Chapter 1

FERRITIN AS AN IRON SOURCE FOR PATHOGENS

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ABSTRACT

In this chapter we analyze the interaction between the host ferritin and pathogenic microorganisms, since this ferric protein can be used by invaders for their growth and, thus, colonization and invasion of tissues, causing disease. Iron is an essential nutrient for all living beings; however, this metal is toxic and must be captured by proteins, among them ferritin, the great intracellular storage of iron in the body. Pathogens living inside humans also need the vital iron; therefore, the iron availability in body tissues plays a crucial role in the host-pathogen relationship. In general, microorganisms living within a mammal have

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Evolved several mechanisms to scavenge iron from the host iron-containing proteins; these mechanisms have been considered to be important virulence factors. Pathogens able to destroy cells and tissues can have easy access to ferritin and make use of its iron. Due to the high amount of iron atoms that ferritin is able to capture, this protein is really a remarkable iron source for every intracellular pathogen. In the literature, there are only a few reports about pathogens using ferritin as an iron source, but this is an exciting growing field of research. Each pathogen has developed its own manner to obtain iron from ferritin: for instance, the bacterium *Neisseria meningitidis* triggers the host ferritin redistribution from cytosol to lysosomes within infected epithelial cells and accelerates the ferritin degradation by lysosomal proteases, thus providing the necessary iron for its own existence. On the other hand, in spite of being ferritin a stable supramolecular complex, *Burkholderia cenocepacia* secretes serine-proteases that degrade ferritin. Some strains of *Escherichia coli* and *Mycobacterium* secrete siderophores, ultra-high affinity iron-binding compounds able to confiscate iron to host ferritin. Another mechanism to get ferritin iron is the reported in *Listeria monocytogenes* and the mucosal pathogenic fungus *Candida albicans*, which use surface reductases to acquire iron from ferritin. In parasitic protozoa, there are practically no reports about the utilization of ferritin as a sole iron source. *Trichomonas vaginalis* uses ferritin iron but the mechanism by which it uptakes iron from this protein is still unknown. Recently, we reported that *Entamoeba histolytica* trophozoites endocytose ferritin by clathrin-coated pits and degrade this protein by means of specific cysteine proteases in the endosome/lysosome pathway. Concluding, pathogenic microorganisms capable of removing and acquiring iron from ferritin can obtain a plentiful source of this crucial metal to survive, colonize and invade the host.

**Keywords:** Bacteria, Ferritin, Fungi, Iron source, Parasites, Protozoa

### 1. Iron Is Vital for Mammals but Also for Microorganisms Living in Mammalian Tissues

Iron (Fe) is a vital element for the cellular metabolism of virtually all living organisms, except some *Lactobacillus* species, which have copper or manganese in their enzymes [Archibald, 1983]. However, iron in the two oxidation forms (Fe2+, Fe+3) is toxic, thus it must not be free in cells and tissues. Free Fe2+ concentration, in the presence of oxygen, produces highly
reactive oxygen species (ROS) by the Fenton reaction, damaging all types of biological macromolecules [Chiancone et al., 2004]. Therefore, in all organisms, iron is part of or is bound to proteins. Specifically, mammals possess diverse mechanisms to maintain, perfectly regulated, the iron concentration in the body [Finch, 1994; Weinberg, 1999a; Sharp and Srai, 2007]. This delicate equilibrium is mainly accomplished by proteins that absorb, transport, bind, recycle, store, and use iron.

In humans, extracellular iron is bound to the iron-transporter transferrin, a protein found in serum that provides iron to all cells, mainly to erythroid precursors. Extracellular iron also binds to lactoferrin, protein that sequesters iron in mucosae and infection sites. Intracellular iron can be found in the heme group of electron-transporter proteins, such as cytochromes, the blood oxygen-transporter hemoglobin, and the muscle oxygen-store myoglobin. This element is also a cofactor in redox processes catalyzed by enzymes and in the Fe-S center of several proteins [Weinberg, 1978; Griffiths et al., 1999; Conrad and Umbreit, 2000]. Excess iron is stored in ferritin, keeping this element available intracellularly when the cell needs it. When the intracellular iron concentration increases, ferritin sequesters iron and detoxifies the cell, avoiding its free form in cytosol [Andrews et al., 1993; Arosio and Levi, 2002]. Ferritin controls the reversibility of the transition phase between Fe^{2+} (soluble) and Fe^{3+} (insoluble) mineralized inside the molecule [Theil, 1990; Koorts and Viljoen, 2007]. Ferritin is a versatile protein, mainly cytosolic, but localized also in other structures such as mitochondrion, nucleus, and in mammalian serum (in this case poor in iron). Ferritin is a heteropolymer composed of 24 subunits of two types, H and L, and the proportion of each subunit depends on the main function of the protein. For example, in the liver and spleen, where 50% of iron corporal reserves are kept (0.4g), ferritin is the most efficient protein for iron incorporation, being able to capture up to 4,500 iron atoms, and it has a proportion of 80% L and 20% H. However, in the brain, heart and lungs, ferritin is devoted to a detoxification function, capturing free iron and avoiding the ROS generation; the optimal proportion for this function is 80% H and 20% L. It is important to mention that ferritin is found in all tissues due to the iron requirements for metabolic processes and the necessity of avoiding the ROS production, toxic for the cell [Theil, 1990; Harrison and Arosio, 1996; Arosio et al., 2009].

In addition to its role as an intracellular protein, ferritin can be an iron-carrier when it resides in extracellular sites. For example, serum ferritin and ferritin secreted by macrophages have a role in iron delivery to several kinds
of cells, and due to its high iron content, the iron supply can be more efficient than that of transferrin. Among the cells benefited by the ferritin-iron delivery pathway are erythroid precursor cells, hepatocytes, brain oligodendrocytes, lymphocytes, and enterocytes, all of which have a receptor to bind ferritin [Wang et al., 2010]. Interestingly, in mouse oligodendrocytes, which do not have a detectable level of transferrin receptor, and then all iron is provided by ferritin, the ferritin-H endocytosis receptor has been identified as TIM-2: this is a transmembrane receptor, expressed in the liver, kidney, and in T and B cells, which regulates T helper type-2 responses and autoimmunity [Chakravarti et al., 2005; Chen et al., 2005].

Iron is also highly important for pathogens, and this element must be perfectly regulated in order to avoid toxicity to these microbes. They possess similar iron-dependent enzymes and proteins for metabolism to those found in humans. In most microbial species, complex systems for acquiring iron from the environment that function in response to iron-starvation have been described. In *Escherichia coli*, the bacterial species most studied in bacterial genetics, regulation of genes that participate in iron metabolism is mainly carried out at the transcriptional level by the repressor protein Fur and at the post-transcriptional level by the non-coding RNA RyhB, whose transcription is negatively regulated by Fe-Fur [Lee and Helmann, 2007].

Interestingly, parasitic protozoa must attain a higher quantity of iron than bacteria in order to grow, colonize, invade, and survive within a host. Some protozoa need even more iron, such as the amitochondrial protists (*Tritrichomonas, Trichomonas, Giardia, Entamoeba*), which have unusually high requirements for iron (50–200 µM), surpassing those of the majority of both eukaryotic and prokaryotic cells (0.4-4 µM) [Weinberg, 1974]. This is due to their energy metabolism, which relies heavily on Fe-S proteins [Tachezy, 1999; Vanacova et al., 2001]. Iron storage is extremely important to all forms of life, thus ferritin is found in the three domains of living beings, *Archaea, Bacteria*, and *Eukarya*. However, some eukaryotic cells like yeasts and protozoa apparently do not have a ferritin molecule, but have evolved other ways to store and maintain their own iron homeostasis [Suchan et al., 2003; Koorts and Viljoen, 2007; Arosio et al., 2009]. Microorganisms therefore appear to be well-equipped for living in an iron-poor environment, and due to their imperious need for iron they have evolved different strategies for the capture of this element and supply themselves the iron requirement.
Table 1. Microorganisms capable of using ferritin as a sole iron source and their ferritin-iron acquisition system

