Metal-dependent gene regulation in the causative agent of Lyme disease

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Borrelia burgdorferi (Bb) is the causative agent of Lyme disease transmitted to humans by ticks of the Ixodes spp. Bb is a unique bacterial pathogen because it does not require iron (Fe^{2+}) for its metabolism. Bb encodes a ferritin-like Dps homolog called NapA (also called BicA), which can bind Fe or copper (Cu^{2+}), and a manganese (Mn^{2+}) transport protein, Borrelia metal transporter A (BmtA); both proteins are required for colonization of the tick vector, but BmtA is also required for the murine host. This demonstrates that Bb’s metal homeostasis is a critical facet of the complex enzootic life cycle between the arthropod and murine hosts. Although metals are known to influence the expression of virulence determinants during infection, it is unknown how or if metals regulate virulence in Bb. Recent evidence demonstrates that Bb modulates the intracellular Mn^{2+} and zinc (Zn^{2+}) content and, in turn, these metals regulate gene expression through influencing the Ferric Uptake Regulator (Fur) homolog Borrelia Oxidative Stress Regulator (BosR). This mini-review focuses on the burgeoning study of metal-dependent gene regulation within Bb.

Keywords: Borrelia burgdorferi, Lyme disease, copper, manganese, zinc, calprotectin

INTRODUCTION

Borrelia burgdorferi (Bb) is the causative agent of a multisystem disorder known as Lyme disease. Bb persists within an enzootic cycle that includes two diverse hosts, a tick vector and a warm-blooded host, typically small rodents. “Hard” ticks of the Ixodes genus are important arthropod hosts for colonization by Bb. Ixodes ticks are slow-feeding ticks (∼48 h for a bloodmeal) that have a 2-year life cycle including three distinct stages: larvae, nymph, and adult (Figure 1). At each stage, ticks will feed once on a warm-blooded host then undergo a molting process, which precedes a period of dormancy that may last months (Figure 1). Because Bb colonization of ticks does not appear to occur through transovarial transmittance, unfed larvae ticks are naïve and acquire Bb during feeding on an infected warm-blooded host. Feeding ticks can acquire Bb at any stage of the usual 2-year life cycle and transmission of Bb can occur during feeding on an animal host at any subsequent stage of the life cycle. Small rodents (especially the white-footed mouse, Peromyscus leucopus) are the primary animal reservoirs for Bb within this enzootic cycle and are sources for the bloodmeal during the larval and nymphal stages (Figure 1). Unlike most bacterial pathogens, Bb lacks lipopolysaccharide (LPS), lipooligosaccharide (LOS), and capsule (Radolf and Samuels, 2010). Bb is highly motile due to the presence of flagella; however, Bb’s flagella are contained within the periplasmic space between the outer and inner membranes. Therefore, Bb’s flagella is not surface exposed and is called an endoflagella. The endoflagella are anchored at each end of the cell and provide Bb with a characteristic corkscrew movement. Despite Bb’s limited metabolism and fastidious nature Bb survives within two hosts, a tick vector and a small rodent host. Other animals, such as humans, are infected by Bb, but are not considered important for persistence of Bb within the enzootic cycle. Of significant interest, Bb is one of the few pathogens that does not require iron (Fe^{2+}) to grow (Posey and Gherardini, 2000). Given the importance of Fe^{2+} in the regulation of virulence within other bacteria, it is not clear which metals Bb utilize for regulating virulence factors. Recent work suggests that metals may play an important role in regulation of virulence within Bb.

