Manganese and Zinc Regulate Virulence Determinants in *Borrelia burgdorferi*

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Borrelia burgdorferi, the causative agent of Lyme disease, must adapt to two diverse niches, an arthropod vector and a mammalian host. RpoS, an alternative sigma factor, plays a central role in spirochetal adaptation to the mammalian host by governing expression of many genes important for mammalian infection. *B. burgdorferi* is known to be unique in metal utilization, and little is known of the role of biologically available metals in *B. burgdorferi*. Here, we identified two transition metal ions, manganese (Mn²⁺) and zinc (Zn²⁺), that influenced regulation of RpoS. The intracellular Mn²⁺ level fluctuated approximately 20-fold under different conditions and inversely correlated with levels of RpoS and the major virulence factor OspC. Furthermore, an increase in intracellular Mn²⁺ repressed temperature-dependent induction of RpoS and OspC; this repression was overcome by an excess of Zn²⁺. Conversely, a decrease of intracellular Mn²⁺ by deletion of the Mn²⁺ transporter gene, *bmtA*, resulted in elevated levels of RpoS and OspC. Mn²⁺ affected RpoS through BosR, a Fur family homolog that is required for *rpoS* expression: elevated intracellular Mn²⁺ levels greatly reduced the level of BosR protein but not the level of *bosR* mRNA. Thus, Mn²⁺ and Zn²⁺ appeared to be important in modulation of the RpoS pathway that is essential to the life cycle of the Lyme disease spirochete. This finding supports the emerging notion that transition metals such as Mn²⁺ and Zn²⁺ play a critical role in regulation of virulence in bacteria.

Borrelia burgdorferi is the causative agent of a multisystem infection of humans known as Lyme disease (1–3). *B. burgdorferi* is maintained in the enzootic cycle through a tick vector, *Ixodes scapularis*, and a mammalian host, e.g., the white-footed mouse, *Peromyscus leucopus*. *B. burgdorferi* can thrive in these two extremely diverse host environments by altering expression of its proteins such as outer surface protein A (OspA) and OspC (for a review, see references 4, 5, 6, and 7). OspA is produced chiefly in unfed ticks and is critical for spirochetal survival in the tick midgut environment (8–12), whereas OspC is produced during tick feeding and is critical for transmission and for the initial stage of mammalian infection (13–18).

It is well established that differential expression of OspC and many other mammalian infection-associated proteins are governed by the RpoN-RpoS pathway (or σ^{54} - σ^S sigma factor cascade). In this pathway, an NtrC-like bacterial enhancer-binding protein (EBP), Rrp2, along with the alternative sigma factor RpoN (σ^{54} or σ^N), directly activates production of the other alternative sigma factor and global regulator RpoS (σ^S), through a –24/–12 σ^{54} -type promoter sequence located upstream of the *rpoS* gene (19–25). In addition, *rpoS* can be transcribed to produce a long form of *rpoS* mRNA from an unknown “housekeeping” σ^{70} -type promoter (26, 27). RpoS directly activates *ospC* (22) and functions as a global regulator that is indispensable for the enzootic cycle of *B. burgdorferi* (19, 20, 23, 24, 28).

Recent findings by two independent research groups on RpoS regulation show that BosR (*Borrelia* oxidative stress response regulator), a member of the ferric uptake regulator (Fur) family of transcriptional regulators, is essential for *rpoS* expression (29–34). BosR has been shown to bind to Zn²⁺ and may function as either an activator or a repressor for several genes involved in the oxidative stress response (29, 30, 35, 36). The requirement of BosR in *rpoS* activation is unexpected, since it is well established that in other bacteria, EBP and σ^{54} (along with the core RNA polymerase) are necessary and sufficient to activate transcription from a –24/

–12 σ^{54} -type promoter (37, 38). However, it has been reported that in other bacteria, Fur is involved in regulation of *rpoS* expression from a σ^{70} -type promoter (39, 40). Recent DNA binding data imply a direct role of BosR in activation of the *rpoS* transcription through binding in the vicinity of the σ^{54} -type promoter of *rpoS* (41). However, how BosR fits into the current dogma of σ^{54} -dependent transcriptional activation remains to be elucidated.

Metals are vital cofactors for essential enzymes in biology. They are also important signals for gene regulation. For instance, the food-borne pathogen *Salmonella enterica* serovar Typhimurium alters the expression of virulence factors when magnesium or iron concentrations are low (42–45). Host immunobiology factors, such as the cytokine interleukin-6 (IL-6) and the regulators STAT-3 and SMAD-7, influence regulation of metal homeostasis (46, 47). Little is known about the role of biologically available metals on gene expression for *B. burgdorferi*. In this study, we showed that environmental signals that are known to affect the RpoN-RpoS pathway dramatically influenced the intracellular Mn²⁺ level, and an increase in the intracellular Mn²⁺ levels significantly repressed the RpoN-RpoS pathway, by reducing the BosR protein level via a yet-to-be identified mechanism. Mn²⁺ repression of BosR and RpoS could be overcome by excess Zn²⁺.

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TABLE 1 Primers used in this study

Name ^a	Sequence (5' to 3')
<i>flaB</i> fwd	ACCAGCATCACTTTCAGGGTCTCA
<i>flaB</i> rev	CAGCAATAGCTTCATCTTGGTTTG
<i>bosR</i> fwd	CATTTTATACATAGCATCAAACCC
<i>bosR</i> rev	TATATACTGTTGCTTTTGATAGGC
<i>A3ospC</i> fwd	TAGCGGGAGCTTATGCAATATCAACC
<i>A3ospC</i> rev	CATCAATTTTTCCTTTAATCCTTCA
<i>297ospC</i> fwd	AAAGGTGGGAATACATCTGC
<i>297ospC</i> rev	TCTTTCACAGCCAGAACAAC
<i>rpoS</i> fwd	ATAAAAAGATATGCGGGTAAAGGG
<i>rpoS</i> rev	TGATTGCTTAATCCAAAATGATGC
<i>bmtA</i> fwd	TAATGGACGCTATGCTTGG
<i>bmtA</i> rev	AATGTATCCAAGCTCTTCAGC
<i>BbbmtA</i> Fwd	TTGTGGAGGCCCTCATGTAG
<i>BbbmtA</i> Rev	GAATATATCAGCGGAAAATTGG

^a Primers were ordered from IDT Integrated DNA Technologies.