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Iron acquisition system from ferritin</th>
<th>Tissue or organ where microorganisms obtain ferritin</th>
<th>Reference</th>
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<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td>Bacillus cereus</td>
<td>Iron acquisition system still unknown, it uses ferritin after direct binding to the surface receptor IlsA</td>
<td>Intestinal cells, oral epithelium, retinal tissue, CNS, blood, liver, striated muscle, skin, heart tricuspid-valve</td>
<td>[Daou et al., 2009]</td>
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<tr>
<td>Burkholderia cenocepacia</td>
<td>Proteolytic mechanism: Secreted serine proteases that degrade ferritin</td>
<td>Lungs, macrophages, epithelial cells</td>
<td>[Whitby et al., 2006]</td>
</tr>
<tr>
<td>Escherichia coli, and Yersinia pestis</td>
<td>Siderophores: Aerobactin is a siderophore used to obtain iron from ferritin</td>
<td>E. coli: intestinal cells, urinary tract, kidneys, CNS, blood</td>
<td>[Brock et al., 1991]</td>
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<td></td>
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<td>Yersinia pestis: blood, lungs, lymph nodes, macrophages</td>
<td>[Perry and Brubaker, 1979; Sikkema and Brubaker, 1989]</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>Surface-associated ferric reductase system: still unidentified</td>
<td>Intestinal cells, macrophages, hepatocytes, epithelial cells, fibroblasts, endothelial cells, neurons</td>
<td>[Deneer et al., 1995; Barchini and Cowart, 1996; Jin et al., 2006]</td>
</tr>
<tr>
<td>Mycobacterium spp</td>
<td>Siderophores: Carboxymycobactin is a siderophore capable to remove iron from ferritin</td>
<td>Lungs, macrophages</td>
<td>[Gobin and Horwitz, 1996]</td>
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Table 1. (Continued)

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<tr>
<th>Microorganism</th>
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<th>Tissue or organ where microorganisms obtain ferritin</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Neisseria meningitidis</td>
<td>Host ferritin redistribution by bacteria and Proteolytic process: Ferritin is hydrolyzed specifically in host lysosomes</td>
<td>Respiratory tract, blood, CNS</td>
<td>[Larson et al., 2004]</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>Iron acquisition system still unknown, only it has been described the use of ferritin as an iron source</td>
<td>Lungs, throat epithelium, skin</td>
<td>[Eichenbaum et al., 1996]</td>
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<td>Parasite</td>
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<tr>
<td>Entamoeba histolytica</td>
<td>Endocytosis through clathrin-coated vesicles and Proteolytic process: Cysteine-proteases through endosomal/lysosomal pathway</td>
<td>Blood, brain, intestinal cell, hepatocyte, lungs</td>
<td>[Lopez-Soto et al., 2009]</td>
</tr>
<tr>
<td>Trichomonas vaginalis</td>
<td>Iron acquisition system still unknown, only it has been described the use of ferritin as an iron source</td>
<td>Vaginal mucosa</td>
<td>[Lehker and Alderete, 1992]</td>
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<td>Fungi</td>
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<td>Candida albicans</td>
<td>Surface reductase: Als3 is the ferritin receptor, the identity of the associated reductase is unknown</td>
<td>Vaginal, oral-pharyngeal, esophageal, and gastrointestinal mucosae; blood, CNS, internal organs like lungs and heart</td>
<td>[Almeida et al., 2008]</td>
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2. FERRITIN CAN BE AN ABUNDANT IRON SOURCE FOR PATHOGENIC MICROORGANISMS

Mammals have multiple sites rich in iron that can potentially support the iron requirement for the growth of pathogens. However, as a general strategy against invading microbes, mammals possess intricate iron-withholding systems for efficiently reducing the iron available to invaders. In this way, under normal conditions, the free-iron concentration in body fluids is negligible (~10^{-18} M) [Weinberg, 1978; Bullen, 1981]. As a consequence, pathogenic microbes surviving inside a host must successfully compete with the host’s iron-containing proteins to supply their own iron needs in order to colonize, invade, and survive. Evidently, the host’s iron availability plays a crucial role in the host-pathogen relationship.

In spite of this host strategy of keeping iron away from microbes, it has been well demonstrated that pathogens have evolved different mechanisms to achieve the host iron. Indeed, they can uptake extracellular and intracellular iron in the ferrous and ferric forms and, importantly, inorganic iron, as well as that bound to proteins. Microbes invading blood can lyse erythrocytes and have access to the four ferrous-iron ions supplied by hemoglobin; in this fluid, pathogens also can uptake the two ferric ions of transferrin. In mucosal tracts and infection sites, pathogens can bind lactoferrin and get its two ferric ions [Weinberg, 1999b]. In an enormous contrast, ferritin could provide more than 1000-fold the iron to pathogens than hemoglobin, transferrin, and lactoferrin, and definitively be a striking iron source for them. Since ferritin is mainly an intracellular protein, microorganisms may destroy the host cells, or invade them, or be phagocytosed by cells to have access to the ferritin iron. Serum ferritin is poor in iron. However, this protein can be iron-loaded in diseases involving iron overload and in infections, as a host response known as hypoferremia of infection, and then serum ferritin becomes a generous iron source for pathogens [Worwood et al., 1976; Arosio et al., 1977; Weinberg, 1978; Wang et al., 2010]. In order to acquire the necessary iron, some bacteria and fungi produce siderophores, ultra-high affinity molecules devoted to scavenging iron from the environment and able to remove iron from host proteins such as transferrin, lactoferrin, and ferritin. In addition, bacteria express receptors that directly bind these three host iron-containing proteins for acquiring iron. Other mechanisms are the production of proteases that cleave these proteins, leading to release of iron, and of reductases, which reduce the ferric iron to the more soluble and assimilable ferrous form.
Next, we will describe some strategies employed by microbes to acquire iron from ferritin, which are summarized in Table 1.

2.1. Bacterial and Fungal Mechanisms for Iron Acquisition from Ferritin

2.1.1. *Neisseria meningitidis* Triggers Host Cell Ferritin Redistribution and Accelerates its Degradation by Lysosomal Proteases

*Neisseria meningitidis* is a human-specific Gram-negative diplococcal bacterium found frequently in the respiratory tract of healthy individuals but can cause bacterial meningitis. Meningococcal disease is a severe life-threatening infection often related with serious complications; it continues to be a major cause of childhood morbidity and mortality worldwide, the majority of cases occurring in developing countries. Infants and children who survive bacterial meningitis often suffer neurological and other disabling sequelae [Cartwright et al., 1987; Orr et al., 2003; Choudhuri et al., 2011; Trivedi et al., 2011]. In order to generate disease, the meningococci (MC) must disseminate to the bloodstream causing septicemia and then cross the brain-blood barrier to the cerebrospinal fluid and trigger meningitis. Meningococci express a wide range of virulence factors including capsular polysaccharide, lipopolysaccharide, and a number of surface-expressed adhesive proteins necessary to evade killing by host defense mechanisms [Nassif and So, 1995].

One key determinant in the MC pathogenesis is their ability to acquire iron from the human host proteins: MC can grow extracellularly and have access to iron of lactoferrin in mucosae and of transferrin in serum, in addition to the uptake of heme of the hemoglobin molecule by the destruction of erythrocytes in blood. MC can also invade and replicate within epithelial cells, and this fact may be a critical factor in both the establishment of a carrier state and the development of meningitis [Schryvers and Stojiljkovic, 1999; Stephens et al., 2007; Jordan and Saunders, 2009; Virji, 2009]. Studies about intracellular replication of MC have been carried out in the human epithelial endocervical cell line A431. Interestingly, desferal inhibited the intracellular replication of MC; since this iron-chelator does not chelate heme iron, MC then could not use the iron incorporated with heme for intracellular growth. In addition, by the study of neisserial mutants defective either in porphyrin synthesis or in heme oxigenase, it was determined that heme is not required for the intracellular growth of MC. Also, a mutant affected in the use of
transferrin replicated normally inside the cell line A431. Therefore, intracellular MC use neither the iron from heme, nor from transferrin, for growth, and thus ferritin was the best candidate for the iron supply [Turner et al., 1998; Larson et al., 2002].