Metal homeostasis is important to maintain the metabolism of bacterial pathogens. This is accomplished through the combined action of metal transporters, both importers and exporters, which control the abundance of specific metals and the ratio of the transition metals within the cell. Although some metal transporters are highly specific for a cognate metal, others are capable of importing several metals with different affinity of each metal. In addition to the importance of metals in bacterial physiology, metals play a critical role in the control of gene regulation within pathogens. The role of metals within Bb is not fully understood. Only a single protein, Borrelia metal transporter A (BmtA) is known to participate in metal transport. Analysis of the intracellular metal content with in vitro grown Bb suggests that BmtA transports Mn^{2+} since this metal is nearly undetectable in ΔbmtA strains (Ouyang et al., 2009a; Troxell et al., 2013). BmtA may also be involved in the import/export of other metals since deletion of bmtA alters the intracellular concentrations of Fe^{2+}, Cu^{2+}, and Zn^{2+} (Wang et al., 2012). The mechanism of BmtA-dependent metal transport is still unknown, but recent evidence indicates that BmtA and Mn^{2+} are involved in regulation of virulence through a Ferric uptake regulator (Fur) homolog named Borrelia Oxidative Stress Regulator (BosR). BosR is redox sensing DNA binding protein that utilizes Zn^{2+} as a cofactor (Boylan et al., 2003; Katona et al., 2004). Discussed here is the role of metals in...
Bb physiology and gene expression as it relates to virulence factors required in vivo.

**Bb: A NON-COMBATANT IN THE WAR FOR Fe^{2+}**

Just as a siege limits the influx of food and supplies to an enemy’s stronghold, during infection the host transports metals away from the locale of pathogens and synthesizes copious amounts of metal-chelating proteins to limit access of these essential micronutrients. The hosts’ ability to produce metal-chelating proteins is essential for nutritional immunity, but recent evidence demonstrates the importance of chelating Zn^{2+} and Mn^{2+} in thwarting bacterial infections (Kehl-Fie and Skaar, 2010). However, as part of the antimicrobial defense present within ticks, an antimicrobial peptide, known as microplusin, inhibits bacterial growth by Cu^{2+} chelation (Silva et al., 2009, 2011). Microplusin is expressed within the hemocoel of ticks (Esteves et al., 2009), implying that this locale is a Cu^{2+} limited environment. Bb does not appear to transport Fe^{2+}, lacks many biosynthetic and catabolic pathways that require Fe^{2+}, and exhibits no defect in growth in the absence of detectable Fe^{2+} (Posey and Gherardini, 2000). Although a recent study indicates there is detectable Fe^{2+} within Bb, the physiological relevance of this finding remains uncertain (Wang et al., 2012). Another study did not detect intracellular Fe^{2+} following *in vitro* cultivation of Bb (Aguirre et al., 2013). Therefore, additional experiments are required to address these discrepancies. At this point, how Fe^{2+} is transported within Bb is unknown. Future work is required to determine the contribution of intracellular Fe^{2+} to Bb gene regulation and metabolism. Instead, because calprotectin inhibits Bb growth by Zn^{2+} sequestration, the existing data suggests that Zn^{2+} is an important metal within the metabolism of Bb. This is supported by the Zn^{2+}-dependent enzymatic activity of peptide deformylase (Nguyen et al., 2007) and the glycolytic enzyme fructose-1,6-bisphosphate aldolase (Bourret et al., 2011). Furthermore, peptide deformylase may be an essential enzyme ( Jain et al., 2005) and, since glycolysis is the sole mechanism for the generation of ATP within Bb, Zn^{2+} may be a critical metal for Bb.

**BicA AND BmtA: TWO PROTEINS WITH NOVEL FUNCTION WITHIN Bb**

Bacteria encode metal binding proteins (ferritins or ferritin-like proteins) that store metals and serve as a facile source of essential metals when encountering a metal-depleted environment. Bb encodes a metal binding protein (NapA or BicA) that exhibits homology to the ferritin-like Dps present in other bacteria. Purified BicA is capable of binding Fe^{2+} or Cu^{2+}, but lacks either metal when isolated (Li et al., 2007; Wang et al., 2012). The majority of studies have focused on the role of Fe^{2+} chelation by the host in nutritional immunity, but recent evidence demonstrates the importance of chelating Zn^{2+} and Mn^{2+} in thwarting bacterial infections (Kehl-Fie and Skaar, 2010). However, as part of the antimicrobial defense present within ticks, an antimicrobial peptide, known as microplusin, inhibits bacterial growth by Cu^{2+} chelation (Silva et al., 2009, 2011). Microplusin is expressed within the hemocoel of ticks (Esteves et al., 2009), implying that this locale is a Cu^{2+} limited environment. Bb does appear to regulate its intracellular Cu^{2+}, but the relevance or need for Cu^{2+} is unknown (Wang et al., 2012). The importance of BicA to the enzootic cycle is restricted to residence within the tick vector (Li et al., 2007), implying that Zn^{2+} and Cu^{2+} are limiting within this host.

