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The BosR regulatory protein of *Borrelia burgdorferi* interfaces with the RpoS regulatory pathway and modulates both the oxidative stress response and pathogenic properties of the Lyme disease spirochete

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Abstract

Borrelia burgdorferi, the Lyme disease spirochete, adapts as it moves between the arthropod and mammalian hosts that it infects. We hypothesize that BosR serves as a global regulator in *B. burgdorferi* to modulate the oxidative stress response and adapt to mammalian hosts. To test this hypothesis, a *bosR* mutant in a low passage *B. burgdorferi* isolate was constructed. The resulting *bosR*::kan^R strain was altered when grown microaerobically or anaerobically suggesting that BosR is required for optimal replication under both growth conditions. The absence of BosR increased the sensitivity of *B. burgdorferi* to hydrogen peroxide and reduced the synthesis of Cdr and NapA, proteins important for cellular redox balance and the oxidative stress response, respectively, suggesting an important role for BosR in borrelial oxidative homeostasis. For the *bosR* mutant, the production of RpoS was abrogated and resulted in the loss of OspC and DbpA, suggesting that BosR interfaces with the RpoS-RpoN-Rrp2 regulatory cascade. Consistent with the linkage to RpoS, cells lacking *bosR* were non-infectious in the mouse model of infection. These results indicate that BosR is required for resistance to oxidative stressors and provides a regulatory response that is necessary for *B. burgdorferi* pathogenesis.

INTRODUCTION

The etiological agent of Lyme disease is the spirochete *Borrelia burgdorferi* and is transmitted by the bite of an infected tick. Infection with *B. burgdorferi* results in a multisystem disorder characterized by a skin lesion known as erythema migrans that is accompanied by a flu-like illness. If treatment is not sought at this stage, the infected individual is at risk for developing later stage pathology including carditis, neurologic issues, and arthritic manifestations, which contribute to the profound morbidity associated with this infection (Nadelman and Wormser, 1998; Steere *et al.*, 2004).

Given the recent advent of borrelial genetic techniques (reviewed in (Rosa *et al.*, 2005), the ability to evaluate individual loci in the context of experimental Lyme infection is now

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possible, assuming that the gene in question is not essential for *in vitro* growth. We have a long-standing interest in the role of the BosR regulatory protein, a homologue of the Fur family of regulators (Boylan *et al.*, 2003; Hyde *et al.*, 2006; Katona *et al.*, 2004; Seshu *et al.*, 2004b), in the ability of *B. burgdorferi* to regulate the oxidative stress response in this pathogen. Within the Fur family, BosR is most similar to PerR, a repressor that regulates genes involved in the oxidative stress response in *Bacillus* spp. (Bsat *et al.*, 1998b; Fuangthong *et al.*, 2002; Herbig and Helmann, 2001a; Mongkolsuk and Helmann, 2002). In contrast to PerR, Boylan et al. demonstrated that BosR activates expression of target borrelial genes involved in the oxidative stress response including *napA* (*dps*) and a CoA disulfide reductase designated *cdr* (Boylan *et al.*, 2003; Boylan *et al.*, 2006). In this regard, the role of BosR resembles that of OxyR, an activator in *E. coli* that promotes the expression of genes required for oxidative stress detoxification (Georgiou, 2002; Storz and Imlay, 1999; Zheng *et al.*, 1998). In biochemical assays, BosR binds to putative operator sequences upstream of *napA* (*dps*), *cdr*, superoxide dismutase (*sodA*), *bosR*, *bb0646*, and *oppA-V*, providing suggestive evidence that BosR functions as a global regulator to many unlinked genes within the *B. burgdorferi* genome (Boylan *et al.*, 2003; Boylan *et al.*, 2006; Katona *et al.*, 2004; Medrano *et al.*, 2007; Seshu *et al.*, 2004b). To date, all genetic analyses related to *bosR* have been limited to non-infectious isolates of *B. burgdorferi* (Hyde *et al.*, 2006; Seshu *et al.*, 2004b); that is, all prior attempts to genetically inactivate *bosR* in low-passage isolates have been unsuccessful.

Using a previously described limiting dilution transformation method (Yang *et al.*, 2004), we have isolated a genetic knockout of *bosR* in low-passage *B. burgdorferi* and have begun characterizing the importance of this regulator in the physiological and pathogenic properties of this spirochete. As expected, the *bosR* mutant in low-passage *B. burgdorferi* is impaired during *in vitro* growth. We also found that the *bosR* mutant is more sensitive to H_2O_2 and exhibits differential production of proteins involved in the oxidative stress response. Unexpectedly, the *bosR* mutant was incapable of synthesizing RpoS, which functions as a master switch for expression of a number of genes during mammalian infection (Burtnick *et al.*, 2007; Caimano *et al.*, 2004; Caimano *et al.*, 2007; Fisher *et al.*, 2005; Hubner *et al.*, 2001; Yang *et al.*, 2000b), including those encoding the well-characterized virulence determinants OspC and DbpA (Blevins *et al.*, 2008; Caimano *et al.*, 2004; Gilbert *et al.*, 2007; Grimm *et al.*, 2004a; Pal *et al.*, 2004; Shi *et al.*, 2008; Tilly *et al.*, 2007; Weening *et al.*, 2008; Yang *et al.*, 2005). As such, BosR is not only involved in the response to reactive oxygen species (ROS), but also interfaces with the pathogenic potential of *B. burgdorferi*, suggesting that BosR plays a crucial role in both borrelial oxidative homeostasis and the adaptive response important for infectivity.

RESULTS

Inactivation of *bosR* in low-passage *B. burgdorferi*

All prior attempts to isolate a *bosR* mutant in infectious *B. burgdorferi* using BSK-II agarose plating were unsuccessful and, given our recent success isolating a *dbpBA* deletion strain using a liquid limiting dilution method (Weening *et al.*, 2008; Yang *et al.*, 2004), this approach was utilized for the inactivation of *bosR* in low-passage *B. burgdorferi*. *B. burgdorferi* strain B31 derivative ML23 was transformed with pJS167 that contains the *bosR*::kan^R allele, used previously to inactivate *bosR* in non-infectious *B. burgdorferi* (Seshu *et al.*, 2004b), to isolate strain JH300 (Fig. 1A). Transformants were obtained and the putative mutants screened for the *bosR*::kan^R allele by both PCR (Fig. 1B) and Southern blot analysis (data not shown). Transformants were identified by the amplification of a 2.5-kb band representing *bosR* interrupted by a 2-kb stable transposon that confers resistance to kanamycin in *B. burgdorferi* (JH300, Fig. 1B). The same oligonucleotide primers amplify the approximate 0.5-kb native *bosR* gene (ML23, Fig. 1B). Those candidates that contained

the desired *bosR*::kan^R allele were then tested for their ability to produce BosR. Three independently isolated mutants were analyzed by Western immunoblot analysis under conditions where the parental strain produced BosR. All putative mutants did not produce BosR and one representative clone, designated JH300, was chosen for further study (Fig. 1C). The same compendium of plasmids was observed for JH300 as is seen for ML23 (i.e., missing cp9 and lp25; data not shown) (Labandeira-Rey and Skare, 2001a; Labandeira-Rey *et al.*, 2003). Subsequently, JH300 was transformed with pCADDY, which contains the *bbe22* (*pncA*) gene on a streptomycin resistant borrelial shuttle vector. This plasmid restores infectivity to the lp25 deficient strain ML23 (Purser *et al.*, 2003) and its transformation into JH300 was required to determine how the inactivation of *bosR* affects borrelial pathogenesis.