Our findings support the notion that Mn²⁺ and Zn²⁺ have a critical role in regulation of virulence in bacteria (48).

MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* strain 297 and the isogenic mutant *bmtA* were kindly provided by M. V. Norgard and Z. Ouyang (49), and strain B31-A3 was kindly provided by P. Rosa (50). For construction of the *bmtA* mutant in the *B. burgdorferi* strain B31-A3 background, 20 µg of the suicide plasmid DNA pOY04 (49) was transformed into B31-A3, and selection for mutants was performed as described previously (12, 51) with 500 µg/ml of kanamycin (Sigma-Aldrich, St. Louis, MO). One clone was obtained, and primers *BbbmtA* Fwd and *BbbmtA* Rev (Table 1) were used to confirm the mutation by PCR. The cultures used were no more than three passages from original stock (4), and spirochetes were grown using standard BSK-II medium (52) at 37°C in a 5% CO₂ incubator.

Medium and growth conditions. BSK-II medium supplemented with 6% rabbit serum was used throughout (53). To reduce the divalent cations in BSK-II medium, Chelex 100 (Bio-Rad, Hercules, CA) was used to treat the medium as previously described (54). Briefly, BSK-II medium was prepared, and 50 g/liter of Chelex 100 resin was added to the medium followed by gentle stirring at 4°C for 1 h. The Chelex-treated medium was centrifuged at 7,000 × g for 30 min, the pH of the supernatant was reduced to 7.5 or 7.0 by the addition of HCl, and then the mixture was sterilized by filtration. This process removed any remaining Chelex 100 resin from the medium. Metal analysis by inductively coupled plasma mass spectrometry (ICP-MS) confirmed that Chelex treatment reduced manganese concentration to below detection. Medium was stored at –80°C until use.

Cultures from –80°C storage were inoculated into BSK-II medium and cultivated at 37°C in a 5% CO₂ incubator until the exponential phase (~1 × 10⁷ cells/ml). Then, cultures were diluted to 10⁵ cells/ml for experiments. Cultures were grown at 37°C for 5 to 7 days, washed with 0.9% NaCl, and treated for denaturing and reducing SDS-PAGE. For additional experiments, cultures were grown at 25°C for 21 days.

SDS-PAGE and immunoblotting. Denatured and reduced samples prepared as described above were resuspended in Laemmli sample buffer (Bio-Rad, Hercules, CA) and boiled. Denatured proteins were separated on Mini-Protean TGX gels (12% acrylamide; Bio-Rad) and transferred to 0.45-µm nitrocellulose membranes (Bio-Rad). Transfer was confirmed by Ponceau S staining of the membrane. Primary antibodies against FlaB, RpoS, and OspC were reported previously (32, 55, 56). The antibody against BosR used in this study was purchased from General Bioscience Corp. (Brisbane, CA). Secondary antibody (peroxidase-conjugated goat anti-mouse antibody; Jackson ImmunoResearch Laboratories, West Grove, PA) was used at

1:1,000. Detection of horseradish peroxidase activity was determined using 4-chloro-1-naphthol and H₂O₂ (Fisher Scientific).

Metal analysis by ICP-MS. To measure intracellular metal content, strains were grown in BSK-II or Chelex-treated BSK-II medium at 37°C for 7 days (initial cell density, 10⁵ cells/ml). Samples (n ≥ 3) were centrifuged, washed two times in phosphate–100 µM EDTA buffer (pH 7.8), and concentrated 100-fold in buffer. Samples were placed in a drying oven at 95°C for 24 h, and a dry weight measurement was recorded. Dry cell pellets were resuspended in 3 N nitric acid and heated in a drying oven as described above. Control samples without bacteria that included the wash buffer and acid treatments were included with each analysis to account for the presence of metals in the buffer reagents, on the surface of lab equipment, etc. Values of metal content obtained with the control samples were subtracted from the values recorded from biological samples. Acid-treated samples were resuspended in 0.5 ml of 3 N nitric acid and sent for analysis at the Analytical Spectroscopy Services Laboratory (ASSL) located at North Carolina State University and analyzed using a Varian 820 ICP-MS.

qRT-PCR. Primers used in quantitative reverse transcriptase PCR (qRT-PCR) are listed in Table 1. To determine the change in transcription of target genes (*flaB*, *bmtA*, *rpoS*, *bosR*, and *ospC*), strains were grown to the stationary phase and samples were split into separate tubes for Western blotting and RNA purification. Total RNA was isolated using TRIzol reagent (Invitrogen). Following purification, total RNA was treated with DNase I (New England BioLabs) for 1 h at 37°C. Then, the DNase-treated RNA was purified using the RNA cleanup protocol with the RNeasy mini-prep kit (Qiagen). cDNA was synthesized as follows: 1 µl of a 10 mM deoxynucleoside triphosphate (dNTP) mixture (2.5 mM each dNTP), 1 µl of 50 µM gene-specific primer, total RNA, and H₂O were added to a final volume of 13 µl. The sample was heated at 65°C for 5 min and then placed on ice for 1 min. Then, 5 µl of 5× first-strand synthesis buffer (Invitrogen), 1 µl of 0.1 M dithiothreitol (DTT; Invitrogen), 1 µl of RNase OUT (Invitrogen), and 1 µl of Superscript reverse transcriptase III (Invitrogen) were added. The sample was incubated at room temperature for 5 min and then at 50°C for 60 min and at 70°C for 15 min. Following cDNA synthesis, 20 or 30 µl of double-distilled water (ddH₂O) was added to the sample. A control receiving no reverse transcriptase was included for each RNA sample.

qRT-PCR was performed, using the RT² SYBR green ROX qPCR Mastermix, as follows: 10 µl of qPCR Mastermix, 0.5 µM primers, 2 µl of cDNA, and ddH₂O to a 20-µl final volume were added to a 96-well plate (Applied Biosystems). An ABI Prism 7000 real-time PCR machine was used with the following PCR parameters: 95°C for 10 min with 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 30 s. Melting curve analysis confirmed the presence of a single PCR product for each sample. A standard curve of *flaB* DNA (10² to 10⁷ copies per reaction mixture) was used to quantify transcripts, and expression data were normalized per 1,000 copies of *flaB*. qPCR with no reverse transcriptase control, targeting *flaB*, confirmed the reduction of ≥5 orders of magnitude in copy number in the samples. These results indicated that genomic DNA contamination was near background level.

