod vectors and a strategy to obtain heme required for *Bartonella* growth. *Bartonella* genomes do not encode for siderophore biosynthesis or a complete iron Fe<sup>3+</sup> transport system. Only genes, sharing strong homology with all components of a Fe<sup>2+</sup> transport system, are present in *Bartonella* genomes. Also, *Bartonella* genomes encode for a complete heme transport system. *Bartonella* must face various environments in their hosts and vectors. In mammals, free heme and iron are rare and oxygen concentration is low. In arthropod vectors, toxic heme levels are found in the gut where oxygen concentration is high. *Bartonella* genomes encode for 3–5 heme-binding proteins. In *Bartonella henselae* heme-binding proteins were shown to be involved in heme uptake process, oxidative stress response, and survival inside endothelial cells and in the flea. In this report, we discuss the use of the heme uptake and storage system of *B. henselae* during its infection cycle. Also, we establish a comparison with the iron and heme uptake systems of *Yersinia pestis* used during its infection cycle.

**Keywords:** *Bartonella*, heme utilization, flea transmission, heme-binding protein, *Yersinia*

## INTRODUCTION

*Bartonella* are  $\alpha$ -proteobacteria that employ arthropods for transmission and erythrocyte parasitism as a common parasitism strategy (Dehio and Sander, 1999; Schulein et al., 2001). Currently, 26 distinct *Bartonella* species have been described (Kaiser et al., 2011). *Bartonella bacilliformis*, *Bartonella quintana*, and *B. henselae* are the three most important human pathogens (Dehio, 2005; Florin et al., 2008; Gupta et al., 2010). Humans are the reservoir host for *B. bacilliformis* and *B. quintana*, in which they cause various clinical manifestations associated with both intra-erythrocytic bacteremia and endothelial cell infection (Hill et al., 1992; Maurin and Raoult, 1996). The cat is the reservoir host for *B. henselae* (Chomel et al., 2009). *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 usually in immunodeficient patients (Spach et al., 1995; Sander et al., 1996).

## *Bartonella* AND THEIR INFECTION CYCLE

Each *Bartonella* species appears to be transmitted by specific bloodsucking arthropod vectors, and is highly adapted to one or several mammalian reservoir hosts, in which it causes long-lasting intra-erythrocytic bacteremia (Schroder and Dehio, 2005). 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; Schulein et al., 2001; Marignac et al., 2010). 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). Endothelial cells (Dehio, 2005) or CD34<sup>+</sup> progenitor cells (Mandle et al., 2005) were proposed as the primary niche. About 4–5 days post-infection, bacteria seeded from the primary niche to the bloodstream are able to invade mature erythrocytes (Schulein et al., 2001). Inside erythrocytes, bacteria multiply until reaching a steady number (eight bacteria per infected erythrocyte for *Bartonella tribocorum* in rat erythrocytes), which is maintained for the remaining lifespan of the infected cell (Schulein et al., 2001). Intra-erythrocytic bacteremia in the *B. tribocorum*-rat model is persistent for 8–10 weeks (Schulein et al., 2001). During this period, bloodsucking arthropods mediate the transmission to other susceptible hosts. This infectious procedure was also observed in a mouse model for *B. grahamii* and *B. birtlesii* (Koesling et al., 2001; Marignac et al., 2010).

## IRON/HEME UPTAKE IN *Bartonella*

Analysis of *Bartonella* genomes indicated that they neither encode for a siderophore biosynthesis pathway, nor for a complete Fe<sup>3+</sup> transport system. *Bartonella* genomes encode for homologs of YfeABCD from *Yersinia pestis* (Perry et al., 2007) and SitABCD from avian pathogenic *E. coli*, both characterized as Fe<sup>2+</sup> and