The growth phenotype of strain JH300

To determine how the loss of *bosR* affects *B. burgdorferi* growth, JH300 was grown both microaerobically and anaerobically and compared with its isogenic parental strain ML23. Microaerobically grown JH300 showed no lag in growth but exhibited a significant decrease in growth rate (as seen by the decreased slope) and did not attain the maximum cell density relative to its parental strain ML23 (Fig. 2A). Anaerobically grown JH300 exhibited a longer lag period than did ML23 (approximately 3 days), but demonstrated a comparable exponential growth rate and achieved the same final cell density seen for ML23 (Fig. 2B). The difference in cell growth for the *bosR* mutant when grown microaerobically and anaerobically suggests that BosR is needed to mediate different adaptive responses under these different growth conditions. It is important to note that for anaerobically grown JH300, there is a significant difference in cell density when a given day of growth is compared; however, once the lag in growth is relieved, the normal rate of growth is observed.

Sensitivity of the *bosR* mutant to hydrogen peroxide

Given the linkage of BosR to the oxidative stress response in *B. burgdorferi* (Boylan *et al.*, 2003; Boylan *et al.*, 2006; Seshu *et al.*, 2004b), we were interested in determining how the *bosR* mutant compared with its parental strain in its resistance to ROS. To this end, *B. burgdorferi* strains JH300 and ML23 were treated with H_2O_2 in concentrations ranging from 1 mM to 50 mM for 4 hours and compared to samples not exposed to H_2O_2. The incorporation of propidium iodide was tracked to assess the toxicity of H_2O_2 treatment since cells with compromised/depolarized cytoplasmic membranes rapidly take up propidium iodide. The resulting samples were then scored by flow cytometry to obtain an unbiased evaluation of cell viability for the samples and treatment tested. Comparison of strain JH300 and its parent ML23 indicated that the cells making BosR (ML23) were more resistant to H_2O_2 than those that did not make BosR (JH300), with the exposure at 5 mM H_2O_2 yielding the most pronounced differential in cell viability (Fig. 3). Statistical analyses indicated that a significant difference is observed across all concentrations of H_2O_2 tested (logit analysis yielded a $P < 0.001$). It is also important to emphasize that each data point shown is based on approximately 40,000 borrelial cells for each strain and concentration of H_2O_2 tested, and summarizes the data set from two independent experiments.

Inactivation of *bosR* alters the production of oxidative stress proteins

To determine how BosR levels affect the production of proteins involved in the oxidative stress response, we assessed the levels of BosR, NapA, Cdr, and SodA in strain JH300 when grown both anaerobically and microaerobically. BosR production was undetectable in JH300 under all tested growth conditions but was abundantly produced in strains MSK5 and ML23 grown anaerobically with CO_2 present (Fig. 4) as previously reported (Hyde *et al.*, 2007). The increase in BosR in MSK5 and ML23 under these conditions is not due to an increase in transcription since *bosR* transcripts were unchanged under both microaerobic and

anaerobic conditions (Hyde *et al.*, 2007), and instead reflects a post-transcriptional regulatory effect that is not yet characterized.

The production of NapA was increased in the low-passage parental strains ML23 and MSK5 (infectious B31 strain containing all known plasmids) relative to the *bosR* mutant JH300 (Fig. 4), when the parental strains were grown anaerobically. The increased production of NapA during anaerobiosis (Fig. 4) is consistent with the increased production of BosR under anaerobic conditions as previously described (Hyde *et al.*, 2007). Although JH300 is capable of producing NapA, the absence of BosR prevents the increased production of NapA during anaerobic growth as is observed for ML23 and MSK5 (Fig. 4). A similar pattern was observed for Cdr (Fig. 4), a protein required for redox balance in the borrelial cytoplasm (Boylan *et al.*, 2006). The results for NapA and Cdr provide further support to the prior contention that BosR functions as an activator for both *napA* and *cdr* (Boylan *et al.*, 2003;Boylan *et al.*, 2006). Given that both NapA and Cdr were produced at greater levels in ML23 and MSK5, it was surprising that SodA levels were not affected by the absence of BosR (Fig. 4). Nevertheless, based on the response seen for both NapA and Cdr, it is clear that BosR is required for optimal production of a subset of borrelial oxidative stress responsive proteins.

The loss of *bosR* abrogates RpoS-dependent regulation

When strain JH300 was grown anaerobically and compared with anaerobically grown ML23 and MSK5, it was apparent that JH300 did not synthesize an abundant 23-kDa protein species (Fig. 5A). Previous work demonstrated that OspC is induced under anaerobic growth (Hyde *et al.*, 2007) and, given this similarity, we asked whether the *bosR* mutant was unable to make OspC and DbpA, as well as RpoS. The profiles observed indicate that the loss of *bosR* in JH300 abrogates the production of detectable levels of RpoS, OspC and DbpA when these cells are grown anaerobically (Fig. 5B). Thus, BosR appears to interface with the activation of *rpoS*, which is an alternative sigma factor needed for both *ospC* and *dbpBA* expression (Caimano *et al.*, 2004;Caimano *et al.*, 2007;Fisher *et al.*, 2005;Gilbert *et al.*, 2007;Hubner *et al.*, 2001;Yang *et al.*, 2005). Since both OspC and DbpA play important roles in borrelial pathogenesis, the linkage of BosR to their production implicates BosR in the pathogenic potential of *B. burgdorferi*. Since RpoS production is dependent on the Rrp2-RpoN system (Boardman *et al.*, 2008;Burtnick *et al.*, 2007;Caimano *et al.*, 2004;Caimano *et al.*, 2007;Fisher *et al.*, 2005;Hubner *et al.*, 2001;Yang *et al.*, 2000b;Yang *et al.*, 2003), the steady-state levels of Rrp2 and its cognate histidine Hk2 were evaluated. The absence of BosR had no effect on the production of either Rrp2 or Hk2 (data not shown for Rrp2 and Fig. 5B).