The role of Mn^{2+} in Bb metabolism is not understood. The gene bmtA, encoding a Mn^{2+} transport protein BmtA, is not essential for *in vitro* growth within virulent Bb strains from the B31 (tick isolated) and 297 (human isolated) lineages despite reducing cellular Mn^{2+} to near undetectable concentrations.
(Ouyang et al., 2009a; Troxell et al., 2013). Bb cultivation in vitro requires a complex growth medium called BSK (Barbour, 1984). Treatment of BSK medium with a chelating resin, called Chelex, results in significant changes of the concentrations of metals. Chelex treatment of BSK reduces Zn\(^{2+}\), but Mn\(^{2+}\) becomes undetectable in the medium. Despite the undetectable Mn\(^{2+}\) in Chelex-treated BSK growth medium, no growth defects are observed for wild-type or ΔbmtA strains during cultivation in this medium (Troxell et al., 2013). BmtA has homology to the GuA family of metal transporters (Guerinot, 2000). BmtA has 8 membrane spanning domains and is predicted to transport cations through a novel mechanism (Ouyang et al., 2009a). To date, only a single protein within Bb is characterized as being Mn\(^{2+}\)-dependent; specifically, the superoxide dismutase (SOD) encoded by sodA (Troxell et al., 2012; Aguirre et al., 2013). The expression of bmtA and the intracellular concentration of Mn\(^{2+}\) are enhanced during cultivation at 25°C, suggesting there may be a requirement for Mn\(^{2+}\) at cooler temperatures (Ojaimi et al., 2003; Troxell et al., 2013). The physiological need for more Mn\(^{2+}\) at 25°C is unknown, but this may be due to the need for defense against reactive oxygen species (ROS) because Bb encodes a Mn-dependent SOD and lower temperatures contain increased concentrations of dissolved O\(_2\) that could lead to enhanced formation of superoxide radical (O\(_2^–\)) (Troxell et al., 2012; Aguirre et al., 2013). However, Bb may encode additional proteins that require Mn\(^{2+}\).

Mn\(^{2+}\) is considered an essential trace element within biology. In bacteria, Mn\(^{2+}\) is critical for defense against several stresses such as oxidative stress, bile stress, and resistance to antibiotics (Anjem et al., 2009; Srinivasan et al., 2012). In addition, Mn\(^{2+}\) is involved in gene regulation through indirect mechanisms. For instance, the alarmon guanosine tetraphosphate (ppGpp) is synthesized and degraded by SpoT/RelA homolog proteins. During conditions of nutrient deprivation, ppGpp is synthesized and binds to the RNA polymerase (RNAP) in order to enhance transcription of genes important for survival or virulence while reducing transcription of genes involved in growth and cell division (Magnusson et al., 2005). SpoT/RelA homologs contain a highly conserved Mn\(^{2+}\) binding site and require Mn\(^{2+}\) as a cofactor for the enzymatic degradation of ppGpp (Sy, 1977; Sun et al., 2010). Bb encodes a SpoT/RelA homolog, bbo198, that is induced during serum starvation and is responsible for both synthesis and degradation of ppGpp (Concepcion and Nelson, 2003; Bugrysheva et al., 2005). This suggests that Bb may require Mn\(^{2+}\) in order to initiate cell growth. Recently, Bb’s peptide deformylase was isolated with bound Mn\(^{2+}\) (Aguirre et al., 2013); however, an enzymatic assay of the Mn-bound enzyme was not conducted. Whether peptide deformylase functions with Mn\(^{2+}\) is unknown, but this enzyme is active with Zn\(^{2+}\) as a cofactor (Nguyen et al., 2007). Future work is needed to determine the metal specificity of BBO198 and the peptide deformylase and to identify Bb proteins that require Mn\(^{2+}\).