In 2004, Larson et al. demonstrated for the first time and through elegantly-designed experiments, that a pathogen is able to use the host intracellular ferritin as an iron source, in assays using the cell line A431 [Larson et al., 2004]. First, this group of research demonstrated that the possibility of *N. meningitidis* used transferrin-derived iron indirectly after its removal from transferrin was null, and additionally, that iron-loaded transferrin inhibited the intracellular replication of MC in a dose-dependent manner. Moreover, the replication rate of MC was higher in iron-starved than in iron-replete cells, showing that intracellular MC replication is stimulated by the need for iron. Next, by using an ELISA, they monitored the levels of ferritin inside the cells, and found that ferritin levels declined rapidly in those cells infected with MC, and this fact was not due to a repression of ferritin transcription or transduction. Furthermore, by experiments of pulse-chase using $[^{35}\text{S}]$-cysteine and methionine, and immunoprecipitation with an anti-ferritin Ab, they determined that the declined ferritin levels were a result of a ferritin degradation triggered by MC infection. After infection of MC to epithelial cells, these bacteria induced aggregation of cytosolic ferritin and this led to the degradation of the ferric protein in a degradative compartment, perhaps lysosomes; this was observed by using indirect immunofluorescent microscopy. Interestingly, when holotransferrin (iron income prevents ferritin turnover), ascorbic acid (avoids the ferritin autophagy and degradation) or leupeptin (lysosomal proteases inhibitor) were added, the redistribution and degradation of ferritin, as well as the intracellular replication of MC, were diminished. With this set of experiments, the determination that ferritin degradation could be providing the iron source critical for growth and colonization of *Neisseria meningitidis* was overwhelmingly demonstrated. In many cell lines, degradation of cytosolic ferritin during iron starvation occurs to release iron in order to meet its own cellular metabolic needs [Ollinger and Roberg, 1997; Radisky and Kaplan, 1998; Tabuchi et al., 2000]. MC disrupt transferrin uptake by epithelial cells, reducing transferrin receptor mRNA and slowing the receptor cycling [Bonnah et al., 2000]. The authors propose that in this way MC induce an iron starvation response in host cells, resulting in the lysosomal degradation of cytosolic ferritin, a mechanism that has not been described in other pathogens [Larson et al., 2004].
2.1.2. *Burkholderia cenocepacia* Secretes Serine-proteases that Degrade Ferritin

*Burkholderia cenocepacia* is a motile, rod-shaped, metabolically diverse Gram-negative β-proteobacterium. This species is one of at least 17 phenotypically similar species known as the *Burkholderia cepacia* complex (Bcc), a group of genetically-related environmental microorganisms that cause chronic opportunistic infections in patients with cystic fibrosis (CF) and other underlying diseases [Vandamme *et al.*, 2003; Vanlaere *et al.*, 2008; Vanlaere *et al.*, 2009]. *B. cenocepacia* is the most common species isolated from CF patients and associated with the epidemic spread among these people [LiPuma *et al.*, 2001; LiPuma *et al.*, 2002]. *B. cepacia* complex organisms possess several factors that play a key role in pathogenesis, such as the ability to survive intracellularly within macrophages and respiratory epithelial cells, cable pili, flagella, a type-III secretion system, surface exopolysaccharide, production of melanin, catalase, up to four types of iron-chelating siderophores, proteases and other secreted enzymes, quorum-sensing systems, and the ability to form biofilms. Not all strains produce each of the proposed virulence factors and, to date, none of these individual factors have been clearly demonstrated to be a major contributor to human disease. Iron acquisition processes could be important in the lung colonization process during cystic fibrosis [Hunt *et al.*, 2004; Visser *et al.*, 2004; Loutet and Valvano, 2010].

The iron metabolism of the lungs differs from that of the rest of the body [Gutteridge *et al.*, 2001]. Each day, the human respiratory tract is exposed to a great quantity of airborne iron [Turi *et al.*, 2004], and, thus, it can suffer iron-mediated oxidative damage. The risk of damage to the lungs is additionally enhanced by the high partial pressure of oxygen and the presence of inhaled microorganisms. Thus, free iron is rapidly sequestered mainly by transferrin, lactoferrin and ferritin. Consequently, in the CF lung, ferritin is significantly up-regulated in comparison with normal healthy lungs [Stites *et al.*, 1998; Stites *et al.*, 1999; Turi *et al.*, 2004].

Whitby *et al.* (2006) demonstrated that the strain *B. cenocepacia* J2315T directly utilized iron from ferritin for its growth *in vitro*; this assessment was done in base to growth kinetics in media where ferritin was the only iron source [Whitby *et al.*, 2006]. Further studies examining the mechanisms of iron uptake from ferritin indicated that iron utilization resulted from a proteolytic degradation of this otherwise stable macromolecular structure. By using different types of protease-activity inhibitors, it was found that ferritin proteolysis was due to secreted serine proteases. Since ferritin concentration is
significantly higher in the CF than in healthy lungs, undoubtedly the ability of *B. cenocepacia* to use host ferritin may contribute to its colonization and persistence in the cystic fibrosis patient [Whitby *et al.*, 2006].

*B. cenocepacia* possesses another important iron acquisition system found under condition of iron depletion. This bacterium produces two main siderophores, ornibactin and pyochelin, acting to scavenge free or protein-bound iron from the surrounding environment. Ornibactin has been reported as the biologically more important siderophore, which is able to compensate for the function of pyochelin. Although it has not been reported that these siderophores are used by *B. cenocepacia* to get iron from ferritin, this mechanism could be possible, as occurs in *Mycobacterium* and other bacterial species. In addition to siderophore-mediated mechanisms of iron uptake, *B. cenocepacia* possesses mechanisms for acquiring iron from heme [Visser *et al.*, 2004].

2.1.3. *Listeria monocytogenes* and the Fungus *Candida albicans* Use Surface Reductases to Acquire Iron from Ferritin

Ferric-reductases, as a mechanism to obtain iron from ferritin, is interesting, since reducing Fe$^{3+}$ to Fe$^{2+}$ not only releases iron from the molecule, but also facilitates the assimilation of iron in the ferrous form by the pathogen.

*Listeria monocytogenes*

As a facultative intracellular pathogen and saprophyte, the Gram-positive bacterium *L. monocytogenes* can live in soil and decaying vegetation, but once it enters an animal or human host, it can cause severe disease. This species is not a usual constituent of the human flora, but its ability to grow at 4 °C allows it to contaminate foodstuffs. The great majority (99%) of the infections caused by *L. monocytogenes* are thought to be food borne [Mead *et al.*, 1999; Swaminathan and Gerner-Smidt, 2007]. Infection causes gastroenteritis in healthy human individuals [Orsi *et al.*, 2011]. However, groups at high risk for contracting invasive listeriosis are immune-compromised individuals such as HIV patients, the elderly, infants, and pregnant women [Schlech, 2000]. The most severe clinical manifestations of invasive human listeriosis include septicemia, encephalitis, meningitis, and spontaneous late-term abortion [Orsi *et al.*, 2011]. This pathogen is capable of invading cells within the host, multiplying within the cytoplasm, and spreading from cell-to-cell using a mechanism that exploits the host cell actin [Ramaswamy *et al.*, 2007].
Iron is required by *Listeria* in relatively large amounts to support growth *in vitro* and during experimental infections [Sword, 1966]. Consistently, a *L. monocytogenes* strain defective in hemin/hemoglobin uptake showed attenuated virulence in mice [Jin et al., 2006]. The ability of this bacterium to acquire and utilize iron could be considered as an important virulence factor, since iron is essential to support its growth and survival in various environmental niches, especially during infection; this is why iron acquisition mechanisms in *L. monocytogenes* are thus diverse, complex and flexible. *L. monocytogenes* does not secrete siderophores; however, it mediates iron acquisition by at least five different systems: (i) ferric citrate uptake by a citrate inducible receptor [Adams et al., 1990]; (ii) utilization of exogenous siderophores (xenosiderophores) or siderophore-like molecules [Simon et al., 1995; Jin et al., 2006] and iron-catecholamine complexes [Coulanges et al., 1997; Coulanges et al., 1998]; (iii) acquisition of iron from hemin and hemoglobin through the ATP-binding membrane permease HupC [Newton et al., 2005; Jin et al., 2006]; (iv) acquisition of iron by a cell surface of 126-kDa transferrin-binding protein [Hartford et al., 1993]; and finally, (v) reduction of ferric to ferrous iron from several sources, one of them ferritin, by a bacterial surface-bound reductase or by an extracellular reductase [Cowart and Foster, 1985; Deneer et al., 1995; Barchini and Cowart, 1996].