It is important to note that RpoS, OspC and DbpA are all present in microaerobically grown ML23 and MSK5 but at levels significantly below that seen for anaerobically grown *B. burgdorferi.* Furthermore, each lane is equivalently loaded as shown in the endoflagellum (EF) control immunoblot and Coomassie blue-stained gel (Fig. 5). Longer exposures did show the presence of RpoS, OspC and DbpA in microaerobically grown ML23 and MSK5 (not shown). However, even at these longer exposures, no detectable levels of RpoS, OspC or DbpA were visualized for JH300 grown either microaerobically or anaerobically.

Role of *bb0646* in the JH300 phenotype

Another possible explanation for the growth phenotype and change in antigen production observed for strain JH300 could be the elimination of the gene downstream from *bosR*, *bb0646*, which is predicted to encode an exported lipase (Katona *et al.*, 2004). To address this possibility, strain DS102 (ML23 *bb0646*::gent^{R}) was compared with strains ML23 and JH300 for various phenotypic traits. Recent results indicated that DS102 exhibited no overt

defect in growth rate relative to its parental strain (Hyde et al., 2010), suggesting that the growth defect seen for strain JH300 was restricted to the loss of *bosR* and not *bb0646*.

To assess the role of BB0646 in antigen production previously attributed to BosR-dependent mechanisms, we tested the ability of DS102 to generate RpoS and other RpoS-dependent proteins. The results indicated that DS102 makes unimpaired levels of BosR, and more importantly RpoS (Fig. 6) and OspC (data not shown). Furthermore, we have recently tested strain DS102 in the mouse model of experimental Lyme borreliosis and determined that strains lacking BB0646 retain infectivity (Shaw and Skare, unpublished observations). Taken together, these data support the notion that the phenotype observed for JH300 is associated with the absence of BosR and is not due to a polar effect involving the loss of *bb0646*.

Infectivity of *B. burgdorferi* lacking BosR

To assess the role of BosR in borrelial pathogenesis, we tested whether *B. burgdorferi* lacking *bosR* were able to infect mice. Although the inability of the *bosR* mutant to produce OspC and DbpA suggested that the strain would be significantly impaired in its ability to infect, it is possible that the effect seen is limited to an in vitro growth characteristic of *bosR*-deficient *B. burgdorferi*. When mouse infections were conducted with JH300 pCADDY and compared with its infectious parental strain ML23 pCADDY, we found that JH300 pCADDY supported no infectivity at either a 10^3 or a 10^5 inoculum dose (Table 3). This is in contrast to the parental strain ML23 pCADDY, which infected all mice at each inoculum dose tested. The largest limitation of this comparison was our inability to genetically complement the *bosR* mutant either in *trans* on a shuttle vector or via *cis* complementation at a heterologous site in the chromosome (Li *et al.*, 2007). Despite this limitation, these infectivity results, coupled with the linkage of BosR to RpoS production, strongly suggest that BosR plays an important role in the pathogenic potential of *B. burgdorferi*.

DISCUSSION

With improved molecular genetic tools to evaluate various genes in *Borrelia burgdorferi*, we are beginning to get a better understanding of loci that are key for the pathogenesis of Lyme borreliosis. Although several important pathways have been identified, most notably the Rrp2-RpoN-RpoS regulatory cascade (Boardman *et al.*, 2008; Burtnick *et al.*, 2007; Caimano *et al.*, 2004; Caimano *et al.*, 2007; Fisher *et al.*, 2005; Hubner *et al.*, 2001; Yang *et al.*, 2000b; Yang *et al.*, 2003; Yang *et al.*, 2005), it remains unclear how *B. burgdorferi* integrates the signals it perceives into a regulatory response. Several *in vitro* correlates have been established that simulate environmental conditions as the spirochete moves from an infected tick to a mammalian host following a blood meal, particularly increases in temperature, fluctuations in pH, dissolved oxygen, CO_2 and other uncharacterized mammalian specific factors (Akins *et al.*, 1995; Akins *et al.*, 1998; Brooks *et al.*, 2003; Carroll *et al.*, 1999; Cassatt *et al.*, 1998; Champion *et al.*, 1994; Das *et al.*, 1997; Fikrig *et al.*, 1997; Hyde *et al.*, 2007; Lybecker and Samuels, 2007; Ojaimi *et al.*, 2003; Ojaimi *et al.*, 2005; Revel *et al.*, 2002; Schwan *et al.*, 1995; Seshu *et al.*, 2004a; Skare *et al.*, 1999; Stevenson *et al.*, 1995; Tokarz *et al.*, 2004; Yang *et al.*, 2000a). Despite these important advances in the molecular and genetic analyses of *B. burgdorferi*, much remains unknown regarding genes that contribute to the pathogenic potential of *B. burgdorferi* as well as the transcriptional regulatory proteins that affect the expression of these genes.

In this study, we demonstrate that the BosR regulatory protein is linked to the pathogenic potential of *B. burgdorferi* since it is needed for the synthesis of RpoS (Fig. 5B), which in turn is required for the expression of the known borrelial virulence determinants, most

notably *ospC* and *dbpBA* (Blevins *et al.*, 2008;Caimano *et al.*, 2004;Gilbert *et al.*, 2007;Grimm *et al.*, 2004b;Pal *et al.*, 2004;Shi *et al.*, 2008;Tilly *et al.*, 2007;Weening *et al.*, 2008;Yang *et al.*, 2005). We also show that BosR is linked to the optimal response to ROS and affects the production of NapA and Cdr, two proteins involved in combating oxidative stress and maintaining the proper redox balance in *B. burgdorferi* (Boylan et al., 2003;Boylan et al., 2006;Li et al., 2007). These data are consistent with the similarity that BosR shares with the oxidative stress regulator PerR from *Bacillus* spp. (Bsat *et al.*, 1998a;Fuangthong *et al.*, 2002;Herbig and Helmann, 2001b;Mongkolsuk and Helmann, 2002). As such, BosR is linked to both physiologically critical and virulence-associated pathways in a manner that we predict is RpoS-independent and RpoS-dependent, respectively. Our working hypothesis is that BosR responds to the redox environment of the cell and modulates gene expression accordingly in either a direct or indirect manner. Since the redox status is likely to change as *B. burgdorferi* traffics through the tick vector and mammalian host, regulation by BosR might represent an important adaptive response that this pathogen uses to colonize such disparate hosts.