Surprisingly, some enhancement in the intracellular concentration of Zn\(^{2+}\) for ΔbmtA has been noted (Ouyang et al., 2009a; Wang et al., 2012). It has been hypothesized that within ΔbmtA there may be compensation for the reduction of Mn\(^{2+}\) by enhancing the transport of Zn\(^{2+}\) and thereby replacing the requirement of Mn\(^{2+}\) with Zn\(^{2+}\). Although future work is required to fully test this hypothesis, the replacement of Mn\(^{2+}\) for Zn\(^{2+}\) in Mn\(^{2+}\)-dependent enzymes causes a pronounced reduction in catalytic efficiency or abrogates enzymatic activity altogether (Ose and Fridovich, 1976; Sobota and Imlay, 2011; Gu and Imlay, 2013). Metal-dependent transcription factors can utilize a variety of metals for function, i.e., Mn\(^{2+}\) or Fe\(^{2+}\) in the case of Fur (Privalle and Fridovich, 1993), and host metal-sequestering proteins exhibit promiscuity in metal binding, which is demonstrated by the Mn\(^{2+}\) or Zn\(^{2+}\) binding site (S1 site) in calprotectin (Damo et al., 2013). This is in contrast to metal-dependent enzymes, which exhibit stringent metal specificity for activity, as is the case for SpoT/RelA homologs and Bb’s SodA (Sy, 1977; Troxell et al., 2012; Aguirre et al., 2013). However, because many of Bb’s putative metalloenzymes are uncharacterized, the possibility exists that a significant number of these proteins can utilize either Mn\(^{2+}\) or Zn\(^{2+}\) within the cell.

**Bb’s Metal Requirement Within the Tick**

The unfed tick is presumed to be a nutrient deprived environment for Bb. Starvation conditions may mimic oxidative stress conditions and factors responsible for defense against ROS are also important for survival during starvation (Jenkins et al., 1988; Nystrom et al., 1996). Bb may require Mn\(^{2+}\) in order to defend against ROS that occurs during onset of the bloodmeal. Although Mn\(^{2+}\) complexed with other biological compounds, such as bicarbonate, are capable of degrading ROS, this requires large concentrations of intracellular Mn\(^{2+}\) that occurs within *Lactobacillus plantarum* (Archibald and Fridovich, 1981, 1982; Stadtmann et al., 1990). In the only report to compare directly the intracellular Mn\(^{2+}\) content of *L. plantarum* with Bb, it was observed that Bb contains 20 to 100-fold lower intracellular Mn\(^{2+}\) compared to *L. plantarum*, indicating this is an unlikely mechanism for ROS defense within Bb (Posey and Gherardini, 2000). However, Bb’s intracellular Mn\(^{2+}\) can fluctuate during *in vitro* growth conditions (Troxell et al., 2013), suggesting that environmental conditions within the tick-mouse life cycle may exist whereby Bb could contain sufficient intracellular Mn\(^{2+}\) to degrade ROS in a manner similar to *L. plantarum*. Although sodA is required for infection of the murine host (Esteve-Gassent et al., 2009), the contribution of sodA within the tick vector is unknown. It is currently unclear if Bb contains a high intracellular Mn\(^{2+}\) within the unfed tick or is starved for metals. Because of the involvement of BiC in Cu\(^{2+}\) and Zn\(^{2+}\) homeostasis and since ΔbicA exhibits a defect within the unfed tick (Li et al., 2007), the results support the notion that these two metals are limiting. In addition, the contribution of BmtA to the unfed tick is unknown.