Statistical analyses. Statistical significance was determined using Student's *t* test, and when multiple comparisons were made, the *P* value was corrected using the Bonferroni correction. One-way analysis of variance (ANOVA) was used to determine significance, and results are shown in Table 2.

RESULTS

Intracellular Mn²⁺ inversely correlates with OspC expression. To investigate whether intracellular metals may influence expression of virulence factors in *B. burgdorferi*, we analyzed the intracellular metal content under various growth conditions. It is well established that elevated temperature induces many virulence factors such as *ospC* via direct activation by RpoS (13, 23, 26, 57–59). Thus, a culture growing at 37°C (at late log or stationary phase)

TABLE 2 Exogenous addition of Mn^{2+} and Zn^{2+} increases the intracellular Mn^{2+} and Zn^{2+} concentrations^a

Medium	Mean intracellular metal content $\pm$ SD (μ mol/g, dry wt)		
	Mn^{2+}	Zn^{2+}	Mn^{2+}/Zn^{2+} ratio
BSK-II	0.18 ± 0.08	0.93 ± 0.14	1:5
BSK-II Chelex	0.03 ± 0.01	0.60 ± 0.02	1:20
+ 10 μ M $MnCl_2$	0.53 ± 0.19	0.45 ± 0.06	1
+ 10 μ M $ZnSO_4$	0.04 ± 0.02	1.20 ± 0.11	1:30
+ 100 μ M $ZnSO_4$	0.01 ± 0.01	1.82 ± 0.06	1:182
+ 10 μ M $MnCl_2$ + 10 μ M $ZnSO_4$	0.69 ± 0.16	0.68 ± 0.21	1
+ 10 μ M $MnCl_2$ + 100 μ M $ZnSO_4$	0.35 ± 0.10	1.36 ± 0.19	1:4

^a *B. burgdorferi* strain 297 was cultivated at 37°C to stationary phase, and intracellular metal contents from two separate experiments with three independent cultures were determined by ICP-MS. Bold values are significantly different from those of the corresponding metal from Chelex-treated BSK-II ($P < 0.05$). One-way ANOVA with Dunnett's multiple-comparisons test was used to determine significance.

has been considered to be under an RpoS “on” condition, whereas a culture incubated at 25°C is regarded as being under an RpoS “off” condition (Fig. 1A). We also examined the level of BosR, because in addition to RpoN and Rrp2, BosR was recently shown to be required for the transcriptional activation of *rpoS* (19, 32, 41, 60). As shown in Fig. 1A, BosR was also induced by elevated temperature, suggesting that temperature-induced *rpoS* activation is, at least in part, through BosR. We then examined the intracellular concentrations of two transition metal ions, Mn^{2+} and Zn^{2+} , in *B. burgdorferi* strain 297 cultivated under either 25°C or 37°C conditions. The results showed no significant difference in the intracellular Zn^{2+} levels between 25°C and 37°C samples (Fig. 1B, right panel). Surprisingly, we detected a 5-fold-lower intracellular Mn^{2+} level when spirochetes were cultivated at 37°C compared to 25°C (Fig. 1B, left panel). At 25°C, the intracellular Zn^{2+} and Mn^{2+} levels were similar, whereas at 37°C, the level of Mn^{2+} was more than 6-fold lower than the level of Zn^{2+} . In other words, the intracellular ratio of Mn^{2+} to Zn^{2+} was $\sim$ 1:1 at 25°C and $\sim$ 1:6 at 37°C. These results suggest that intracellular Mn^{2+} is temperature regulated and inversely correlates with RpoS activation.

To validate the measurement of intracellular metal concentrations, a *bmtA* mutant of strain 297 that was previously reported by Ouyang et al. (49) was included in the experiments. *BmtA* is involved in metal homeostasis by functioning as an Mn^{2+} transporter in *B. burgdorferi* (49). Consistent with the previous finding, the *bmtA* mutant exhibited 12-fold reduction of intracellular Mn^{2+} compared to the parental strain 297 grown at 37°C (Fig. 1B) (49). However, in contrast to wild-type spirochetes, the *bmtA* mutant cultivated at 25°C had low intracellular Mn^{2+} levels (Fig. 1B), suggesting that the increased level of Mn^{2+} observed in wild-type spirochetes at 25°C is *BmtA* dependent.

Culture temperature influences expression of *bmtA*. Because the increased intracellular Mn^{2+} content at 25°C was *BmtA* dependent, we further examined the influence of culture temperature on *bmtA* expression. As expected, the expression of *ospC* at 25°C was reduced >200 -fold compared to that at 37°C (Fig. 1C). Under the same conditions, *bmtA* expression was increased >3 -fold by cultivation at 25°C compared to 37°C (Fig. 1C). This is

consistent with previously published microarray data (61). Thus, expression of *bmtA* appears to be temperature regulated, which contributes to the increased Mn^{2+} level by lowered temperature.