The most direct approach to evaluate the importance of BosR is by genetically inactivating or deleting *bosR*; however, prior attempts to inactivate *bosR* in infectious *B. burgdorferi* were unsuccessful. All of the previous attempts to isolate *bosR* mutants used agarose plating and the ability to visualize colonies may have been impaired since the mutant exhibits a delayed growth rate. Because transformations involving *B. burgdorferi* are inefficient, one can dilute a transformation population in liquid media under appropriate selection and obtain clonal isolates (Yang *et al.*, 2004). When this method was used to select *bosR* mutants, several ML23 *bosR*::kan^R clones were isolated, characterized, and a representative strain designated as JH300 (Fig. 1).

The growth defect observed for JH300, especially when grown microaerobically, may have contributed to the difficulty in isolating a *bosR* mutant previously, given the inability of this strain to replicate to high densities (Fig. 2). One striking feature of JH300 is its increased ability to attain maximal densities when the cells are grown anaerobically. This is consistent with the idea that a critical detoxification function for BosR would not be required under anaerobiosis when the levels of dissolved oxygen, and thus levels of ROS, would be reduced.

The increased sensitivity of the *bosR* mutant to H_2O_2 is consistent with a role of BosR in regulating the oxidative stress response; however, we predicted that the differential would be more pronounced (Fig. 3). The high level resistance to H_2O_2 observed in the parental strain is consistent with recent results reported by Boylan et al. indicating that borrelial cells are more refractory to H_2O_2-mediated oxidation than other bacteria (Boylan *et al.*, 2008), mostly due to their lack of iron and thus the concomitant absence of Fenton-based DNA damage (Posey and Gherardini, 2000). Boylan et al. also showed that *B. burgdorferi* are more sensitive to alkyl peroxides that can oxidize polyunsaturated lipids that these spirochetes incorporate into their cell envelope (Boylan *et al.*, 2008). The difference in H_2O_2 sensitivity between the *bosR* and wild type strains observed here suggests that BosR does neutralize the oxidative stress imposed by H_2O_2, due instead to damage incurred by the oxidation of borrelial proteins and lipids, but not nucleic acids. Another protein whose levels we thought would be adversely affected by BosR levels was SodA since purified BosR was found to bind to a putative *sodA* operator domain (Seshu et al., 2006). However, the *bosR* mutant showed no change in the level of SodA (Fig. 4). Recently, Esteve-Gassent et al. demonstrated that *sodA* mutants show an increased sensitivity to superoxide anion generating compounds relative to their isogenic parent (Esteve-Gassent *et al.*, 2009). While it is possible the *bosR* and *sodA* mutants may have similar sensitivity phenotypes to other oxidative stressors, including H_2O_2, it is equally as plausible that the response seen to H_2O_2

and other peroxides would be distinct given the fact the SodA provides a specific enzymatic function (i.e., conversion of superoxide anion to H_2O_2) whereas BosR presumably regulates many unlinked genes involved in the oxidative stress response (i.e., NapA and Cdr) and thus would likely have a more profound overall effect on the borrelial ROS detoxification. It is important to note that the analysis described herein only accounts for steady-state levels of SodA. It is possible that BosR levels affect *sodA* transcription and the lack of an observed effect may be due to the stability of the borrelial SodA protein.

The observation that the oxidative stress responsive proteins NapA and Cdr are induced when the wild-type (MSK5) and parental strain (ML23) are grown anaerobically (Fig. 4) is somewhat counterintuitive to the proposed functions of these proteins; that is, one might predict that the NapA and Cdr would be selectively produced under microaerobic growth and made at lower levels when borrelial cells are grown under hypoxic conditions. This observation may reflect differences in CO_2 content in the growth of these spirochetes. Higher concentrations of CO_2 (5%) simulate, in part, mammalian host conditions and serve as an environmental cue independent of oxygen (Hyde *et al.*, 2007;Klengel *et al.*, 2005;Mitchell, 2005). A CO_2 effect was observed for NapA production such that when CO_2 was limited, independent of the presence or absence of oxygen, less NapA was synthesized relative to conditions where CO_2 levels were increased (Hyde *et al.*, 2007). Thus, a CO_2 regulatory effect is operative that limits NapA production when the media is purged of all gases, including CO_2 (Hyde *et al.*, 2007). Under these conditions one might expect that NapA and Cdr production would also be altered in the *bosR* mutant but, more importantly, would not be able to respond as effectively to make more of these proteins when either CO_2 or O_2 levels are increased.

Perhaps the most interesting characteristic of the *bosR* mutant is its link to RpoS, and thus OspC and DbpA, proteins that are important to establish infection (Blevins *et al.*, 2008; Caimano *et al.*, 2004; Grimm *et al.*, 2004b; Pal *et al.*, 2004; Shi *et al.*, 2008; Tilly *et al.*, 2007; Weening *et al.*, 2008; Yang *et al.*, 2005). The fact that OspC and DbpA levels are dependent on BosR in wild type *B. burgdorferi* suggests that BosR in some way interfaces with the Rrp2/RpoN/RpoS two-component regulatory system in a manner that results in the production of key virulence determinants in *B. burgdorferi* (Fig. 5B). Based on the BosR-dependent production of RpoS seen in ML23 and MSK5 relative to JH300 (Fig. 5B), BosR is required for *rpoS* expression, probably in an indirect but perhaps in a direct manner, or is involved (indirectly) in a post-transcriptional regulatory event that promotes the synthesis of RpoS. The connection of BosR to RpoS induction via BosR is not known but may involve the following: (*i*) alteration of the phosphorylation status of Hk2 (the cognate histidine kinase genetically linked to *rrp2*) or other phosphorylated intermediates that activate Rrp2 and subsequently *rpoS* (Boardman *et al.*, 2008); (*ii*) expression of *rpoN*; and/or (*iii*) induction of DsrA, a small regulatory RNA required for efficient translation of *rpoS* transcripts into RpoS (Lybecker and Samuels, 2007).

In regard to the potential role of Hk2 in the BosR-specific induction of *ospC* and *dbpBA*, it is important to note that the absence of BosR in JH300 did not affect the production of Hk2 or Rrp2 (Fig. 5B for Hk2 and data not shown for Rrp2). This is consistent with the idea that the activation of these proteins does not require increased transcription since these two-component regulatory proteins are generally constitutively expressed and the resulting proteins activated post-translationally via a reversible phosphorylation event. Furthermore, the production of RpoS can occur independent of Hk2 given that an *hk2* mutant can still transcribe *rpoS* via Rrp2 (Burtnick *et al.*, 2007). The genes that may contribute to the phosphorylation status of Rrp2, in either an Hk2-dependent or independent manner, are not known.