*L. monocytogenes* can utilize ferritin as an iron source to support its growth in iron deficient media [Jin et al., 2006]. Reduction of Fe$^{3+}$ from ferritin could be after direct contact of the bacterial surface with ferritin [Deneer et al., 1995], or through an extracellular iron reducing activity [Barchini and Cowart, 1996]. This reduction does not involve ferritin degradation to release iron [Deneer et al., 1995]. Slow reduction of iron was also observed from lactoferrin and transferrin, whereas the reduction to Fe$^{2+}$ from ferritin proceeded relatively rapid [Deneer et al., 1995]. However, the responsible reductase and the ferritin receptor remain to be identified.

The ferric reductase activity of *L. monocytogenes* using ferric ammonium citrate or Fe-NTA as an iron source was characterized, showing that the surface bound iron reductase and the secreted one could be the same enzyme, requiring FMN and NADH cofactors [Deneer et al., 1995; Barchini and Cowart, 1996]. After iron reduction, ferrous iron was not released into the medium, suggesting that it is immediately internalized by bacteria. The reducing activity was not affected by the stage of growth, iron depletion or iron repletion [Deneer et al., 1995], but it was increased when cells were grown in aerobic conditions or low temperature (4 °C). This could be analogous to what happens with other virulence determinants of *L.*
monocytogenes [Czuprynski et al., 1989; Stephens et al., 1991]. Therefore, prolonged storage of Listeria at refrigeration temperatures would lead to increased ferric reductase activity and thereby contribute to increased virulence when consumed by a host. However, it is not possible to know if such characteristics are also applicable to the ferritin reducing activity, since it is not known if this is the same activity responsible for reducing iron from ferric ammonium citrate/Fe-NTA.

The listerial determinants favor the escape from the phagosome, which is considered to be iron-limiting, and permit proliferation in the host-cell cytosol, where iron-saturated ferritin is located [Gold et al., 2001; Schaible and Kaufmann, 2004]. It has been suggested that L. monocytogenes can assess the intracellular iron concentration through the mechanism of iron-sensing: in the case of iron-limitation, this resulted in the increased expression of PrfA-regulated virulence factors listeriolysin (LLO) to lyse the membrane and ActA for phagosomal escape, movement in host cytosol and cell-to-cell spread, and in the case of iron-repletion, this resulted in the up-regulation of internalin proteins (InlA and InlB) required for invasion [Bockmann et al., 1996; Conte et al., 1996; Conte et al., 2000; Gray et al., 2006; Lungu et al., 2009]. All the mentioned data allow us to conclude that L. monocytogenes is a well-adapted species capable of acquiring iron in both life styles, as saprophyte and as pathogen inside the host.

Candida albicans

This polymorphic yeast is the most important fungal pathogen of humans; however, it is normally a benign colonizer of human mucosal surfaces [Wilson et al., 2009]. C. albicans is an opportunistic pathogen for immune-compromised people. It is responsible for painful mucosal infections, such as vaginitis in women and oral-pharyngeal thrush in AIDS patients [Kim and Sudbery, 2011]. The severity of candidiasis increases dramatically in people with predisposing factors, such as harshly impaired immunity, cancer, disruption of natural barriers, presence of indwelling catheters, dialysis, and solid organ transplantation [Ruhnke and Maschmeyer, 2002; Perlroth et al., 2007]. In such vulnerable patients it causes severe, life-threatening bloodstream infections and subsequent infections in internal organs [Kim and Sudbery, 2011]. The extraordinary ability of C. albicans to successfully infect virtually every anatomical site reflects a remarkable potential to adapt to various microniches within the human host [Wilson et al., 2009]. One of the key features of C. albicans is its ability to grow in different morphological forms, either as ovoid yeasts, filamentous hyphae, or as pseudohyphae
During the pathogenesis of oral infections, three different sub-stages have been identified: an early/colonization phase, characterized by adhesion of yeasts to upper layers of the oral tissue and fungal proliferation; an invasion phase, associated with hyphae formation and penetration of these layers; and a late phase, associated with extensive tissue destruction [Wilson et al., 2009].

Several studies in mice and a number of clinical observations have shown the importance of iron to *C. albicans* virulence [Abe et al., 1985; Iglesias-Osma et al., 1995]. Iron overload may inhibit T-helper (Th) cell development in mice with candidiasis, thus negatively affecting the course and outcome of the infection [Mencacci et al., 1997]. An undeniable role of iron in the virulence of this microorganism was demonstrated when the *FTRI* gene encoding for a surface iron permease, essential for iron uptake, showed to be crucial for *C. albicans* virulence in an experimental animal model of infection [Ramanan and Wang, 2000]. *In vitro* *C. albicans* was found to require approximately 0.2-0.5 μM iron for its complete unrestricted growth in a chemically defined medium [Sweet and Douglas, 1991; Holbein and Mira de Orduna, 2010]. Furthermore, transition to the hyphal growth, one of the important virulence factors, requires a greater amount of iron than that needed for yeast growth [Sweet and Douglas, 1991]. Therefore, one key factor for adaptation to the host environment is the ability of *C. albicans* to acquire iron within the host’s iron-restricted sites. In consequence, *C. albicans* possesses more than one type of iron acquisition system: for hemoglobin [Santos et al., 2003], for heterologous siderophores [Heymann et al., 2002; Hu et al., 2002] and the reductive uptake system for acquisition of transferrin, ferritin, and free iron [Hammacott et al., 2000; Knight et al., 2002; Knight et al., 2005]. All three iron acquisition systems appear to be independent from each other [Almeida et al., 2008], perhaps because each system is specifically adapted for an environmental niche or because redundancy is desired for this crucial function.

Ferritin promoted the *in vitro* growth of *C. albicans*. Utilization of iron from ferritin was independent of the siderophore and hemoglobin uptake systems and from secreted aspartic proteases. Ferritin use was also proposed occurring *in vivo* using oral epithelial cells enriched in intracellular ferritin, which are more susceptible to tissue damage by wild-type *C. albicans* than cells depleted of ferritin. The reduced damage of iron-depleted epithelial cells correlated with reduced invasion of *C. albicans* hyphal in these cells [Almeida et al., 2008].
Ferritin iron uptake requires the reductive pathway since mutants lacking the high-affinity permease Ftr1 or the copper transporter Ccc2, both essentials for this pathway, were not able to grow in ferritin. This system is located in the plasma membrane and has three components. The first component is a ferric-reductase, which is able to reduce insoluble extracellular ferric (Fe$^{3+}$) ions into soluble Fe$^{2+}$ ions; in the case of ferritin, this reduction step would be required to remove iron from the protein. The second and third components form a protein complex consisting of a ferroxidase and an iron permease, which together transport Fe$^{3+}$ into the cell. Although the identity of the ferric-reductase is still unknown, 17 homologous genes encoding putative surface ferric-reductases have been identified in the *C. albicans* genome [Almeida *et al.*, 2009]. Ferric reductases already characterized in *C. albicans*, like Fre10, have been shown to act over structurally different substrates, increasing the possibility that an intermediate electron carrier performs reduction of ferric iron from ferritin [Knight and Dancis, 2006; Jeeves *et al.*, 2011]. Since Fre10 has FAD- and NADPH-binding motifs, this could mean that it uses an intermediate molecule, like O$_2$ or Flavin, to mediate the electron transfer in analogy to what happens in other ferric reductases [Knight *et al.*, 2002; Schroder *et al.*, 2003; Knight and Dancis, 2006; Jeeves *et al.*, 2011]. Fre10 is increased in response to iron-restricted conditions, and regulated by the transcriptional repressor complex Tup1-Sfu1p (amongst other proteins), in iron replete conditions [Knight *et al.*, 2002; Lan *et al.*, 2004; Pelletier *et al.*, 2007].

The second component is a multi-copper oxidase. Reduced ferrous iron generated by surface reductase activity is toxic, and because of that Fe$^{2+}$ needs to be re-oxidized to Fe$^{3+}$ by multi-copper oxidase activity, thus preventing any damage [De Luca and Wood, 2000; Kosman, 2003]. This re-oxidation to Fe$^{3+}$ may also serve to provide increased substrate specificity to iron import since iron permeases have shown to be highly specific for free Fe$^{3+}$ [Schroder *et al.*, 2003]. The *C. albicans* genome contains five putative multi-copper oxidase genes, two of which, *FET3* and *FET99*, have been characterized [Eck *et al.*, 1999; Knight *et al.*, 2002]. Because of the copper requirement of the oxidase activity, the intracellular copper transporter Ccc2 is essential for this reductive pathway and for ferritin iron uptake [Weissman *et al.*, 2002].