Since the level of RpoS is regulated by culture temperature, we examined the possibility that RpoS may negatively regulate *bmtA* transcription. To test this, wild-type *B. burgdorferi* strain 297 and the isogenic *rpoS* mutant were grown to stationary phase, and protein and RNA were prepared from each biological replicate. As shown previously, wild-type *B. burgdorferi* produced OspC that can be easily detected in Coomassie blue-stained gels under the optimal conditions (37°C, stationary phase) (26, 28, 55, 56, 62, 63) (Fig. 1D, right panel). Inactivation of *rpoS* abolished *ospC* expres-

FIG 1 Intracellular manganese content inversely correlates with temperature-dependent activation of RpoS and OspC. (A) Immunoblot analysis demonstrating an RpoS “on” condition when wild-type *B. burgdorferi* strain 297 was cultivated at 37°C and an RpoS “off” condition when cultivated at 25°C. The level of BosR correlated well with expression of RpoS. The constitutive active FlaB serves as a control. Results are representative of two separate experiments with 3 replicates per experiment. (B) Metal analysis by inductively coupled plasma mass spectrometry (ICP-MS). The parental strain 297 (WT) and the isogenic *bmtA* mutant were grown in standard BSK-II medium at 37°C or at 25°C. Samples were harvested at the stationary phase and analyzed for intracellular Mn^{2+} (left panel) and Zn^{2+} (right panel). Data represent the means $\pm$ standard deviations (SD) of 3 independent samples from two separate experiments. (C) Expression of *bmtA* is temperature dependent. Wild-type *B. burgdorferi* strain 297 was grown in standard BSK-II medium at 37°C or 25°C and harvested at the stationary phase. RNAs were extracted from 3 independent cultures, and expressions of *flaB*, *ospC*, and *bmtA* were determined by qRT-PCR. Data were normalized to 1,000 copies of *flaB*, and the fold changes are shown with expressions at 37°C set to 1. Significance was determined by Student's *t* test. (D) Expression of *bmtA* is RpoS independent. Wild-type *B. burgdorferi* 297 and the isogenic *rpoS* mutant were grown in standard BSK-II medium at 37°C and harvested at the stationary phase. Expressions of *flaB*, *ospC*, and *bmtA* were determined by qRT-PCR from total RNA extracted from 3 independent cultures. Cell lysates were separated by SDS-PAGE, and a representative Coomassie blue-stained gel is shown in the right panel. The arrowhead indicates the band corresponding to OspC.

FIG 2 Manganese and zinc in BSK-II medium can be reduced by Chelex treatment. The manganese (A) and zinc (B) contents of BSK-II medium and Chelex-treated BSK-II medium were measured as described in Materials and Methods. ND, not detected. Data are from three separate batches of Chelex-treated BSK-II. Significance was determined by Student's *t* test.

sion at both RNA and protein levels (Fig. 1D) (19, 64). However, the expression of *bmtA* was virtually unchanged in the *rpoS* mutant (Fig. 1D, left panel). Thus, the regulation of *bmtA* appears to be independent of RpoS. These results suggest that the increased *bmtA* expression and the Mn^{2+} level at 25°C were not the result of downregulation of RpoS. Rather, the increased Mn^{2+} level at 25°C may negatively regulate RpoS levels.

Mn^{2+} negatively regulates OspC and RpoS but is overcome by excess Zn^{2+} . The inverse correlation of the intracellular Mn^{2+} levels with RpoS levels suggests that Mn^{2+} may negatively regulate RpoS. If so, an increase in intracellular Mn^{2+} level should inhibit RpoS and OspC production, even in spirochetes grown under RpoS “on” conditions (37°C). To address this, manipulation of the metal status in the complex BSK-II medium is required. To this end, we treated standard BSK-II medium with Chelex-100 resin to chelate divalent cations from the medium. We determined the effectiveness of the Chelex-100 treatment by measuring Mn^{2+} and Zn^{2+} levels before and after treatment. BSK-II medium contains a low concentration of Mn^{2+} , which can be reduced to an undetectable concentration ($<0.5 \mu\text{g/liter}$, $\sim 10 \text{ nM}$) upon treatment with Chelex-100 resin (Fig. 2A). Furthermore, Zn^{2+} , which is substantially higher than Mn^{2+} in BSK-II, can be reduced 6-fold by Chelex treatment (Fig. 2B). Chelex treatment did not completely remove all the metals, but the dramatic reduction of Mn^{2+} allows us to investigate the potential role of Mn^{2+} in RpoS and OspC activation.

B. burgdorferi strain 297 grown in Chelex-treated or untreated BSK-II medium exhibited no discernible differences at 37°C in either growth rate or final cell density (Fig. 3A), despite virtually no detectable amount of Mn^{2+} in the medium. This was not surprising, given that the *bmtA* mutant showed little effect on cell growth (49). However, the level of OspC was enhanced when spirochetes were cultivated in the Chelex-treated BSK-II medium (Fig. 3B). This result implied that cultivation of spirochetes in an Mn-limited medium enhanced OspC levels. To determine whether this enhanced OspC production with Chelex-treated medium was due to the reduction of Mn^{2+} rather than to a reduction of other divalent cations, Chelex-treated BSK-II was then supple-

FIG 3 Influence of RpoS and OspC production by Mn^{2+} and Zn^{2+} in *B. burgdorferi* strain 297. (A) Growth curves of wild-type *B. burgdorferi* strain 297 in standard or Chelex-treated BSK-II medium. The means with SD from two separate experiments are shown with duplicates of each sample. (B) Protein profiles of spirochetes grown in standard or Chelex-treated medium. Cells were harvested at the late log phase, and cell lysates were subjected to SDS-PAGE analysis. The bands corresponding to OspC are indicated by an arrow. Results are representative of the two separate experiments. (C and D) Effects of Mn^{2+} and Zn^{2+} on RpoS and OspC levels. Strain 297 was grown at 37°C in Chelex-treated BSK-II medium (pH 7.5) with or without added $10 \mu\text{M}$ $MnCl_2$ and/or $ZnSO_4$ (C) or $100 \mu\text{M}$ $ZnSO_4$ (D). Equivalent amounts of protein were separated by SDS-PAGE (D, top panel) and immunoblotted with antibodies against FlaB, RpoS, or OspC (C and D, bottom panels). FlaB served as a loading control. *, a 10-fold dilution of each sample was used in this immunoblot assay to avoid saturation of the OspC signal. Data shown in panels C and D are representative from two separate experiments performed with samples in triplicate.

mented with various concentrations of $MnCl_2$ or $ZnSO_4$. As shown in Fig. 3C, addition of $10 \mu\text{M}$ $MnCl_2$ to this medium dramatically reduced the production of both RpoS and OspC (Fig. 3C, second lane from left), but addition of $10 \mu\text{M}$ $ZnSO_4$ did not reduce production of OspC (Fig. 3C, third lane from left). These results suggested that Mn^{2+} , not Zn^{2+} , played a repressive role in the production of OspC and RpoS.