While we were encouraged by the lack of infectivity seen for JH300 (Table 3), we were disappointed that we were unable to genetically complement this *bosR* mutant. Attempts to genetically complement JH300 with *bosR* expressed from its native promoter on a borrelial shuttle vector were unsuccessful, as were *cis* complementation approaches at a heterologous site in the chromosome. The inability to complement the *bosR* mutant with a shuttle vector construct may be due to a negative gene dosage effect due to the multicopy status of these plasmids. That is, if the balance of functional BosR is critical for cellular homeostasis, then increased levels of the regulator could result in anomalous expression of target genes with deleterious effects on *B. burgdorferi* growth. These concerns would presumably be allayed in a *cis* complementation strategy. However, since we designed our construct to recombine into a non-native sequence in the borrelial chromosome (Li *et al*., 2007), additional upstream sequences not found in Li et al. configuration, may be necessary for the native expression of *bosR*.

The lack of infectivity observed for JH300 was expected due to the absence of RpoS production and is consistent with the non-infectious phenotype of the *B. burgdorferi rpoS* mutant (Caimano *et al.*, 2004). Based on the data obtained, we hypothesize that BosR regulated genes can be categorized as those that (i) maintain oxidative homeostasis in an RpoS-independent manner; (ii) affect the pathogenesis of *B. burgdorferi* due to RpoS-dependent regulatory events; and (iii) alter the pathogenic potential of the spirochete in an RpoS-independent context. Addition of inducible *rpoS* back into a *bosR* mutant, under conditions that synthesize native levels of RpoS, might provide a modality to determine how the BosR-dependent, RpoS-independent gene subset contributes to the infectivity potential of *B. burgdorferi*.

Another consideration for the *bosR*::kan^R mutation in JH300 is the potential polar effect seen on the downstream gene *bb0646*, which encodes a putative lipase (Katona *et al.*, 2004). To address the potential impact of the absence of *bosR* and *bb0646* in JH300 relative to the loss of *bb0646* alone in strain DS102, we compared some phenotypic traits of these two *B. burgdorferi* mutants. Strain DS102 exhibited no difference in growth rate relative to its parental strain ML23 (data not shown) and, in stark contrast to JH300, the absence of the putative lipase did not affect the production of RpoS (Fig. 6) or OspC (data not shown). These results suggest that the loss of RpoS and OspC in strain JH300 is due to the absence of BosR and not BB0646 and is consistent with the contention that a global regulator such as BosR would be associated with the regulatory response and/or production of several target proteins whereas a protein with a specific enzymatic function like BB0646 would not be involved in this type of regulation.

Recently an independently isolated strain was characterized whereby the native *bosR* promoter was replaced with a previously developed IPTG inducible promoter system (P_{flac}; Gilbert *et al*., 2007). Specifically, a P_{flac}-*bosR* construct was integrated into the borrelial chromosome (and linked with an antibiotic marker) replacing the native *bosR* locus (Hyde et al., 2010). When IPTG was selectively restricted, a conditional *bosR* mutant was obtained such that this strain exhibited reduced growth commensurate with that observed here for strain JH300 and did not make detectable levels of BosR. Conversely, if IPTG was added back to the culture, the growth of the cells mirrored that observed for strain ML23 and BosR was readily observed (Hyde et al., 2010). In addition, the induction of *bosR* was required for maximal resistance to H_2O_2 and resulted in the increased synthesis of NapA and SodA. Moreover, growth in the presence of IPTG and the concomitant production of BosR promoted the synthesis of OspC and DbpA, suggesting that BosR interfaced with *rpoS* (Hyde et al., 2010). *B. burgdorferi* cells grown in the absence of IPTG were non-infectious in immunodeficient mice (the parent strain lacks plasmid lp28-1 that is required for persistent infection; Labandeira-Rey *et al.*, 2003) as observed for JH300 in

immunocompetent mice described here (Table 3). Taken together with the data presented here, these results serve as an independent corroboration of the role of BosR in both oxidative stress and pathogenic properties of *B. burgdorferi* and further validate the importance of the BosR regulator in borrelial biology.

Herein we describe the isolation and characterization of a knockout mutant of *bosR* in a low passage isolate of *B. burgdorferi*. Initial characterization indicated that BosR is required for the normal replication of *B. burgdorferi* when the cells are cultured either microaerobically or anaerobically. Furthermore, *B. burgdorferi* cells to which BosR is absent are more sensitive to H_2O_2 (Fig. 3), suggesting that BosR regulatory events are required for the high level resistance to ROS seen for *B. burgdorferi* (Boylan *et al.*, 2008). Moreover, *bosR* mutants are non-infectious presumably because the production of BosR is required for the optimal expression of genes involved in both the oxidative stress response (i.e., *napA* and *cdr*) and virulence (*rpoS* and *rpoS*-regulated genes). Array-based analyses will be helpful in identifying genes regulated by BosR to better define the loci needed for the borrelial oxidative detoxification response and to determine if and how BosR regulated RpoS-dependent and RpoS-independent genes factor into the pathogenesis of *B. burgdorferi*.

EXPERIMENTAL PROCEDURES

Bacterial Strains

B. burgdorferi strains, B31 derivatives, were grown in BSK-II medium supplemented with 6% normal rabbit serum (NRS) either microaerobically or anaerobically as described (Table 1) (Hyde *et al.*, 2007). Borrelial strains were grown in the appropriate antibiotic for selective pressure as needed: kanamycin at 300 μg/ml; streptomycin at 50 μg/ml; and gentamicin at 50 μg/ml. The Institutional Biosafety Committee at Texas A&M University approved the use of infectious *B. burgdorferi* described in this study.

Escherichia coli Mach1™-T1^R cells were used for all cloning steps and were transformed with appropriate PCR-amplified products cloned into pCR8/GW/TOPO (Invitrogen Corp., Carlsbad, CA). The *E. coli* cells were grown with aeration in LB media at 37 C. For experiments involving *E. coli*, antibiotics were used at the following concentrations: spectinomycin at 100 μg/ml and kanamycin at 50 μg/ml.

Plasmid constructs

A streptomycin-resistant shuttle vector carrying the *bbe22* and *bbe23* region of lp25 to restore infectivity was constructed and designated pCADDY (Table 1). To obtain pCADDY, pBBE22 and pKFSS1 were digested with *Nde*I and *Aat*II to remove the *aphI* (confers resistance to kanamycin; kan^R) and *aadA* (confers resistance to streptomycin; str^R) genes, respectively. The pKFSS1 fragment containing *aadA* was then cloned into the pBBE22 backbone replacing the *aphI* gene. The construct was screened by restriction digest and candidates confirmed by sequence analysis.

Transformation of *B. burgdorferi*

B. burgdorferi strains ML23 and JH300 were made competent, electroporated, and transformants isolated with antibiotics (kanamycin or streptomycin as appropriate depending on the construct), as previously described (Samuels, 1995; Seshu *et al.*, 2004b; Seshu *et al.*, 2006; Weening *et al.*, 2008). Electroporated cells were allowed to recover overnight in 15 ml of complete BSK media. Following an overnight incubation, the final volume was increased to 45 ml and 180 μl of 0.5% phenol red was added along with the appropriate antibiotic to allow for improved visibility due to a decrease in pH and to impose a selective pressure, respectively. Cultures were then distributed in 180 μl volumes across two 96 well

plates and incubated for several weeks until a change in the pH indicator is detected, suggestive of growth (Yang *et al.*, 2004). Putative transformants were then screened by PCR, Southern, and Western analysis.