The third component is the ferric permease. *C. albicans* has two iron permeases that are encoded by two highly homologous genes. The high-affinity iron permease gene, *FTR1*, is induced and essential for growth upon iron deprivation and for iron acquisition from ferritin and transferrin
Fungal cells lacking FTR1 lost their ability to damage oral epithelial cells and were completely avirulent in a mouse model of systemic infection [Ramanan and Wang, 2000; Almeida et al., 2008]. These data demonstrate that the Ftr1 protein is an essential component of the reductive pathway both in vitro and in vivo and is thus involved in iron uptake in low-iron environments (such as within the host) and in iron acquisition from at least two different host proteins, including ferritin, making this permease crucial for C. albicans virulence [Almeida et al., 2009]. Additionally, the fungus was only able to use ferritin as an iron source under conditions which allowed acid production (glucose, but not casaminoacids as a carbon source) and acidification of the surrounding environment (low concentrations of buffer at pH 7.4). The reduction of ferric iron from the ferritin core may be facilitated under acidic pH, since it is known that ferritin is unstable at acidic pH and natural recycling of iron from ferritin occurs in the acidic environment of lysosomes [Radisky and Kaplan, 1998; Dominguez-Vera, 2004; Kidane et al., 2006]. A second potential link exists among the external pH, hyphal formation, and iron acquisition. It is well known that the external pH influences hyphal formation [Soll and Mitchell, 1983; Davis et al., 2000]. Also, the balance between the soluble Fe²⁺ ion and the insoluble ferric form Fe³⁺ shifts towards the insoluble form in alkaline pH, therefore acidification facilitates iron acquisition. A close association between C. albicans cells and ferritin is required for the release of iron from ferritin and subsequent uptake into the fungal hyphae, but not yeast-phase cells. Hyphae can bind both purified ferritin, and ferritin contained within epithelial cells, which was demonstrated by electron microscopy [Almeida et al., 2008].

Potential ferritin receptors were searched for among those genes known to encode hyphal-specific proteins that are cell surface localized. Only in the Δals3 gene mutant was the ferritin binding dramatically reduced. Also the presence of both ALS3 transcriptional factors, Tec1 and Bcr1, was necessary for C. albicans cells to bind ferritin. To confirm that ALS3 gene is coded as the ferritin receptor, strains of S. cerevisiae, which does not normally bind ferritin, engineered to express C. albicans Als3 (but not the closely related proteins Als1 or Als5), were able to bind ferritin. Most interestingly, hyphae of a Δals3 mutant grew poorly on media containing ferritin as the sole source of iron, while uptake of free iron was normal. These observations together demonstrate that Als3 functions as a ferritin receptor and gives to C. albicans the capacity to obtain iron from the host ferritin [Almeida et al., 2008].
ALS3 (Agglutinin-Like Sequence) gene encodes a hyphal-specific cell wall protein, which belongs to a family of adhesins (Als family). It is a multifunctional protein playing a key role in multiple processes that are necessary for the organism to colonize the host and cause disease [Liu and Filler, 2011]. In addition to its function as ferritin receptor, it plays an important role in biofilm formation on prosthetic surfaces. Als3 is one of two known C. albicans invasins. Consistent with Als3 being required for the uptake of ferritin iron, and all its other functions, it was found that the Δals3 mutants lost their capacity to damage epithelial cells compared to wild type.

C. albicans hyphae, grown under iron-limiting conditions, or in the presence of excess iron, bound ferritin similarly. Also, they were able to bind ferric ferritin and apoferritin with similar efficiency, indicating that iron within the ferritin shell was dispensable for the binding of ferritin. Thus, these data indicate that the binding of ferritin by C. albicans is morphology associated, but not iron-regulated [Almeida et al., 2008]. These agree with the fact that iron starvation did not increase the expression of Als3 [Liu and Filler, 2011].

Taken together, these data suggest that host ferritin can be used as an iron source by C. albicans. The mechanism proposed could be the direct binding by Als3 on the hyphae surface; iron release is then mediated by acidification and its uptake facilitated by the reductive pathway. Therefore, by using as a ferritin receptor, a protein expressed exclusively in hyphae, C. albicans employs an additional morphology specific and unique iron uptake strategy based on ferritin, while invading the host cells where ferritin is located [Almeida et al., 2008].

2.1.4. Mycobacterium tuberculosis Siderophore Carboximycobactin Is Able to Remove Iron from Host Ferritin

Tuberculosis is a common and often lethal infectious disease caused by various strains of mycobacteria, usually Mycobacterium tuberculosis. Tuberculosis generally infects the lungs but can also affect other parts of the body. Bacteria spread among individuals primarily by aerosolized respiratory secretions. In the host, this microorganism is able to multiply both intracellularly in mononuclear phagocytes, especially in lung macrophages, and extracellularly in lung cavities [Gobin and Horwitz, 1996]. The initial foci of mixed inflammation that develop in the lung following aerosol exposure are called primary lesions [Smith et al., 1970; Ho et al., 1978]. A clinical and pathologic feature that characterizes tuberculosis lesions resulting from primary infection is calcification of granulomas of the lung and lymph node, which often appear on chest radiographs as discrete mineralized densities
[Stead et al., 1968]. Dystrophic calcification replaces the foci of necrosis, which represents irreversible tissue damaged that can persist for the life of the patient.

Iron is essential for growth of \textit{M. tuberculosis} and \textit{M. leprae}. Pathogenic mycobacteria, in order to grow and cause disease within a host, must therefore compete against the host for its supply of iron. A general reaction exhibited by bacteria that face an iron-deficient environment is the synthesis of molecules devoted to iron acquisition. Like many bacteria, mycobacteria synthesize siderophores to capture iron. For mycobacteria, siderophores are of the hydroxamate and mixed-ligand type (hydroxamate mixed with phenolic siderophore structure). Mycobacteria, together with some species of \textit{Nocardioides} and \textit{Rhodococcus} are unique amongst microorganisms in synthesizing both an intracellular siderophore, termed mycobactin, that is cell wall-associated [Snow, 1970], and carboxymycobactin, an extracellular siderophore [Ratledge, 2004]. \textit{M. tuberculosis} siderophores are salicylate containing molecules. Two forms of mycobactin are produced, which differ in the length of an alkyl substitution and, hence in polarity and solubility. The more polar form is the carboxymycobactin that is released into the medium, whereas mycobactin, which is the less polar form, remains cell wall associated [Rodriguez, 2006]. Carboxymicobactin has the capacity to remove iron bound to the host iron-binding proteins transferrin, lactoferrin, and ferritin. Purified carboxymycobactin rapidly removed iron from transferrin; it was either 95 or 40% iron-saturated, and from human lactoferrin. Carboxymycobactin also removed iron, but at slower rate, from the iron storage protein ferritin [Gobin and Horwitz, 1996]. The uptake of carboxymycobactin is a process which is not energy-linked. Although nothing substantive is known about the energy-independent process, it could involve the participation of a porin protein such as that described in \textit{M. smegmatis} and \textit{M. chelonae} [Trias et al., 1992; Trias and Benz, 1994] and which also may occur in \textit{M. tuberculosis} [Liu et al., 2009]. Calder and Horowits (1998) identified two iron-regulated proteins from \textit{M. tuberculosis} that may be participating in the uptake of ferri-carboxymycobactin [Calder and Horwitz, 1998]. The two proteins, Irp10 and Mta72, could function as a two-component metal transport system. Recently, other researchers [Rodriguez and Smith, 2006] identified two genes \textit{(irtA} and \textit{irtB}) encoding an ABC transporter. IrtA and IrtB proteins are required for efficient utilization of iron from ferri-carboxymycobactin. These researchers postulate that IrtAB is a transporter of ferri-carboxymycobactin. Later, iron could be released from the carboxymycobactin by a reductase mechanism to other iron containing...
proteins, such as bacterioferritin or could be transferred to mycobactin as the cell wall store of iron [Ratledge, 2004]. Gobin and Horwitz (1996) have shown that exchange of iron occurs from ferri-carboxymycobactin into the mycobactin within the cell envelope of *M. tuberculosis*, even though both molecules probably have the same binding affinities for iron [Gobin and Horwitz, 1996]; this can be explained because the latter molecule could be at a greater concentration within the envelope. Mycobactin releases the iron by means of a ferric-mycobactin reductase in which the ferric iron is reduced in the presence of NAD (P) H to ferrous iron [Brown and Ratledge, 1975; McCready and Ratledge, 1979]. The exact mechanism of iron transfer is not understood, although it has been shown that salicylate can function as an acceptor of Fe (II) after reduction of mycobatin.