We further investigated whether Zn^{2+} would influence the effect of Mn^{2+} by supplementing both $ZnSO_4$ and $MnCl_2$ to the Chelex-treated medium. The result showed that reduced RpoS and OspC production by Mn^{2+} could not be rescued or enhanced by addition of equimolar amount of Zn^{2+} (Fig. 3C, fourth lane from the left). However, when a 10-fold excess of $ZnSO_4$ ($100 \mu\text{M}$) to $MnCl_2$ ($10 \mu\text{M}$) was added, the production of RpoS and OspC was restored (Fig. 3D, fifth lane from left). This result suggested that Zn^{2+} played a positive role in RpoS production and overcame the inhibitory effect of Mn^{2+} .

Further analyses confirmed that the intracellular Mn^{2+} and Zn^{2+} levels were correlated positively with exogenous availability of Mn^{2+} and Zn^{2+} (Table 2). When grown in Chelex BSK-II me-

FIG 4 Influence of Mn^{2+} and Zn^{2+} on RpoS and OspC production in *B. burgdorferi* strain B31. Strain B31-A3 was grown at 37°C in Chelex-treated BSK-II medium adjusted to pH 7.0, with addition of various amounts of Mn^{2+} and/or Zn^{2+} . Samples were harvested at the late logarithmic phase, and cell lysates were detected by Coomassie blue-stained SDS-PAGE gel (A) or by immunoblotting using antisera against FlaB, RpoS, and BosR (B). Data are representative of two separate experiments performed in triplicate.

dium, the intracellular Mn^{2+} concentration was dramatically reduced compared to that observed in growth in BSK-II medium (Table 2), which confirmed that this medium is Mn limited compared to BSK-II. Supplementing Chelex BSK-II medium with 10 μM $MnCl_2$ increased the intracellular Mn^{2+} level of *B. burgdorferi* 20-fold in comparison to that of spirochetes grown in Chelex BSK-II medium (Table 2). In contrast to Mn^{2+} , intracellular Zn^{2+} levels were moderately affected (3-fold increase upon supplementation with 100 μM $ZnSO_4$). This result further suggests that intracellular Mn^{2+} concentration is highly sensitive to environmental Mn^{2+} levels and that Mn^{2+} may serve as an environmental sensor for modulating gene expression.

The above-described experiments were conducted with the infectious *B. burgdorferi* strain 297. Since it is known that different strains of *B. burgdorferi* express various levels of RpoS and OspC (64), the influence of Mn^{2+} and Zn^{2+} on RpoS and OspC was further confirmed using *B. burgdorferi* strain B31-A3 (50). Because we found that B31-A3 often expresses a low level of RpoS and OspC relative to strain 297 when cultivated under the standard conditions (BSK-II medium, pH 7.5, 37°C), we therefore cultivated *B. burgdorferi* strain B31-A3 in Chelex-treated BSK-II that was adjusted to pH 7.0, since reduced culture pH enhances RpoS and OspC expression of *B. burgdorferi* (56, 65). As shown in Fig. 4, OspC induction was readily observed on the Coomassie

blue-stained gel in strain B31-A3 grown under such conditions (Fig. 4A). Similar to what was observed for strain 297, addition of $MnCl_2$ to the medium inhibited production of OspC (Fig. 4A) and RpoS (Fig. 4B) in strain B31-A3, suggesting that the repressive effect of Mn^{2+} on RpoS is not strain dependent. The positive effect of Zn^{2+} on RpoS production was stronger in strain B31-A3 than that in strain 297: excess Zn^{2+} (100 μM) not only was able to overcome the inhibitory effect of Mn^{2+} on RpoS but also further enhanced OspC production, in the absence or presence of Mn^{2+} (Fig. 4A, fourth and fifth lanes from left). These results provide further evidence that Mn^{2+} exhibits a repressive role in RpoS production, whereas Zn^{2+} exerts a positive role.

Mn^{2+} negatively regulates RpoS through BosR. It is well established that expression of *rpoS* is governed by two transcriptional factors, Rrp2 and BosR (Fig. 1A) (31, 32, 60). Since BosR is a Zn-dependent transcriptional activator of *rpoS*, we focused on BosR (29). We cultivated strain B31-A3 in Chelex-treated BSK-II at pH 7.0 with and without the addition of metals. Addition of $MnCl_2$ suppressed not only OspC and RpoS but also the BosR level (Fig. 4B). Similarly, excess Zn^{2+} also enhanced the level of BosR (Fig. 4). Given that BosR is an essential activator for *rpoS*, we conclude that the molecular mechanism for metal-dependent regulation of RpoS is through affecting the level of BosR.

Deletion of *bmtA* enhances BosR level. To gather further genetic evidence supporting the finding that Mn^{2+} suppresses BosR, we examined the *bmtA* mutant that was previously generated in strain 297 by Ouyang et al. (49). *BmtA* is an Mn^{2+} transporter in *B. burgdorferi*; deletion of *bmtA* resulted in reduced intracellular levels of Mn^{2+} (49) (Fig. 1). If Mn^{2+} plays a negative role in the RpoS pathway via BosR, deletion of *bmtA* should lead to elevated OspC and BosR levels. Indeed, when the spirochetes lacking *BmtA* were grown in BSK-II medium at 37°C, the $\Delta bmtA$ strain had higher levels of OspC and BosR than did parental and complemented strains when harvested at the same cell density (late log phase) (Fig. 5A). We confirmed that the $\Delta bmtA$ mutant had increased *ospC* promoter activity by measuring the β -galactosidase activity in a $\Delta bmtA$ strain harboring a previously described shuttle vector that encodes an *ospC* promoter fused with the *lacZ* reporter (pBHospCp-*lacZBb*) (data not shown). It is known that elevated pH (pH 8) inhibits the activation of the RpoS pathway in wild-type *B. burgdorferi* (56, 65). However, the *bmtA* mutant was even

FIG 5 Deletion of *bmtA* resulted in elevated OspC and BosR production. Wild-type *B. burgdorferi* strain 297 (A) or strain B31-A3 (B) and the isogenic *bmtA* mutant or the complemented strain (*bmtA-comp*) were grown in BSK-II medium (pH 7.5) at 37°C and harvested at the late logarithmic phase. Equal amounts of whole-cell lysates were separated by Coomassie blue-stained SDS-PAGE gel (left panels), or probed for FlaB or BosR protein by immunoblotting analyses (right panels). Data shown are representative of two separate experiments performed in duplicate.