**Regulation of σ\(^{57}\) by Zn\(^{2+}\) and Mn\(^{2+}\) Within Bb**

*Bb* is capable of surviving within two diverse hosts through changes in gene expression, specifically outer surface lipoproteins that modulate adaptation within each host. Outer Surface Proteins A (OspA) and C (OspC) are lipoproteins produced by Bb within the tick and animal host, respectively. Bb contains a limited genome that contains a relatively small number of transcription factors and sigma factors: Bb encodes only three sigma factors the housekeeping σ\(^{70}\), and two alternative sigma
In addition, *B. burgdorferi* genome encodes only one bacterial enhancer binding protein (bEBP), known as Rrp2, which is involved in σ^5^4 activation. The requirement of the Rrp2-RpoN-RpoS pathway (or Rrp2-σ^5^4-σ^S^ sigma factor cascade) in the regulation of ospA and ospC demonstrates the importance of this regulatory network (Hubner et al., 2001; Yang et al., 2003; Caimano et al., 2004; Fisher et al., 2005; Gilbert et al., 2007). Rrp2 and σ^5^4 directly activates transcription of rpoS (Smith et al., 2007; Blevins et al., 2009). σ^S^ then activates transcription of ospC by direct binding to the promoter of ospC (Yang et al., 2005) and also represses expression of ospA (Caimano et al., 2007). In addition, BosR, a Fur/PerR-like family transcription factor and the Fur protein (Blevins et al., 2009). 

The requirement for Mn^2+^ within the mammalian host (Hyde et al., 2009; Ouyang et al., 2009b). Moreover, the intracellular Mn^2+^ can fluctuate 20-fold during *in vitro* growth conditions and the temperature-dependent inverse concentration of intracellular of Mn^2+^ is reminiscent of the inverse regulation of ospA and ospC within *B. burgdorferi* (Stevenson et al., 1995; Obonyo et al., 1999; Yang et al., 2000; Alverson et al., 2003). To test if Mn^2+^ could suppress regulation by σ^S^, MnCl_2^ was added to cultures growing under conditions of σ^S^ activation. The addition of MnCl_2^ increases intracellular Mn^2+^ and reduces the expression of rpoS and σ^S^-activated ospC (Troxell et al., 2013). The addition of excess ZnSO_4^ increases the intracellular Zn^2+^, increases the level of BosR protein, and abrogates the repression of rpoS by Mn^2+^.

**FIGURE 2** Known and putative roles of Mn^2+^, Cu^2+^, and Zn^2+^ in gene regulation and metabolism of *B. burgdorferi*. A schematic of the importance of transition metals within *B. burgdorferi* is shown with a magnification of a section from a single *B. burgdorferi* cell. Extracellular Mn^2+^ is transported through BmtA and supplies the appropriate cofactor for the Mn-SOD and possibly the SpotReIA homolog BB0198 (designated by a pink arrow). In addition, Mn^2+^ reduces the level of BosR protein (designated by a pink blunted line), which controls transcription of the alternative sigma factor, rpoS (not shown). The putative role of Mn^2+^ as a cofactor for additional unknown enzymes is shown with a pink box. Zn^2+^ transport is uncharacterized in *B. burgdorferi*, but is presumed to be transported by a membrane bound protein. The requirement for Zn^2+^ within *B. burgdorferi* is likely to include enzymes within glycolysis, such as fructose 1,6-bisphosphatase (BB0445), and the peptide deformylase (BB0065) shown in the white box. Zn^2+^ is a known cofactor for the DNA binding protein BosR. Therefore, the intracellular Mn^2+^-Zn^2+^ can modulate the level of BosR protein. The transporter for Cu^2+^ and the role of Cu^2+^ within *B. burgdorferi* is unknown, but BicA may be involved in transport and homeostasis (blue box). Moreover, the contribution of Cu^2+^-Zn^2+^ to gene regulation within *B. burgdorferi* is unknown, but is predicted to involve redox sensing transcription factors (Changela et al., 2003; Gomez-Santos et al., 2011). Future work is required to elucidate the complete role of these metals in gene regulation and physiology of this important vector borne pathogen.
not influence transcription of bosR, but reduced the level of the BosR protein (Troxell et al., 2013). In addition, deletion of bmtA in two infectious strains does not alter bosR transcription, but enhances temperature-dependent activation in the level of BosR, which results in increased transcription of rpoS and ospC. As an earlier study shows, the BosR protein level is increased by CO2 despite the inability of dissolved CO2 to regulate transcription of bosR (Hyde et al., 2007). These combined results suggest that either metals or CO2 may control the level of the BosR protein, which activates transcription of rpoS. Correlation of the intracellular Mn2+/Zn2+ indicates that the ratio between these two metals play an important role in the level of BosR protein and rpoS regulation. Collectively, these results support the hypothesis that a combined reduction in intracellular Mn2+ while increasing Zn2+ regulates σ5 by dramatically enhancing the level of BosR protein.