The importance of iron in tuberculosis is shown in an *in vivo* study by Basarba et al. (2008) in which they showed that ferric iron accumulates both intra- and extra-cellularly in the primary lung lesions of guinea pigs aerosol-infected with *M. tuberculosis* H37Rv strain [Basaraba et al., 2008]. Iron was accumulated within macrophages at the periphery of the primary granulomatous lesions while extra-cellular ferric iron was concentrated in areas of lesion necrosis. Accumulation of iron within primary lesions was preceded by an increase in expression of H-ferritin, lactoferrin and receptors for transferrin, primarily by macrophages and granulocytes. The authors also found that the increased expression of intra-cellular H-ferritin and extra-cellular lactoferrin, more so than transferrin receptor, paralleled the development of necrosis within primary lesions. On the other hand, primary lung lesions from guinea pigs vaccinated with *Mycobacterium bovis* BCG prior to an experimental infection, reduced iron accumulation as well as H-ferritin, lactoferrin, and transferrin receptor expression. Then the amelioration of primary lesion necrosis was coincident with lack of extra-cellular ferric iron and lactoferrin accumulation.

### 2.1.5. *Escherichia coli* and *Yersinia pestis* Are Able to Obtain Iron from Ferritin

*Escherichia coli* and *Yersinia* spp are Gram-negative bacteria that produce intestinal diseases. *E. coli* secretes two types of siderophores, enterochelin (catechol) and aerobactin (hydroxamate). Brock et al. (1991) described that both siderophores may acquire iron from different sources, enterochelin scavenging predominantly transferrin-bound iron and aerobactin obtaining iron preferentially from cell or tissues (ferritin) [Brock et al., 1991]. In the case of *Yersinia*, it comprises three pathogenic species. The most notorious
member is *Yersinia pestis*, the causative agent of bubonic and pneumonic plague [Perry, 1993]. *Y. pestis* possesses systems to obtain iron during transient intracellular or extracellular growth in mammals. Although *Y. pestis* uses a wide variety of heme containing compounds as iron sources, some molecules, such as ferritin, support the growth of this bacterium [Perry and Brubaker, 1979; Sikkema and Brubaker, 1989]. *Y. pestis* possesses a functional Fur protein that could participate in ferric iron transport as well as hemin, heme/hemopexin, heme/albumin, ferritin, hemoglobin and hemoglobin/haptoglobin utilization [Staggs and Perry, 1991]. Kirillina et al. (2006) proposed that the inorganic iron ABC transporter Yfe of *Y. pestis* is responsible for uptake of available iron in the spleen and liver, possibly for ferritin stores or other intracellular iron reservoirs [Kirillina et al., 2006].

### 2.1.6. *Streptococcus pyogenes* Uses Ferritin as an Iron Source

*Streptococcus pyogenes* (β-hemolytic group-A *Streptococcus*) is a cause of significant morbidity and mortality worldwide. This Gram-positive respiratory and skin pathogen can be carried by humans asymptotically or cause uncomplicated pharyngitis; however, *S. pyogenes* can also cause life-threatening diseases such as streptococcal toxic-shock syndrome and necrotizing fasciitis. *S. pyogenes* is also an important contributor to mortality associated with the influenza virus [Simonsen et al., 2000; Morens and Fauci, 2007; Dmitriev and Chaussee, 2010].

*S. pyogenes* encodes a set of virulence factors necessary for the pathogenesis, such as adhesion to cells and evasion of the immune system. The *S. pyogenes* adhesion process is achieved by bacterial adhesins to several host molecules such as integrins, fibrinogen, collagen and extracellular matrix proteins [Cunningham, 2000; Nobbs et al., 2009]. Another important virulence factor is the secreted lytic enzymes capable of hydrolyzing important host cell molecules involved in modulation of the human immune system [Collin and Olsen, 2003; Chiang-Ni and Wu, 2008]. There is only one report about the use of ferritin as an iron source by this pathogen [Eichenbaum et al., 1996]. This research group observed that 5 mg of ferritin were able to support the growth of the *S. pyogenes* culture in an iron-deprived condition. However, the iron acquisition mechanism by which this bacterium uses ferritin is still unknown. *S. pyogenes* secretes molecules known to produce cellular lysis such as proteases and streptolysin; in this way, several iron containing proteins from infected cells can be released, such as ferritin, hemoglobin, and myoglobin, which could be used as iron source by this bacterium. Surely *S. pyogenes* will be studied in this aspect in the close future since it may be of interest to know
whether ferritin use is a virulence mechanism utilized by this important pathogen to invade the human host.

2.1.7. Bacillus cereus Uses Als3 as a Ferritin Binding Protein

*Bacillus cereus* is a Gram-positive, motile, aerobic-to facultative, spore-forming rod widely distributed environmentally. The bacterium exists as a spore former and vegetative cell in nature and only as a vegetative cell when colonizing the human body [Bottone, 2010]. *B. cereus* is generally regarded as a pathogen causing food-borne gastroenteritis due to the production of diarrheal or emetic toxins [Granum and Lund, 1997; Kotiranta et al., 2000]. In addition to food poisoning, *B. cereus* causes a number of systemic and local infections in both immunologically compromised and immunocompetent individuals. Among those most commonly infected are neonates, intravenous drug abusers, patients suffering traumatic or surgical wounds, and those with indwelling catheters. The spectrum of infections include fulminant bacteremia, central nervous system (CNS) involvement (meningitis and brain abscesses), endophthalmitis, pneumonia, and gas gangrene-like cutaneous infections, to mention a few [Bottone, 2010].

Larva infection of the lepidopterans, like *Galleria mellonella*, is used as a model to study *B. cereus*, since this bacterium can cause infection in insects, mice and other mammals [Salamitou et al., 2000; Vilas-Boas et al., 2007]. A mutant strain of *B. cereus*, lacking a ferric dicitrate transporter, showed attenuated virulence in a lepidopteran infection model, highlighting the importance of iron and iron uptake systems to the virulence of *B. cereus* [Harvie and Ellar, 2005]. Accordingly, *B. cereus* has developed strategies to use iron-containing proteins from the host. It is able to use hemoglobin (2 µM), hemin (16.5 µM) and ferritin (0.3 µM) as iron sources for growth in iron-depleted media. The use of transferrin and lactoferrin is controversial, and might be strain dependent [Sato et al., 1999; Park et al., 2005; Daou et al., 2009].

*B. cereus* lacking IlsA protein showed significant growth defects in iron-depleted media that had been supplemented with hemoglobin, hemin, or ferritin. *ilsA* mutants had no problem with the uptake of inorganic iron, suggesting that IlsA is involved in iron acquisition from these three host proteins during infection [Daou et al., 2009]. In vivo, disruption of *ilsA* decreased the growth and virulence of *B. cereus* after oral inoculation or injection into the insect hemocoel [Fedhila et al., 2006; Daou et al., 2009]; these observations indicate that IlsA is an important factor required for adaptation within an insect host. Using a plasmid with a construction fusioning
the *ilsA* promoter (*pIl*A) with the reporter gene of the green fluorescent protein (*GFP*), it was observed that *ilsA* is highly expressed in the hemocoel during infection of the insect larvae. Actually, in insects, notably in *G. mellonella*, ferritin is present in high amount in hemolymph and in hemocytes and may play a role in iron transport in addition to iron storage [Kim *et al.*, 2001; Ji-Eun *et al.*, 2005]. In others hosts, where ferritin is mostly intracellular, an extracellular pathogen such as *B. cereus* would need to release ferritin in order to have access to it. In fact, it has been shown that *B. cereus* produces a large variety of cytotoxic proteins (Hbl, Nhe, CytK, Clo, and HlyII), all of them able to lyse various eukaryotic cells [Lindback *et al.*, 1999; Andreeva *et al.*, 2006; Fagerlund *et al.*, 2008].

*ilsA* (regulated leucine-rich surface protein) gene possesses a Fur-binding box in the promoter region. The protein has an N-terminal peptide signal, and three conserved domains: NEAT domain (N-terminal iron transport-associated domain), LRR (Leucine-Rich Repeat), and SLH (S-Layer Homology) [Fedhila *et al.*, 2006]. *ilsA* is expressed in iron-depleted conditions, which is consistent with the presence of a Fur binding box for iron regulation in its promoter [Fedhila *et al.*, 2006]. It is present in the surface of the bacteria, accumulating at the division site [Daou *et al.*, 2009]. The surface localization may be due to a SLH-domain that presumably binds the protein to peptidoglycan [Fouet and Mesnage, 2002; Fedhila *et al.*, 2006].