FIG 6 Manganese downregulates BosR at the posttranscriptional level. (A) Addition of MnCl₂ downregulates BosR at the posttranscriptional level. Strain B31-A3 was grown at 37°C in Chelex-treated BSK-II medium (pH 7.0) with or without addition of 10 μ M MnCl₂. Spirochetes were harvested at the late log phase and subjected to qRT-PCR (left panel) and immunoblotting analyses (right panel). qRT-PCR data are from 3 independent samples with significance determined by Student's *t* test. A representative result from immunoblotting of samples is shown. (B and C) The *bmtA* mutant in a 297 background (B) or a B31 background (C) enhances BosR protein at the posttranscriptional level. The parental strains and the isogenic *bmtA* mutant were grown in BSK-II medium (pH 7.5) at 37°C and harvested at the late logarithmic phase. Spirochetes were then subjected to qRT-PCR (left panel) and representative immunoblotting analyses (right panel) as described for panel A.

able to overcome the inhibitory effect by pH 8 and produced OspC (data not shown). On the other hand, the *bmtA* mutant remained incapable of expressing OspC when cultivated at 25°C despite the low intracellular Mn²⁺ level (data not shown), suggesting that some additional factor(s) was required for activating the pathway at lower temperature. Nevertheless, these results indicate that deletion of *bmtA* resulted in enhanced activation of the RpoS pathway, and this effect was through an increased BosR level.

To further confirm that inactivation of *bmtA* enhanced BosR and RpoS levels, we constructed a new *bmtA* mutant in *B. burgdorferi* strain B31-A3 (see Fig. S1A in the supplemental material). Deletion of *bmtA* in B31-A3 did not alter the growth kinetics compared to the parental strain (see Fig. S1B in the supplemental material). However, similar to the *bmtA* mutant in the strain 297 background, the *bmtA* mutant in B31-A3 showed dramatic up-regulation of OspC and BosR levels compared to its parental strain (Fig. 5B). These combined data provided strong genetic evidence to support the conclusion that Mn²⁺ negatively regulates the RpoS pathway through influencing the level of BosR.

Mn²⁺ represses BosR at the posttranscriptional level. To gain insight into how metals may affect BosR levels, we investigated whether BosR is regulated at the transcriptional or posttranscriptional level. First, we compared the BosR protein level and mRNA level upon addition of Mn²⁺. Strain B31-A3 was cultivated in Chelex-treated BSK-II at pH 7.0 with or without added MnCl₂. Spirochetes were harvested at stationary phase, and expression levels of *bosR* and *rpoS* mRNA or their proteins were determined by qRT-PCR and immunoblotting. As shown in Fig. 6A, Mn²⁺ dramatically reduced *rpoS* expression at both mRNA and protein levels. However, although the BosR protein level was dramatically reduced, the *bosR* mRNA level was not significantly influenced by the addition of MnCl₂ (Fig. 6A). We also compared the protein and RNA levels of BosR in both of the *bmtA* mutants generated in the 297 and B31-A3 backgrounds. Both *bmtA* mutants showed dramatic increases in the BosR protein level but little change in the *bosR* mRNA level (Fig. 6B and C). Taken together, we conclude that Mn²⁺ suppresses BosR expression at the posttranscriptional level.

DISCUSSION

Metalloenzymes are essential for biological systems, and the metal status is tightly regulated within the cell. Many bacteria encode

metal-dependent transcription factors that sense the intracellular metal status and regulate gene expression in response to stimuli. Fur is one of the most studied metal-dependent transcription factors regulating metal homeostasis and virulence (66, 67). Very little is known about the function and regulation of metals in the Lyme disease spirochete. In this study, we demonstrated that two transition metal ions, Mn²⁺ and Zn²⁺, were important in the regulation of RpoS, a central regulator that governs differential expression of many virulence genes in *B. burgdorferi* (19, 20, 23, 24, 26, 28). We showed that Mn²⁺ suppression of RpoS was through the Fur family homolog BosR, an essential activator for *rpoS* transcription. Furthermore, we demonstrate that Mn²⁺ influences BosR at its protein level. This is the first report that any biologically relevant metal ion influences virulence factor expression in *B. burgdorferi*.

Several lines of evidence support the conclusion that the intracellular Mn²⁺ concentration is dramatically regulated and controls the transcription of *rpoS* via BosR. First, cultivation of spirochetes at 25°C dramatically increased the intracellular Mn²⁺ concentration of *B. burgdorferi* (Fig. 1). Second, addition of exogenous Mn²⁺ increased the intracellular Mn²⁺ (Table 2). Such a dramatic change in intracellular Mn²⁺ levels suggests that Mn²⁺ can function as an environmental sensor. Third, the increased intracellular Mn²⁺ dramatically repressed transcription of temperature and pH-induced *rpoS* and *ospC* (Fig. 3, 4, and 6). Such repression could be relieved by addition of excess Zn²⁺. Likewise, a biochemical approach consisting of reducing the Mn²⁺ content of BSK-II with Chelex treatment enhanced OspC production in wild-type bacteria (Fig. 3A). Finally, we provided genetic evidence that the *bmtA* mutant with decreased intracellular Mn²⁺ had enhanced transcription of *rpoS* and *ospC* in two strain backgrounds (Fig. 5 and 6). Thus, Mn²⁺ appears to exert a negative regulation on RpoS, via negative regulation of BosR. Together with our previous finding that Mn²⁺ exerts positive regulation on SodA, an Mn²⁺-dependent superoxide dismutase (SOD) of *B. burgdorferi* (54, 68), we postulate that Mn²⁺ inversely regulates the levels of BosR and SodA and plays an important role in regulating virulence factors of *B. burgdorferi* as summarized in the model (Fig. 7).