PCR

PCR was done using Invitrogen Supermix High Fidelity as described previously with the oligonucleotide primers listed in Table 2 (Weening *et al.*, 2008).

SDS-PAGE and Western Immunoblotting

B. burgdorferi cultures were grown to 5×10^7 cells/ml and samples were taken for Western analysis. Borrelial protein lysates were resolved by SDS-12.5% polyacrylamide gel electrophoresis (PAGE) as previously described (Hyde *et al.*, 2007). Proteins were resolved by SDS-PAGE and transferred to PVDF membrane for Western analysis as indicated previously (Hyde *et al.*, 2007; Seshu *et al.*, 2004a; Seshu *et al.*, 2004b; Seshu *et al.*, 2006; Weening *et al.*, 2008). Antibodies were used at the following dilutions: murine anti-flagellum (Affinity Bioreagent, Golden, CO) at 1:20,000; rabbit anti-NapA at 1:100,000 (generously provided by Frank Gherardini) (Boylan *et al.*, 2003); rabbit anti-Cdr at 1:1000 (generously provided by Frank Gherardini) (Boylan *et al.*, 2006); rabbit anti-OspC at 1:1,000 (generously provided by Richard Marconi); rabbit anti-DbpA at 1:10,000 (generously provided by Magnus Höök); and mouse anti-Rrp2 and anti-Hk2 each at 1:40, respectively (generously provided by Xiaofeng Yang). To detect Rrp2 or Hk2, an additional amplification step was required using 1:1000 goat anti-mouse Ig followed by a subsequent incubation with 1:1000 anti-goat conjugated horseradish peroxidase. Finally, secondary antibodies, e.g., anti-mouse or anti-rabbit with conjugated horseradish peroxidase, as appropriate, were used in conjunction with chemiluminescent substrates to detect the requisite antigen-antibody complexes.

Hydrogen peroxide assays and flow cytometry analysis

ML23 and JH300 cultures were inoculated at 10^5 cells/ml and incubated under anaerobic conditions until reaching a cell density of 5×10^7 cells per ml. For each assay conducted, 3 independent cultures were grown in triplicate. 5×10^7 cells were pelleted and resuspended in modified BSK-II lacking BSA and treated with 0, 1, 5, 10, or 50 mM hydrogen peroxide for 4 hours in a 1 ml final volume. Samples were centrifuged and washed with phosphate-buffered saline (PBS) containing 2% BSA and incubated with 100 ng/μl propidium iodide (PI) for 15 minutes in the dark. Following the incubation, the samples were fixed with 4% paraformaldehyde for 10 minutes, pelleted, and washed in PBS, 2% BSA. The resulting samples were resuspended in 1 ml PBS, 2% BSA and analyzed with a FACSCalibur flow cytometer in conjunction with CellQuest acquisition software (Becton Dickinson Immunocytometry Systems, San Jose, CA). The log amplification was used to collect forward scatter, side scatter, and PI fluorescence with the latter collected through a 650 nm longpass filter. The list mode data acquired for each strain, growth condition, and concentration of H_2O_2 tested was based on approximately 20,000 events (in duplicate) as indicated by light scatter gates. The subsequent data was analyzed with FlowJo (versions 8.7 and 8.8, Treestar, Inc., Ashland, OR), using forward and side light scatter to gate *B. burgdorferi*. Controls included heat-killed and untreated samples.

Statistical Analysis

The percentage of killed *B. burgdorferi* at various concentrations of H_2O_2 was analyzed using a logit regression model. The data analysis was done in R version 2.9.1 (http://CRAN.R-project.org) using the generalized linear models function in conjunction with a binomial error structure. The response data consisted of a two-column array whereby

column 1 contained the number of cells killed and column 2 contained the number of viable cells. The explanatory variable used was $\log_{10}[H_2O_2]$. The various fractional cell kills following H_2O_2 exposure were predicted from the model for each strain analyzed, as were 95% confidence intervals around each concentration. When compared with $\log_{10}[H_2O_2]$ by itself, the inclusion of the group factor significantly improved the fit of the model tested to $P < 0.001$, based on χ^2 analyses of deviance.

Infectivity Studies

C3H mice at 8 weeks of age were infected intradermally with 10^3, 10^4, or 10^5 of *B. burgdorferi* strain ML23 pCADDY or JH300 pCADDY. Following 14 days of infection, the mice were sacrificed and skin, spleen, heart, bladder, lymph node, and tibiotarsal joint were aseptically removed and cultivated in BSK-II media supplemented with 6% NRS and appropriate antibiotics. The presence of *B. burgdorferi* was scored by darkfield microscopy (Gilbert *et al.*, 2007; Labandeira-Rey and Skare, 2001b; Labandeira-Rey *et al.*, 2003; Seshu *et al.*, 2006; Weening *et al.*, 2008). All animal work performed was reviewed and approved by the University Laboratory Animal Care Committee at Texas A&M University.

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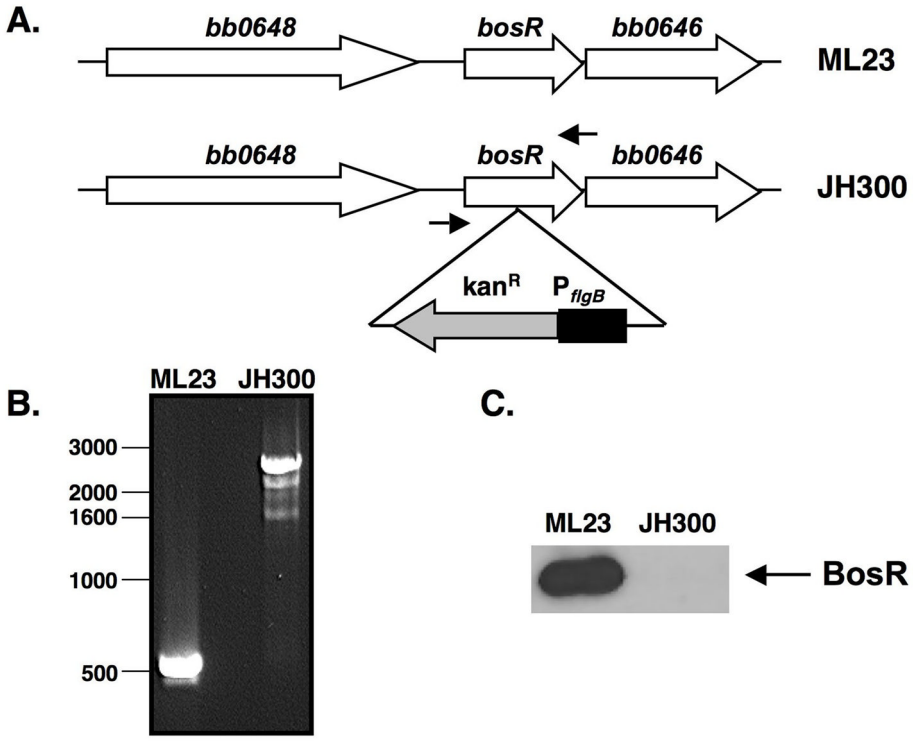

FIG. 1.