Direct interaction between ferritin and IlsA was demonstrated using binding studies with ferritin immobilized on ELISA plates and the purified recombinant protein GST-IlsA. By using Surface Plasmon Resonance (SPR), it was shown that these interactions occur with soluble proteins in real time and under flow conditions. Therefore, *B. cereus* should use hemoglobin, hemin and ferritin, after direct binding to the surface receptor IlsA [Daou *et al.*, 2009]. The NEAT domain is suggested to mediate heme binding to IlsA. However, the interaction with ferritin is much less evident. LRR domains are known to bind structurally unrelated protein ligands [Kobe and Kajava, 2001; Bierne *et al.*, 2007], so it could be speculated that LRR domains are involved in IlsA binding to ferritin. Although both heme and ferritin use the same receptor IlsA, the mechanism of iron uptake should be different for each protein. The authors hypothesize that IlsA might be able to destabilize the ferritin structure, via a possible interaction with the LRR domains [Daou *et al.*, 2009]. The structural modification may permit other factors, such as reductases-like, as occurs in *Listeria* spp. [Deneer *et al.*, 1995], or proteases-like, as occurs in *Burkholderia* [Whitby *et al.*, 2006], to liberate iron (Fe³⁺) that could be captured by siderophores produced by *B. cereus*, such as
petrobactin or bacillibactin [Wilson et al., 2006] and transferred by an iron uptake-system into the cytosol [Daou et al., 2009]. Actually, bacillibactin, like related tris-catecholate siderophore, binds iron with high affinity ($K_a=10^{-47.6}$) [Dertz et al., 2006] that is markedly greater than that of ferritin [Ratledge and Dover, 2000]. However, this is only a hypothesis and needs to be confirmed.

2.2. Parasitic Protozoa Must Get Iron from the Host Resources to Survive

Parasitic protozoa are broadly spread pathogens responsible for important and often fatal diseases worldwide [Lambert and Barragan, 2010]. Parasites and their corresponding hosts have co-evolved during their life in the Earth, in such a way that parasites have been adapted to only live inside a host, and in several cases, in an exclusive host. Some parasites spend one part of their life cycle in an invertebrate vector and the other part in a vertebrate host in which express the pathogenicity determinants [Bannister et al., 2000; Sacks, 2001; Macedo et al., 2004; Lodge and Descoteaux, 2005; Garcia et al., 2007; Carvalho et al., 2009]. Parasites can live in intracellular niches [Simpson et al., 2006; Ronnebaumer et al., 2008; Ueno et al., 2009; Landfear, 2011], but some species can live extracellularly in fluids and secretions [Felleisen, 1999]. In both sites, parasites express nutrient uptake systems such as those to get iron, among other diverse virulence mechanisms [Burchmore and Barrett, 2001; Azema et al., 2004; Ronnebaumer et al., 2008; Blume et al., 2009; Landfear, 2011]. As such, parasitic life requires a repertoire of adaptations to assure entry/exit from the cell and evade the immune responses to prevent clearance [Sibley, 2011]. The mechanisms of intracellular iron transport and its delivery to organelles are poorly understood in parasitic protists. Several authors propose that iron is transported within the cell through a complex with low-molecular mass ligands; this mobile iron is referred as labile-iron pool (LIP), which consists of weakly-bound iron associated with compounds of low molecular mass (5-30 kDa)). Parasitic protozoa must also store the iron excess; however, in the parasites studied, a ferritin has not been found; instead, perhaps iron is stored in cytosol as LIP [Suchan et al., 2003].

2.2.1. Ferritin as an Intracellular Iron Source for Parasitic Protozoa

Host ferritin is an abundant intracellular-iron store and could be an important source of this element for parasites growing inside a cell. In addition, ferritin could be easily accessible to parasites that produce
lytic enzymes that destroy cells. As protist unicellular protozoa, the use of cationic ferritin has facilitated the study of binding and endocytosis of this protein in experiments with electronic microscopy; due to ferritin being electron-dense it has been used as a microscopy tracer. Reports about pathogenic parasites using ferritin as an iron source are few, including *Trichomonas vaginalis* and *Entamoeba histolytica*. In the case of *Tritrichomonas foetus* and *Trypanosoma brucei*, there are reports about endocytosis of ferritin, but its use as an iron source has not been demonstrated to date.

### 2.2.1.1. *Tritrichomonas foetus* Can Endocytose Ferritin

*Tritrichomonas foetus* is a sexually transmitted protozoan that infects the female genital tract of cattle resulting in abortion, endometritis, and infertility [Manning, 2010; Pereira-Neves *et al.*, 2011]. This parasite causes significant economic losses to cattle producers worldwide. Also, some strains of this parasite have been recognized as causing diarrhea in cats [Gookin *et al.*, 1999] and mild rhinitis in swine [Lun *et al.*, 2005]. As an amitochondrial anaerobic parasite, *T. foetus* depends on high iron requirements (50–100 µM) [Tachezy *et al.*, 1996], surpassing those of eukaryotic cells, although comparable to other anaerobic amitochondrial protists. To face its iron requirement, *T. foetus* is able to utilize lactoferrin, transferrin, or LIP complexes [Tachezy *et al.*, 1996; Tachezy *et al.*, 1998]. Involvement of iron in *T. foetus* virulence has been examined in an experimental mouse infection in which the administration of ferric ammonium citrate to infected mice increased the mortality rate [Kulda *et al.*, 1999].

Previous studies of endocytic processes in *T. foetus* have described that this parasite has a high endocytic activity through vesicles of different size and shape [Affonso *et al.*, 1994]. At 4°C, the temperature at which the endocytic processes are stopped, ferritin was only observed in patches; however, at 37°C, no labeling of the plasma membrane was observed. Cytoplasmic positive compartments were observed as pleomorphic or round structures generally filled with ferritin; the internalization of ferritin was followed for 60 min. Thus, this parasite is capable of internalize ferritin [Affonso *et al.*, 1997]. The mechanism by which ferritin is endocytosed is still unknown.

### 2.2.1.2. *Trypanosoma brucei* Endocytoses and Digests Ferritin

*Trypanosoma brucei* causes African trypanosomiasis or sleeping-sickness in humans and nagana in cattle [Roberts *et al.*, 2005]. An estimated 60 million people are infected [WHO, 2001] and 48,000 deaths were reported in 2002.
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[WHO, 2004]. In addition, 46 million cattle are at risk of contracting nagana with a high cost to people working in livestock production [Kristjanson et al., 1999]. The life cycle of this parasite requires a tsetse fly (Glossina spp.) that feeds on blood from an infected host. The parasite metacyclic stage is the infective form to the mammalian host. After transmission, protozoa multiply extracellularly in the blood, lymphatic system and interstitial spaces and then, during latter stages of infection, in the central nervous system [Breidbach et al., 2002; Roberts et al., 2005; Peacock et al., 2011]. Bloodstream trypanosomes are highly pleomorphic forms [short stumpy (SS), intermediate short-stumpy (ISS) and dividing long-slender (LS) forms]; during the life cycle, the LS forms transform in SS forms in the blood and SS transform into the midgut forms in the fly [Langreth and Balber, 1975]. The endocytosis of transferrin by T. brucei has been well-demonstrated [Schell et al., 1991]: holotransferrin, after being bound to its receptor at the flagellar pocket (the main site of uptake of exogenous proteins), is endocytosed and delivered to an endosomal system and, subsequently, to dissociation of the receptor-ligand complex [Maier and Steverding, 1996]. The unoccupied receptor is recycled [Steverding et al., 1995], whereas transferrin is transported to lysosomes and proteolytically degraded [Grab et al., 1992; Steverding et al., 1995]; Steverding determined that accumulation of only 40,000 iron atoms/cell during the generation-doubling time of 10 hours appears to be sufficient to support their multiplication [Steverding, 1998]. A single trypanosome contains 1.4 x 10^6 iron-atoms as determined by atomic absorption spectrometry [Schell et al., 1991]; thus bloodstream forms of T. brucei are capable of storing iron.