The dramatic changes of intracellular Mn²⁺ within *B. burgdorferi* observed in this study are not unique among bacterial pathogens. The intracellular Mn²⁺ content is very low under routine culture conditions for *Escherichia coli* and *S. Typhimurium* but

FIG 7 Model depicting the role of Mn^{2+} and Zn^{2+} in the regulation of the RpoS pathway through BosR. Expression of *rpoS* is modulated by two transcriptional factors, Rrp2 and BosR. Rrp2 activity is modulated via phosphorylation by acetyl~P or Hk2 (80, 99). For the purpose of simplification, factors that influence RpoS through acetyl~P such as CsrA, OppA5, acetate, and the mevalonate pathway are not included in the model (63, 80, 84–86). Environmental and host signals, such as temperature and blood, can modulate *rpoS* expression by altering the intracellular Mn^{2+} and Zn^{2+} status and subsequently BosR levels. A low intracellular Mn^{2+} and high Zn^{2+} status promotes a high level of BosR protein (not *bosR* transcript), leading to the increased level of RpoS and RpoS-controlled products such as OspC. In addition, temperature can also regulate RpoS levels via small RNA DsrA at low cell density (26).

increases dramatically under specific conditions (69, 70). In these organisms, repression of Mn^{2+} transport is accomplished by redox-sensing transcription factors, mainly DtxR or Fur homologs, which promote Mn^{2+} uptake under conditions of oxidative stress (1, 69, 70). Our metal analysis with $\Delta bosR$ in *B. burgdorferi* did not reveal enhanced intracellular concentration of Mn^{2+} (data not shown), suggesting that regulation of Mn^{2+} transport in *B. burgdorferi* appeared to be distinct from that of other bacterial pathogens. Regarding Zn^{2+} , the relatively constant intracellular Zn^{2+} levels within *B. burgdorferi* (Table 2) are also consistent with the tightly regulated intracellular Zn^{2+} in other bacteria (71–76). Expression of Zn^{2+} transporters is typically repressed by the Fur homolog Zinc uptake regulator (Zur) or by family members of the Fur-like protein DtxR. Other related pathogenic spirochetes, *Treponema pallidum* and *Treponema denticola*, encode a DtxR protein called TroR (77, 78), which utilizes both Mn^{2+} and Zn^{2+} as corepressors (78, 79). BLASTP analysis within *B. burgdorferi* failed to identify a TroR homolog, indicating that regulation of Zn^{2+} transport within this spirochete may be unique. Interestingly, the finding in this study suggests that there is a connection between the intracellular concentration and transport of Mn^{2+} and Zn^{2+} (Table 2).

Temperature is one of the important environmental cues that induce RpoS and OspC production in *B. burgdorferi* (6, 7, 13). Previously, the small RNA DsrA was shown to link temperature sensing to RpoS production, by controlling the translation of the long form of the *rpoS* transcript (derived from a non- σ^{54} -type promoter at low cell density) (26) (Fig. 7). How temperature induces *rpoS* transcription

from the σ^{54} (RpoN)-type promoter (the short and major form of *rpoS* transcript) remains unclear. It is known, however, that this RpoN-dependent transcription of *rpoS* requires Rrp2 and BosR (19, 32, 41, 60). It has been proposed that phosphorylation of Rrp2 is the signal that activates the RpoN-dependent transcription (60, 80), but whether temperature influences Rrp2 phosphorylation and subsequently controls *rpoS* transcription has not been tested, largely due to the lack of a method to detect Rrp2 phosphorylation in the cell. Nevertheless, we showed in this study that BosR protein was dramatically induced by elevated temperature (Fig. 1A). This observation indicates that temperature influences the RpoS regulon, at least in part, through regulation of the level of BosR. Thus, temperature influences the level of RpoS in at least two ways (Fig. 7): one is σ^{54} (RpoN) independent at low cell density, through DsrA; the other is σ^{54} (RpoN) dependent, through altering the intracellular Mn^{2+} and Zn^{2+} levels and subsequently the BosR level. What is the physiological significance of increased Mn^{2+} concentration within *B. burgdorferi* when cultivated at lower temperature (25°C)? We speculate that the increased solubility of O_2 in water at 25°C compared to 37°C may lead to an increased formation of O_2^- , and increased intracellular Mn^{2+} concentration would enhance the expression of SodA to protect against O_2^- stress at lower temperature. An increase in the cytosolic levels of Mn^{2+} and/or Zn^{2+} has been reported in other bacteria in response to reactive oxygen stress (81).

In addition to temperature, many environmental factors, such as cell density, pH, CO_2 , and host blood, have been shown to influence RpoS levels (13, 35, 56, 61, 65, 82, 83). However, how these multiple factors collectively influence the *rpoS* expression has not been fully elucidated. We previously showed that multiple environmental factors may influence the intracellular concentration of acetyl~P, a high-energy small phosphate important for phosphorylation and activation of Rrp2 (80). Subsequently, several studies showed factors or pathways that influence RpoS through acetate and acetyl~P, including CsrA, OppA5, and the mevalonate pathway (63, 84–86). More recently, Jutras et al. proposed that the temperature effect on RpoS actually is due to the change of cell growth rate (100). Given that it is known that increase in growth rate leads to enhanced levels of acetyl~P (87, 88), the effect of growth rate on RpoS is likely through acetyl~P. Based on the findings in this study, we hypothesize that these environmental cues may also collectively influence the intracellular Mn^{2+} and Zn^{2+} levels and further affect RpoS levels. For cell density, although the growth phase regulation of Mn transport was not examined in this study, it has been reported that in other organisms Mn^{2+} transport genes are upregulated during transition from the lag phase to the exponential phase of growth (89). This may explain why induction of RpoS requires both elevated temperature and increased cell density (56). For pH, lowered pH (pH 7) enhanced RpoS and OspC expression (13, 56, 65). We showed that addition of Mn^{2+} was capable of inhibiting pH-induced BosR and RpoS activation (Fig. 4). The effect of mammalian blood on *rpoS* expression in feeding ticks may also function via Mn^{2+} and Zn^{2+} levels. When blood is added to BSK-II medium, RpoS-activated genes are induced (82). The high concentration of Zn^{2+} relative to low concentration of Mn^{2+} in human blood (which contains about 100 μM Zn^{2+} [90–92]) may account for this observation. Increased CO_2 has been shown to enhance BosR, RpoS, and OspC levels (83). We postulate that increased CO_2 may suppress Mn^{2+} transport and further lead to reduced intracellular Mn^{2+} level. It is known that Mn^{2+} import is influenced by dissolved gases such as O_2 and during oxidative stress (69, 93). Thus, Mn^{2+} transport in *B. burgdorferi* may be negatively regu-