Mn<sup>2+</sup> inner membrane transporters (Sabri et al., 2006; Anjem et al., 2009). *Bartonella* genomes encode for a complete heme uptake system. This heme uptake system is comprised of HutA, an outer membrane heme transporter, HutB, HutC, and HmuV the three components of an inner membrane ABC transporter and a cytoplasmic heme degrading enzyme (HemS) (Parrow et al., 2009; Liu et al., 2012a). HutA from *B. quintana* contains the FRAP and NPFL domains conserved in heme transporters like HemR of *Yersinia enterocolitica* and HumR of *Yersinia pestis* (Parrow et al., 2009). Also, it was shown that HutA from *B. quintana* can apparently transport heme when expressed in *E. coli hemA* mutant strain (Parrow et al., 2009). This activity is TonB-dependent (Parrow et al., 2009). *B. tribocorum* and *B. birtlessii hutA* mutants are unable to establish bacteremia in their reservoir hosts. This result suggests that HutA is required for *Bartonella* heme uptake in mammals (Saenz et al., 2007; Vayssier-Taussat et al., 2010). After its transport into the cytoplasm, heme must be degraded to release iron. *Ex vivo*, HemS of *B. henselae* promotes the release of iron from heme when expressed in *E. coli* (Liu et al., 2012a). *In vitro*, HemS of *B. henselae* binds heme and degrades it in the presence of suitable electron donors, such as ascorbate or NADPH-cytochrome P450 reductase (Liu et al., 2012a). Interestingly, HemS activity was shown to be involved in the oxidative stress response of *B. henselae* (Liu et al., 2012a). All these above data corroborate previous *ex vivo* results demonstrating that *Bartonella* can use heme as an iron source (Sander et al., 2000). In *B. quintana*, expression of the *hutA*, *hems*, *hutB*, *hutC*, and *hmuV* is repressed by heme in an Irr-dependent manner (Parrow et al., 2009). Over expression of *fur* in the presence of heme repress *tonB* expression (Parrow et al., 2009).

### HEME-BINDING PROTEIN IN *Bartonella*

*Bartonella* express 3–5 outer membrane heme-binding proteins (Battisti et al., 2006; Minnick and Battisti, 2009). Heme-binding proteins of *Bartonella* are a group of 30–40 kDa porin-like outer membrane proteins that lack similarity with known heme receptors (Minnick et al., 2003). HbpA of *B. quintana* was shown to bind heme *in vitro* (Carroll et al., 2000). However, it did not confer a heme-binding phenotype when expressed in *E. coli* (Carroll et al., 2000). Zimmermann et al., identified a prominent heme-binding protein Pap31 (HbpA), through a heme-binding blot performed with membrane proteins from *B. henselae*. They showed that expressing Pap31 in an *E. coli K12 hemA* mutant strain restored its growth when heme was added at 30 μM and above (Zimmermann et al., 2003). The activity of HbpA as a heme transporter was questioned by other authors. Recently, HbpA of *B. quintana* was shown to be unable to complement the *E. coli hemA* mutant in the presence of heme (Parrow, 2010). Complementation assays using the *E. coli hemA* mutant strain on solid medium in the presence of different heme concentrations also showed that HbpA of *B. birtlessii* cannot transport heme (Biville, unpublished results).

The heme uptake activity of four Hbps of *B. henselae*, expressed in an *E. coli* model strain, was investigated. All Hbps of *B. henselae* can bind heme *in vitro*. No heme transport activity

was associated with expression of Hbps in *E. coli* (Liu et al., 2012b). In contrast, Hbps increase heme uptake efficiency when co-expressed with a heterologous heme transporter in an *E. coli* model strain (Liu et al., 2012b). Binding of heme by Hbps was proposed to increase its concentration around the bacteria and, as a consequence, facilitate its uptake. Another potential role for heme binding by Hbps was to provide an antioxidant barrier via heme's intrinsic peroxidase activity (Minnick and Battisti, 2009). Decreasing each Hbp amount using knockdown increases *B. henselae* sensitivity to hydrogen peroxide (Liu et al., 2012b). This antioxidant role of heme-binding proteins was evidenced to play an important role for survival of *B. henselae* in human endothelial cells and in the flea *Ctenocephalides felis*, where reactive oxygen species are produced. The expression levels of genes encoding for heme-binding proteins vary with oxygen, temperature and heme concentration (Battisti et al., 2006). One regulator, Irr, was shown to bind an "H-box" located in the promoter region of *hbp* genes (Battisti et al., 2007). Also, over-expression of RirA increased expression level of *hbpA*, *hbpD*, and *hbpE* (Battisti et al., 2007). Based on their regulatory pattern in *B. quintana*, *hbp* genes were divided into two groups. The first contained *hbpB* and *hbpC*, over expressed under conditions that mimic the gut arthropod environment [high heme concentration and low temperature (30°C), high O<sub>2</sub> concentration] (Battisti et al., 2006). The transcription of *hbpA*, *hbpD*, and *hbpE* was increased at low heme concentrations at 37°C. HbpA, HbpD, and HbpE were suggested as being required when the free heme concentration is low, such as in blood circulation in the mammalian host. 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 (Roden et al., 2012).