Schematic of the *bosR*-containing operon and screening of *bosR*::kan^R mutant, JH300, in *B. burgdorferi*. A. The schematic depicts the chromosomal region of *B. burgdorferi* containing *bosR* and the genetic disruption of *bosR* with the insertion of P_{flgB}-kan^R resulting in strain JH300. The arrows indicate the location of oligonucleotide primers utilized to screen clones for presence of kan^R cassette. B. PCR screen of JH300 was performed using primers specific for *bosR* that amplified a 586 bp product in ML23 or an approximate 2.5-kb product in JH300, indicating the insertion of P_{flgB}-kan^R. Lane 1: ML23; Lane 2: JH300. Markers (in bp) are shown on the left. C. BosR is not produced in strain JH300. Cultures were grown to 5×10^7/ml and cell lysates were resolved by SDS-PAGE, immobilized on PVDF membranes, and probed with antisera specific for BosR.

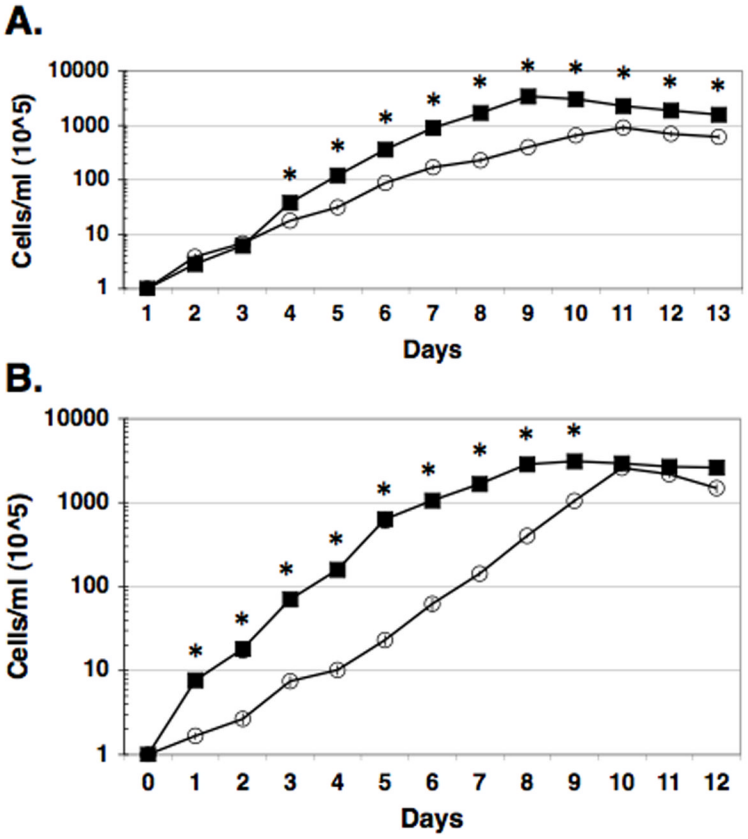

FIG. 2.

The *bosR*::kan^R mutant, JH300, displays a delayed growth phenotype relative to the parental, ML23 under microaerobic and anaerobic conditions. Independent cultures were grown in triplicate, cell densities averaged and standard error indicated by bars. A. ML23 (dark squares) and JH300 (open circles) were grown under microaerobic conditions (3.48 ppm dissolved O_2, 1% CO_2). B. ML23 (dark squares) and JH300 (open circles) were grown under anaerobic conditions (0.087 ppm dissolved O_2, 5% CO_2). Asterisks indicate significant differences in cell density for a given day ($P < 0.001$).

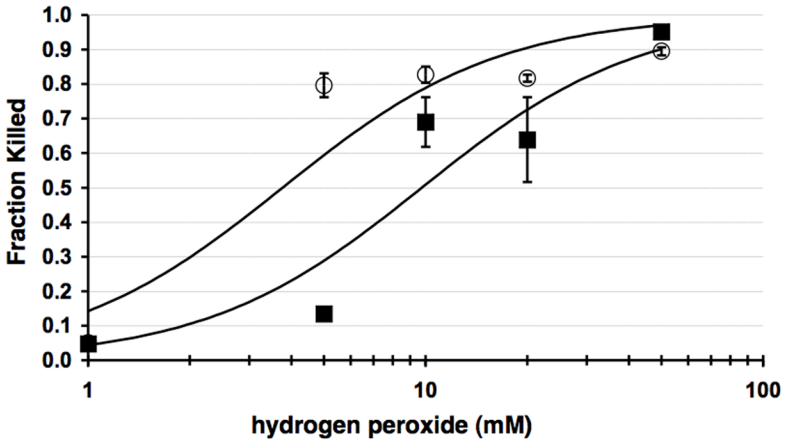

FIG. 3.

BosR is required for optimal protection from H_2O_2. Two independent anaerobic cultures were grown to exponential phase and treated with various concentrations of H_2O_2 up to 50 mM each in triplicate. JH300 (open circles) is significantly more sensitive to killing by H_2O_2 relative to parental, ML23 (solid squares) with a *P* value across all H_2O_2 concentrations tested of less than 0.001. The lines shown represent the best fit to the logit regression model for the *bosR* mutant (JH300, top line) and the parental strain (ML23, bottom line) for the data obtained. Please note that the data points for the 1 mM H_2O_2 treatment are of equal value for both strains tested and thus overlap one another.

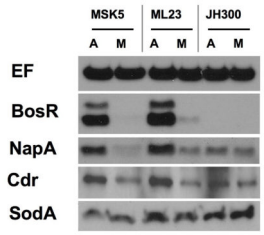

FIG. 4.

BosR is required for maximal expression of NapA and Cdr. Wild type infectious MSK5, parental strain ML23 and JH300 (ML23 *bosR*::kan^R) were grown under both anaerobic (A) and microaerobic (M) growth conditions. Samples were probed with antibodies specific for the antigen indicated. Endoflagellum (EF) levels were used as a control to normalize for equivalent protein loading.