lated by CO₂, which further increases the level of BosR. This prediction is supported by evidence from other bacterial pathogens whose genes responsible for Mn²⁺ transport are repressed by metal-dependent transcription factors under anaerobic conditions (70, 93).

Little is known about metal utilization in *B. burgdorferi*. It was reported that *B. burgdorferi* does not require or transport iron (94). Interestingly, a recent report by Wang et al. showed that *B. burgdorferi* contains both iron and copper (62). Our data in this study and previous studies by others (49, 94) indicate that Zn²⁺ is a substantial metal within the Lyme disease spirochete. The precise role of Zn²⁺ in the physiology of this pathogen is not completely understood; however, its abundance suggests that Zn²⁺ may be essential. In addition to BosR, other possible Zn²⁺-binding proteins have been identified in *B. burgdorferi*, including NapA (BB0690), the peptide deformylase (BB0065), and the glycolytic enzyme fructose-1,6-bisphosphate aldolase (BB0445) (95, 96). Both BB0065 and BB0445 are likely essential genes. Mn²⁺ is present at lower concentrations than Zn²⁺ in *Borrelia* when replicating at 37°C, suggesting a limited number of Mn²⁺-dependent proteins within *B. burgdorferi*. The ability to delete the Mn²⁺ transporter, *bmtA*, and the fact that the $\Delta bmtA$ mutant can grow in BSK-II medium as well as within dialysis membranes implanted in rats (49) suggest that Mn²⁺ may not be a prominent cofactor for *B. burgdorferi* physiology. Moreover, the $\Delta bmtA$ mutant's growth was indistinguishable from that of the B31-A3 parent strain in Chelex-treated BSK-II (see Fig. S1C in the supplemental material). However, despite a 12-fold reduction, we were able to detect a low concentration of Mn²⁺ within the $\Delta bmtA$ strain (Fig. 1). The concentration of Mn²⁺ detected in the $\Delta bmtA$ mutant was >2- to 3-fold above the Mn²⁺ present within the wash buffer, indicating that Mn²⁺ values from the $\Delta bmtA$ mutant were well above background. Thus, the essentiality of Mn²⁺ cannot be ruled out. For example, the *B. burgdorferi* genome carries genes encoding two likely essential glycolytic enzymes that may require Mn²⁺ as cofactors, including BB0004, encoding a putative phosphoglucomutase (EC 5.4.2.2) that catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate (97), and BB0658, encoding a putative phosphoglycerate mutase (Pgm; EC 5.4.2.1) (98). The fact that the $\Delta bmtA$ mutant is unable to infect mice or colonize ticks (49) supports the theory that Mn²⁺ may be essential in these Mn²⁺-limiting environments.

What is the mechanism underlying the negative regulation of BosR by Mn²⁺? Regulation of BosR by CO₂ has been previously observed at the posttranscriptional level (30, 61, 83). In this study, we showed that Mn²⁺ suppressed *bosR* at the posttranscriptional level, i.e., that the *bosR* mRNA level was not affected by Mn²⁺ (Fig. 4B). Given that BosR is a Zn-binding protein, increased intracellular Mn²⁺ levels may also affect the function of BosR. Nevertheless, our study demonstrates that the major effect of Mn²⁺ on BosR is at the level of BosR protein, by affecting either the translation of BosR or the stability of BosR protein. One attractive hypothesis is that there is a yet-to-be-identified Mn²⁺-dependent protease that governs the turnover rate of the BosR protein. We are currently in the process of testing this possibility.

In summary, we have demonstrated that the two transition metals Mn²⁺ and Zn²⁺ play reciprocal roles in regulation of RpoS in *B. burgdorferi* by affecting the level of BosR at the posttranscriptional level. Whether the activity of RpoN or Rrp2 phosphorylation is also affected by Mn²⁺ or Zn²⁺ remains to be determined. Our data also led us to postulate that many environmental cues

and host signals may collectively influence the Mn²⁺/Zn²⁺ ratio and subsequently modulate the BosR protein and *rpoS* transcription levels (Fig. 7). Given that human blood contains a high concentration of Zn²⁺ relative to a low concentration of Mn²⁺ (90–92), it is likely that the tick midgut during the feeding is under low Mn²⁺ and high Zn²⁺ conditions that favor the production of BosR and activation of the RpoS pathway. This model cannot explain the situation in the feeding larvae when spirochetes migrate from mammals to larval midguts, in which the RpoS pathway is not activated even in the presence of mammalian blood. The mechanism underlying the difference in gene regulation of *B. burgdorferi* between feeding nymphs and feeding larvae remains unclear but may be due to differences in temperature shift and growth rate changes that spirochetes encounter between the processes of acquisition (from mammals to larvae) and transmission (from nymphs to mammals) (4, 100). Nevertheless, this study demonstrates that Mn²⁺ is a global regulatory element in *B. burgdorferi* and supports the emerging notion that Mn²⁺ and Zn²⁺ play a critical role in regulation of virulence in bacteria.

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