### IRON SOURCES AND OXIDATIVE STRESS ENCOUNTERED DURING *Bartonella* INFECTION CYCLE

Inside the arthropod gut, *Bartonella* encounter high heme and oxygen levels. Such conditions can generate a massive oxidative stress. Thus, inside the arthropod gut, bacteria confront oxidative stress after a blood meal (Graca-Souza et al., 2006). Inside mammals, getting iron required for bacterial growth is a challenge since 99.9% of total body iron is sequestered inside the cells (Wandersman and Stojiljkovic, 2000). Outside the cells, iron is bound to transferrin in the serum or to lactoferrin in mucosal secretions (Cassat and Skaar, 2013). Another iron source in mammals is heme that is mainly contained in hemoproteins like hemoglobin. After erythrocyte lysis, most hemoglobin is bound by haptoglobin. Hemoglobin degradation allows the release of heme that is sequestered by hemopexin to prevent its toxicity (Wandersman and Stojiljkovic, 2000). Thus, obtaining iron from mammals requires transport systems allowing uptake of heme or iron bound to proteins. After inoculation in mammals, in the primary niche proposed to be endothelial cells, the intracellular iron source is iron Fe<sup>2+</sup>. When bacteria reach the blood stream the iron sources encountered are iron loaded transferrin, heme loaded hemopexin and hemoglobin bound to haptoglobin. Free heme is present at a low concentration (0.5 μM). *Bartonella* does not encode for a complete iron

uptake system and cannot transport heme bound to hemopexin. Thus, the bacteria must use heme, stored on their surface, as an iron source. Inside erythrocytes the hemoglobin concentration is high and heme can be stored in heme-binding proteins. During their life cycle, *Bartonella* encounter heme rich environments and heme poor environments. To face this alternation, heme-binding proteins are hypothesized to play a crucial role in storing heme in the heme rich environment, and delivering it in iron/heme poor environment. The complex regulatory expression pathway of heme-binding proteins supports this hypothesis. *Bartonella* express one heme transport system HutA and control heme entry and toxicity using heme storage proteins, differentially expressed according to the bacterial infection cycle. The *Bartonella* heme uptake and storage pathways are regulated by Irr, RirA, and Fur similar to  $\alpha$ -proteobacteria (Johnston et al., 2007). This iron/heme uptake pathway contrasts with that of another flea-borne pathogen *Yersinia pestis*.

### IRON/HEME UPTAKE AND HEME-BINDING PROTEINS IN *Yersinia pestis*

*Yersinia pestis*, the causative agent of plague, is transmitted mainly by infected flea bites (Chouikha and Hinnebusch, 2012) (Figure 1). Like many highly-virulent pathogenic bacteria, *Y. pestis* possess siderophore-mediated iron acquisition systems. Yersiniabactin (Ybt), the siderophore synthesized and secreted by *Y. pestis*, can remove iron from various host iron-binding proteins, such as transferrin and lactoferrin (Fetherston et al., 2010). Fe-Ybt assimilation occurs through the TonB-dependent outer membrane receptor Psn (Lucier et al., 1996). After passage through the outer membrane, Ybt-bound iron is transported into the bacterial cell by the YbtP-YbtQ ABC transporter (Fetherston et al., 1999). In *Y. pestis*, iron  $Fe^{2+}$  is transported by the YfeABCD and FeoABC systems (Perry et al., 2007). Also, *Y. pestis* encode for two heme transport systems. The *hmu* locus (*hmu*RSTUV) allows *Y. pestis* to use heme as well