Why does Bb require bmtA for the enzootic cycle? The presence of excess Mn2+ suggests there is a collection of unknown targets that require Mn2+ for activity. One function may be to control σ5 activation during the enzootic cycle. Precise regulation of ospC and other outer surface proteins, such as vlsE, is required for infection of the murine host; constitutive activation of either surface protein results in rapid elimination of Bb by either innate cells or the humoral response of the host (Liang et al., 2002; Xu et al., 2006, 2008a,b). In the absence of any defined metabolic requirement for Mn2+ within Bb, the importance of Mn2+ to the enzootic cycle could be to control regulation of highly immunogenic outer surface proteins. Nevertheless, the limited genome of Bb encodes several homologs that may require Mn2+ for activity. Furthermore, BmtA appears to influence not only the intracellular Mn2+ concentration, but also the concentrations of Cu2+ and Zn2+. How Zn2+ and Cu2+ are transported within Bb is unknown, but it is likely that these metals are required for proper regulation of virulence genes and unknown metabolic genes. Future work will no doubt shed light on the importance of these metals as cofactors and their influence on gene regulation. This is summarized in Figure 2, which depicts the known and putative roles of Mn2+, Cu2+, and Zn2+ within Bb.

CONCLUSIONS

Unlike most bacterial pathogens, Bb does not require Fe2+ for growth, which presents a unique model system to study metal-dependent gene regulation and stress responses. The bloodmeal is rich in Zn2+/Cu2+ and relatively poor in Mn2+, which suggests that Bb's intracellular Zn2+/Cu2+ content may increase through unidentified transporters (Figure 2). Because Mn2+ regulates the BosR protein level, but not bosR transcription (Troxell et al., 2013), the low Mn2+ content in blood may further enhance expression rpoS, which is required for Bb to exit the tick midgut and reach the salivary glands during tick feeding (Fisher et al., 2005; Dunham-Ems et al., 2012). How does Bb coordinate the regulation of transport of Mn2+, Cu2+, and Zn2+ during the enzootic cycle? What is apparent from in vitro work is that the intracellular Mn2+/Zn2+ ratio regulates transcription of the alternative sigma factor, rpoS, which controls activation of genes required for infection of mammals. A caveat to these studies is the heavy reliance on in vitro experiments due to the difficulties of measuring intracellular metal content while detecting changes in gene expression during in vivo studies. Infection studies with ΔbicA and ΔbmtA demonstrate the importance of these genes within the enzootic cycle, but the mechanism for why Bb requires them is unknown. Although the contribution to the unfed tick is known for bicA, the contribution of bmtA to survival within the dormant tick is unknown. How BicA and BmtA control metal homeostasis or gene expression in vivo would greatly improve our understanding of their importance in infection. Moreover, the identification of a dedicated Zn transport system and Cu transport system within Bb would provide additional and much needed clarity. It is clear that we are only beginning to understand the importance of metals in the metabolism and gene regulation within the Lyme disease spirochete.

ACKNOWLEDGMENTS

Bryan Troxell was supported by NIH T32 AI060519. Funding for this work was partially provided by NIH grants AI083640 and AI085242, Indiana INGEN and METACyt grants of Indiana University, funded by the Lilly Endowment, Inc (to X. Frank Yang).

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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: 30 July 2013; accepted: 29 October 2013; published online: 15 November 2013.

Citation: Troxell B and Yang XF (2013) Metal-dependent gene regulation in the causative agent of Lyme disease. Front. Cell. Infect. Microbiol. 3:79. doi: 10.3389/fcimb.2013.00079

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

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