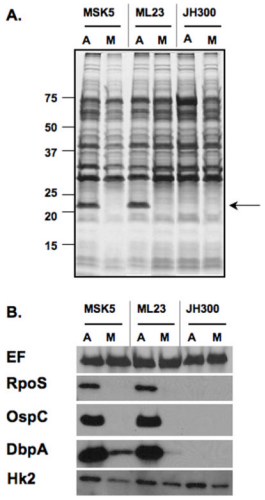

FIG. 5.

BosR alters the synthesis of RpoS and virulence determinants regulated by the Rrp2-RpoN-RpoS pathway. Wild type infectious MSK5, parental strain ML23, and JH300 (ML23 *bosR*::kan^{R}) were grown under anaerobic (A) and microaerobic (M) growth conditions to 5×10^{7} cells/ml. A. Coomassie-stained SDS-PAGE gel of cell lysates. The arrow indicates the presence of a 23-kDa protein in MSK5 and ML23 that is absent in JH300. B. Samples were probed with antibodies specific for the antigen indicated. As in Fig. 4, endoflagellum (EF) is used as a control to normalize for equivalent protein loading.

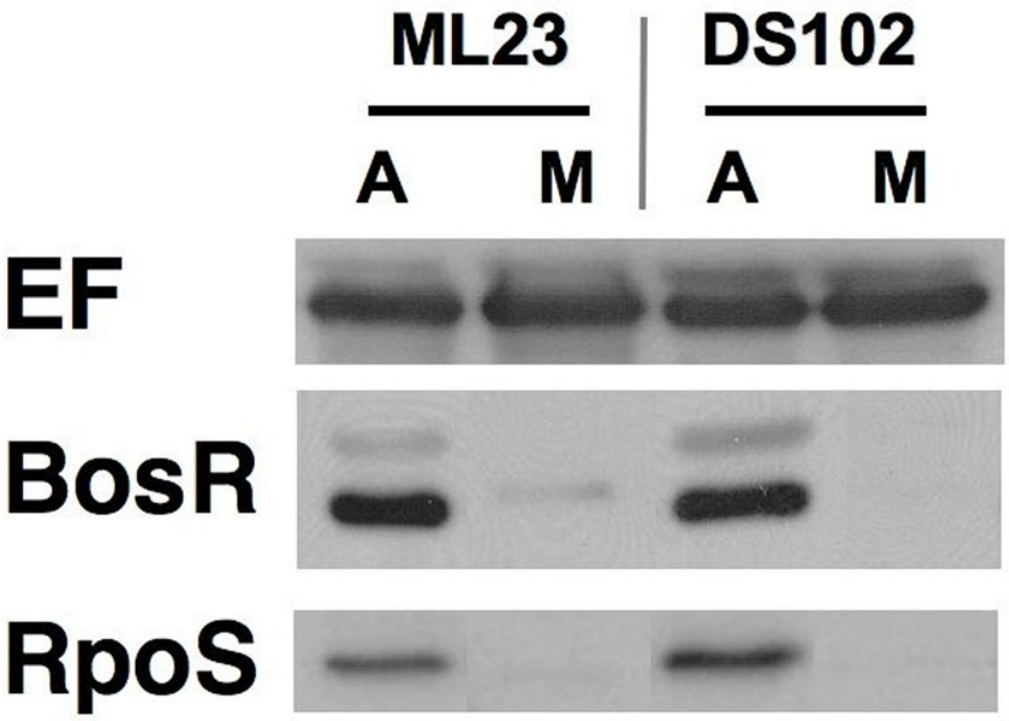

FIG. 6.

ML23 *bb0646*::gent^R does not alter the synthesis of RpoS. The parental strain ML23 and DS102 (ML23 *bb0646*::gent^R) were grown anaerobically and microaerobically, resolved by SDS-PAGE, immobilized on PVDF membrane and probed with antisera for BosR and RpoS. Endoflagellum (EF) was used as a control for equivalent protein loading.

Table 1

Strains and Plasmids used in this study

B. burgdorferi strains used in this study:		
Strain	*Genotype*	*Reference*
MSK5	B31 derivative, all plasmids present	(Labandeira-Rey and Skare, 2001a)
ML23	missing lp25	(Labandeira-Rey and Skare, 2001a)
DS102	ML23, *bb0646*::gent^R	(Hyde *et al*., 2010)
JH300	ML23, *bosR*::kan^R	This study
E. coli strains used in this study:		
Strain	*Genotype*	*Source*
Mach-1™-T1^R	Φ80*lacZ*ΔM15 Δ*lacX*74 hsdR (r_k^-, m_k^+) Δ*recA*1398 *endA*1 *tonA*	Invitrogen
Plasmids used in this study:		
Plasmid	*Resistance*	*Comments/Source/Reference*
pCR8/GW/TOPO	spec^R	Gateway PCR cloning/entry vector; Invitrogen
pJS167	kan^R	suicide vector containing *bosR*::kan^R construct (Seshu *et al.*, 2004b)
pBBE22	kan^R	borrelial shuttle vector pBSV2 containing *pncA* fragment to restore infectivity in ML23 (Purser *et al.*, 2003)
pKFSS1	spec^R/strep^R	streptomycin resistant borrelial shuttle vector (Frank *et al.*, 2003); confers spec^R in *E. coli*
pCADDY	spec^R/strep^R	shuttle vector derived from pBBE22 (Purser *et al.*, 2003) replacing the kanamycin resistance with streptomycin resistant determinant; confers spec^R in *E. coli*

Table 2

Oligonucleotides used in this study.

Designation	Oligonucleotide sequence (5′-3′)	Description
bosRFBamHI	GGATCCTGCTCCAAATCCATGAATA	Amplifies *bosR* with 58 bp upstream sequence resulting in a 586 bp product
bosRRPstI	CTGCAGTTTAAATGTTGAAAAAGATA	

Table 3

Infectivity data for C3H/HeN mice infected with *B. burgdorferi* ML23 pCADDY and JH300 pCADDY.

	Number of culture positive/total number								
Strain	**Inoculum Dose**	**Lymph Node**	**Skin**	**Heart**	**Spleen**	**Bladder**	**Joint**	**All sites**	**No. of mice positive/total mice**
ML23 pCADDY	10^5	5/5	5/5	5/5	1/5	5/5	5/5	26/30	5/5
	10^3	3/5	3/5	2/5	1/5	3/5	3/5	15/30	5/5
JH300 pCADDY	10^5	0/5	0/5	0/5	0/5	0/5	0/5	0/30	0/5
	10^4	0/3	0/3	0/3	0/3	0/3	0/3	0/18	0/3
	10^3	0/5	0/5	0/5	0/5	0/5	0/5	0/30	0/5