as host hemoproteins including hemoglobin, myoglobin, heme-albumin, heme-hemopexin, and hemoglobin-haptoglobin as an iron source (Hornung et al., 1996) (Figure 2). The second heme-protein acquisition system consisted of a heme receptor HasR, HasA hemophore, and HasA-dedicated ABC transporter factor HasDE, as well as a TonB homolog, HasB. However, the Has system appears not to allow the bacteria to use hemoglobin as an iron source under laboratory conditions (Rossi et al., 2001). In *Y. pestis*, the iron and heme uptake systems are Fur regulated (Gao et al., 2008). *Y. pestis*'s main transmission mode depends on the flea foregut blockage (Hinnebusch et al., 1996; Chouikha and Hinnebusch, 2012) resulting in continuous attempts to feed (Figure 1). Thus, the contaminated blood is regurgitated back into the mammalian host, where the bacteria rapidly establish an infection (Hinnebusch et al., 1996). Blockage of the flea foregut requires the activity of *hmsHFRS* gene products which synthesize and export the polysaccharide extracellular matrix required for formation of biofilm (Bobrov et al., 2008). Biofilm formation at the surface of proventricular spines is required for infection of the proventriculus (Jarrett et al., 2004). The Hms system is responsible for absorption of exogenous heme or Congo red when the bacteria are grown at 26°C (Burrows and Jackson, 1956; Surgalla and Beesley, 1969). However, the Hms system never encodes for a heme uptake system. Moreover, the stockpiled heme is not used under iron-deficient conditions (Lillard et al., 1999). Consistently, a deletion of the Hms system has no effect on *Y. pestis* virulence in mice infected by a subcutaneous route. This indicated that the Hms system is not required for the virulence of bubonic plague in a mouse model (Lillard et al., 1999). The Hms heme storage system is essential for establishing an infection within the flea midgut and to block the proventriculus (Hinnebusch et al., 1996). Heme absorbed by the Hms system protects *Y. pestis* from the nitric oxide and superoxide anion toxicity, but not from the  $H_2O_2$  killing effect (Lillard et al., 1999). HmsH and HmsF were