Concerning cationic ferritin, it was demonstrated the uptake and digestion of this protein by transmission electron microscopy [Langreth and Balber, 1975]. Ferritin was observed to be up taken rapidly in the flagellar pocket of the SS bloodstream trypanosomes at 25 °C and then appeared in the cell in large vesicles, which appeared invaginate from the pocket. Since ferritin was not observed in these vesicles at 0 °C, the process requires energy. After that, ferritin was observed in large vesicles fusioned with smooth straight tubules of the collecting system and at a later time in acidic vesicles with digestive activity, probably lysosomes. In this way, T. brucei is most probably capable of using ferritin iron, which is released at acidic pH, since it is known that ferritin is unstable at acidic pH and natural recycling of iron from ferritin occurs in the acidic environment of lysosomes [Goswami et al., 2002; Nadadur et al., 2008; Zhang and Enns, 2009].
2.2.1.3. *Trichomonas vaginalis* Utilizes Ferritin in Cultures in Vitro

*Trichomonas vaginalis* is a sexually transmitted extracellular flagellated single-cell parasitic protozoan that lives in the female lower reproductive tract and the male urethra. Unique genetic and structural features place the parasite at the base of the eukaryotic phylogenetic tree and suggest an intriguing evolution toward mucosal parasitism [Wolner-Hanssen *et al.*, 1989; Shafir *et al.*, 2009]. *T. vaginalis* selectively adheres to the human vaginal epithelial cells, surviving for years in the hostile vaginal environment that is typically acidic, contains a plethora of microbicidal innate-immune factors, and is reinforced by the presence of a complex commensal bacterial consortium [Lehker and Sweeney, 1999; Van der Pol, 2007]. Iron concentration is a key factor in the virulence of this parasite, since its metabolic activity, cytoadherence, and resistance to complement lysis are increased by iron [Tsai *et al.*, 2002; Garcia *et al.*, 2003].

In 1992, it was reported that *T. vaginalis* is capable of using ferritin as an iron source in cultures *in vitro*. The group of research demonstrated that ferritin and lactoferrin (each 250 µM), but not transferrin, were used by this parasite *in vitro* as iron sources and suggested that these iron-containing proteins could be used as an iron source necessary for growth and colonization of the vaginal mucosa. The iron acquisition system from lactoferrin and ferritin is very important for the infection process, due to the iron-limiting environment in the vagina. Currently, the iron acquisition process from host cellular ferritin by *T. vaginalis* is unknown [Lehker and Alderete, 1992].

2.2.1.4. *Entamoeba histolytica* Possesses a Ferritin Binding-Protein and Endocytoses Ferritin by Clathrin-coated Pits

*Entamoeba histolytica* is an enteric parasitic protozoan that causes amoebiasis, a cosmopolitan infection that affects only human beings. Cyst is the infective stage transmitted by the fecal-oral route through the intake of contaminated water and foods. When cysts are ingested they can pass throughout the acidic pH of stomach, and in terminal ileum excystation occurs producing the invasive stage or trophozoites. These primitive eukaryotic cells are able to adhere and invade the colon-intestinal mucosa causing dysentery, fever and abdominal pain; severe cases can lead to ulcerative colitis. By unknown reasons, trophozoites can travel to the liver via portal vein, producing liver abscesses, which can be fatal if not treated. Amoebae can also invade other organs, especially the brain and lungs [Espinosa-Cantellano and Martinez-Palomo, 2000]. Amoebiasis is the third cause of death by parasites
worldwide. An estimate of 50 million people are symptomatically infected and 100,000 people die annually, mainly in developing countries because of the poor hygienic conditions [Stanley, 2003; Ali et al., 2008]. In response to unknown stimuli, amoebae undergo morphological and biochemical changes leading to the formation of cysts, which are eliminated in the feces, closing the cycle [Martinez-Palomo, 1987].

E. histolytica requires a large amount of iron for growth in vitro (80-100 µM Fe), and this fact probably reflects its iron necessity in vivo. This parasite is able to use several host iron-containing proteins for growing in vitro, such as hemoglobin (Hb), ferric transferrin (holoTf) and ferric lactoferrin (holoLf) [Serrano-Luna et al., 1998; Reyes-Lopez et al., 2001; Leon-Sicairos et al., 2005]. Recently, our group reported the interaction between the amoebic trophozoites and ferritin. By using growth kinetics in iron-chelated medium to which different concentration of ferritin was added as a sole iron source, we found that E. histolytica cultures developed at an optimal concentration of 100 µM Fe provided by ferritin. Uptake of this molecule could be mediated by a ferritin binding-protein (EhFbp), since the binding of FITC-ferritin to amoebae was dependent on the ligand concentration, time, and temperature of incubation. Furthermore, the binding was highly specific for ferritin, since other iron-proteins such as Hb, holoTf and holoLf did not compete with ferritin for the binding. Trophozoites surface was saturable at 46 nM of ferritin [Lopez-Soto et al., 2009].

To investigate whether ferritin is endocytosed by trophozoites, we used some inhibitors of diverse endocytosis pathways and demonstrated the ferritin endocytosis by confocal laser-scanning microscopy (CLSM) using FITC-ferritin. Amoebae quickly internalized ferritin (in the first 2 min of interaction). Ferritin entrance was found constrained only by inhibitors of clathrin-coated pits such as chloroquine, NH₄Cl, sucrose, and chlorpromazine. Filipin, which disrupts caveolae structures, or wortmannin, which affects fluid-phase endocytosis, did not inhibit ferritin endocytosis. This result was confirmed by electronic microscopy: cationic ferritin was observed inside clathrin-covered vesicles by using an anti-bovine brain clathrin Ab. Interestingly, after 30 min of incubation, ferritin co-localized with an anti-rat LAMP-2 Ab in amoebic lysosomes by CLSM, suggesting its degradation in these organelles. In amoebic extracts analyzed by electrophoresis of gels copolymerized with ferritin (substrate gels), three internal neutral cysteine-proteases (100, 75, and 50 kDa) of E. histolytica were observed cleaving ferritin [Lopez-Soto et al., 2009]. The results together suggest that E.
_Histolytica_ possesses vesicular proteins related to clathrin and lysosome-like structures that are used by trophozoites for ferritin endocytosis and degradation, a process that seems to be similar to that used by mammalian cells. However, the amoeba receptor and thus its similarity to the mammalian receptor, remain yet to be identified. This mechanism of endocytosis for use of ferritin as an iron source has not been described in other parasites.

As we mentioned before, trophozoites from this parasite are able to invade the human liver causing fatal hepatic abscesses. Furthermore, cysteine-proteases, amoebopores, cytolysins and other factors contribute to liver tissue degradation in hepatic amoebiasis [Perez-Tamayo _et al._, 2006]. Therefore, although the use of this iron-rich protein was not investigated _in vivo_, ferritin could be exposed to the environment by the alteration of liver tissue and being used as an iron source for amoebae, recrudescing the extra-intestinal phase of the disease.

### Conclusion

As we can see, microorganisms have developed numerous ways to acquire iron from ferritin. The mechanism could be as simple and general as the iron acquisition from a xenosiderophore, which the same microorganism does not even need to synthesize, or it could be much more elaborate, like _Neisseria_, which manipulates the host cell so magnificently in order to get ferritin iron. When the microorganism has several mechanisms in order to get iron from ferritin, it could seem a redundant function; however, it is only implying the importance of this rich iron source for the infection success.

Only a few ferritin receptors or surface binding proteins have been studied in microorganisms; moreover, there is no obvious structural homology between them, for example, Als3 from _B. cereus_ and IlsA from _C. albicans_, or even between the microorganism receptors and the mammalian receptor Tim2. Thus, depth structural studies related to the interaction between those receptors and ferritin are needed to elucidate this aspect.

Also, we can perceive that microorganisms do not need to produce specific proteins dedicated to bind ferritin to acquire its iron; in the case of Als3 it also binds hemin and hemoglobin to resolve this trouble. Interestingly, Als3 is a multifunctional protein with many roles in addition to capturing iron. This fact is in agreement with what has been found in some pathogens’ transferrin receptors, like staphylococcal GAPDH or _E. histolytica_ EhADH2.
Both are enzymes working like receptors of a host iron-protein; this is telling us how microorganisms are “economic beings,” taking advantage of proteins with other functions to acquire the host iron.

Finally, given the importance of the iron acquisition mechanisms and the fact that they have no parallel in the host, inhibition of iron binding and transport might be an attractive therapeutic target.

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