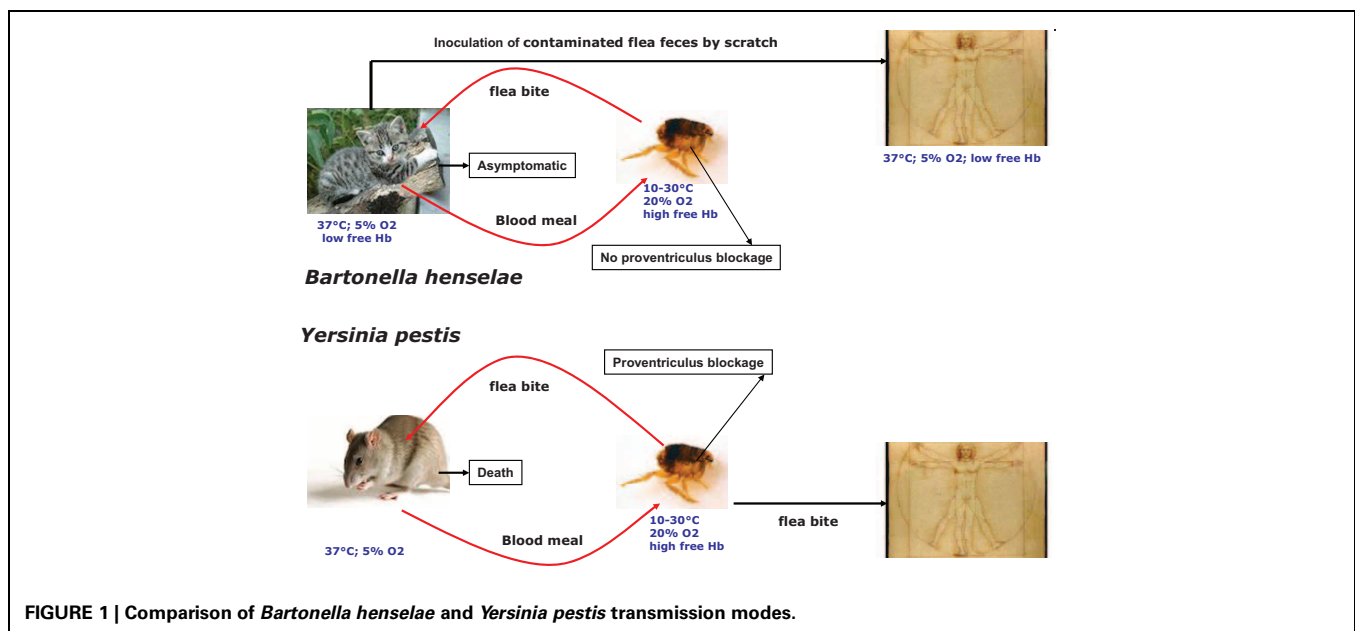
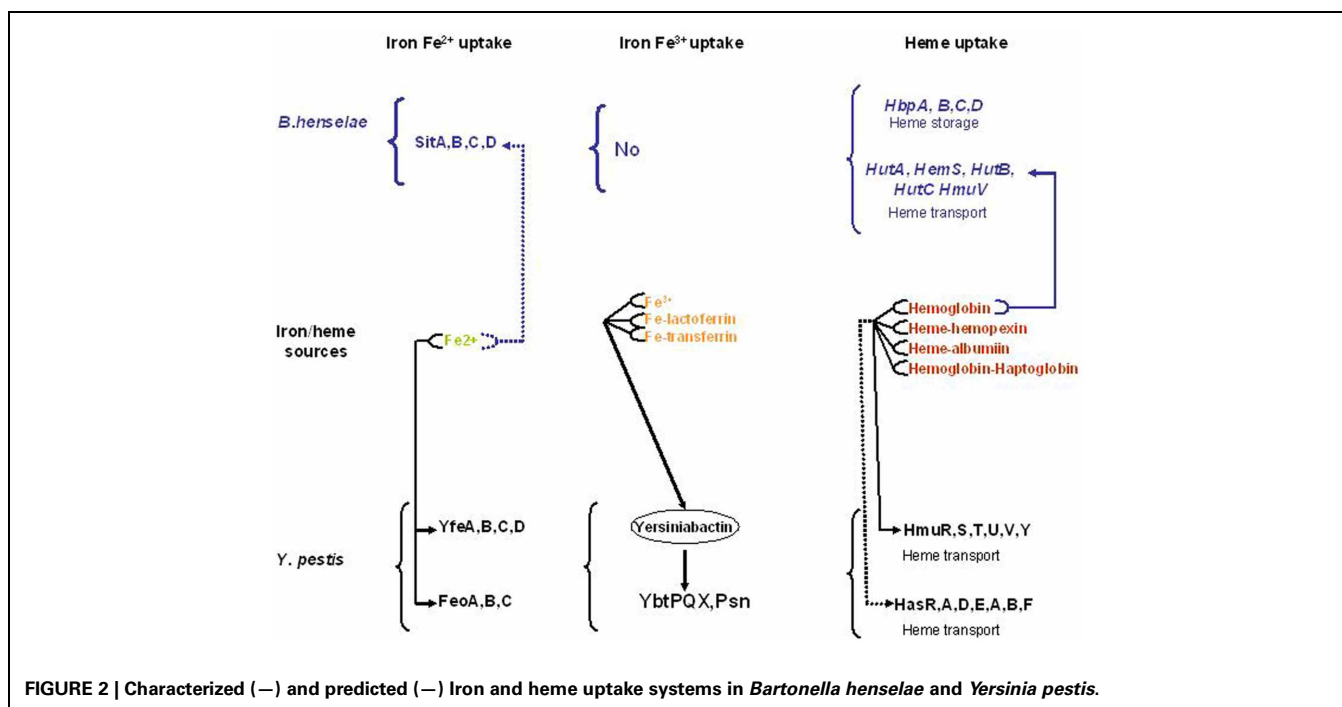


FIGURE 1 | Comparison of *Bartonella henselae* and *Yersinia pestis* transmission modes.



**FIGURE 2 |** Characterized (—) and predicted (---) Iron and heme uptake systems in *Bartonella henselae* and *Yersinia pestis*.

characterized as outer membrane proteins (Pendrak and Perry, 1991) while HmsR, HmsS, and HmsT are inner membrane proteins (Perry et al., 2004). HmsF and HmsR possess domains found in polysaccharide-modifying enzymes and glycosyltransferases, respectively, and HmsT belongs to the family of GGDEF proteins (Lillard et al., 1997; Ausmees et al., 2001; Perry et al., 2004). Transcription of heme acquisition operon *hmsHFRS* and *hmsT* is not regulated by the iron status inside the cells and the availability of exogenous heme (Perry et al., 2004). The temperature-dependent regulation of HmsH, HmsF, HmsR, and HmsT amount is post transcriptional and is related to a decreased stability of these proteins at 37°C (Perry et al., 2004).

## CONCLUSION

The two flea-borne pathogens *B. henselae* and *Y. pestis* can invade the flea gut and mammals. As a consequence, they must adapt their physiology to face the change related to these two environments. Inside the flea gut, bacteria can use heme as an iron source and must face its toxicity. Flea bite is responsible for *Y. pestis* transmission and *B. henselae* can also be transmitted by flea feces contamination (Figure 1). This transmission mode is possible since *B. henselae* is able to survive several days within feces. Inside the flea gut, *Y. pestis* forms a biofilm in the proventriculus responsible for blocking. Blocking and biofilm formation have not been described for *B. henselae*. Inside mammals, *Y. pestis* can use different iron and heme sources (Figure 2). In contrast, the sole iron/heme source for *B. henselae*, outside the cells, is heme contained in hemoglobin (Figure 2). Inside the cells, *B. henselae* could use the reduced form of iron. For *B. henselae*, the ability to invade erythrocytes is thus a good opportunity to obtain heme. In *Y. pestis*, no heme transport activity was associated with HmsH, HmsF, HmsR, and HmsT. Moreover, heme absorbed by

the Hms system is not used as an iron source (Lillard et al., 1999). In contrast, heme-binding proteins from *B. henselae* were shown to increase the heme uptake efficiency. Inside the flea, the presence of all heme-binding proteins is required for *B. henselae* survival. The pathogenesis of *Y. pestis* is not affected by the absence of a heme storage phenotype. In contrast, disruption of one heme-binding protein in *Bartonella* impairs bacteremia establishment. In regard to the cell invasion *B. henselae* and *Y. pestis* exhibit a similar phenotype since HpbA knockdown in *B. henselae* and Hms<sup>-</sup> phenotype in *Y. pestis* decrease the ability to invade cells (Lillard et al., 1999; Liu et al., 2012b). Both in *Y. pestis* and *B. henselae* the heme storage phenotype was evidenced to be involved in protection against oxidative stress. In *B. henselae*, all heme-binding proteins were associated with protection against oxidative stress generated by exposure to hydrogen peroxide. In *Y. pestis*, Hms<sup>-</sup> phenotype decreases the ability to face exposure to paraquat.

For *Bartonella*, the ability to store heme looks like it is more crucial than that for *Y. pestis*. This could explain why small *Bartonella* genomes (Alsmark et al., 2004) encode for 3–5 highly homologous heme-binding proteins whose expression level is submitted to a complex regulatory pathway (Battisti et al., 2007). Since heme storage is a very important process for *Bartonella*, this can explain why decreasing the amount of only one heme-binding protein generates some defects in spite of the presence of others heme-binding proteins. In addition to the regulatory events managing expression of the various *hbp* genes, it cannot be excluded that heme-binding proteins coordinate their activity. A precise biochemical characterization of heme-binding proteins will give fruitful information about a possible cooperative activity of these outer membrane proteins.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 July 2013; paper pending published: 01 August 2013; accepted: 19 September 2013; published online: 18 October 2013.

Citation: Liu M and Biville F (2013) Managing iron supply during the infection cycle of a flea borne pathogen, *Bartonella henselae*. *Front. Cell. Infect. Microbiol.* 3:60. doi: 10.3389/fcimb.2013.00060

This article was submitted to the journal *Frontiers in Cellular and Infection Microbiology*.

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