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Analysis of the RpoS regulon in *Borrelia burgdorferi* in response to mammalian host signals provides insight into RpoS function during the enzootic cycle

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Summary

Borrelia burgdorferi (*Bb*) adapts to its arthropod and mammalian hosts by altering its transcriptional and antigenic profiles in response to environmental signals associated with each of these milieus. In studies presented here, we provide evidence to suggest that mammalian host signals are important for modulating and maintaining both the positive and negative aspects of mammalian host adaptation mediated by the alternative sigma factor RpoS in *Bb*. Although considerable overlap was observed between genes induced by RpoS during growth within the mammalian host and following temperature-shift, comparative microarray analyses demonstrated unequivocally that RpoS-mediated repression requires mammalian host-specific signals. A substantial portion of the *in vivo* RpoS regulon was uniquely upregulated within dialysis membrane chambers, further underscoring the importance of host-derived environmental stimuli for differential gene expression in *Bb*. Expression profiling of genes within the RpoS regulon by quantitative reverse transcription polymerase chain reaction (qRT-PCR) revealed a level of complexity to RpoS-dependent gene regulation beyond that observed by microarray, including a broad range of expression levels and the presence of genes whose expression is only partially dependent on RpoS. Analysis of *Bb*-infected ticks by qRT-PCR established that expression of *rpoS* is induced during the nymphal blood meal but not within unfed nymphs or engorged larvae. Together, these data have led us to postulate that RpoS acts as a gatekeeper for the reciprocal regulation of genes involved in the establishment of infection within the mammalian host and the maintenance of spirochetes within the arthropod vector.

Introduction

In order to be maintained within its enzootic cycle, *Borrelia burgdorferi* (*Bb*), the causative agent of Lyme disease, must be able to grow within two distinctly different environments, the arthropod vector (*Ixodes* ticks) and a mammalian host, typically small rodents. Over the past decade, convincing evidence has emerged to that *Bb* accomplishes this feat by altering its transcriptional and polypeptide profiles in response to the environmental signals associated

with each of these milieus, a dynamic process collectively referred to as host adaptation (Akins *et al.*, 1998; Anguita *et al.*, 2003; Pal and Fikrig, 2003; Stevenson *et al.*, 2006). The transcriptome changes associated with growth *in vivo* have been of particular interest as it is presumed that the polypeptides encoded by these differentially-expressed loci are required to fulfil virulence-related functions or the physiological adjustment to growth within the mammalian host. Although the majority of differential-expression studies in *Bb* have focused on genes whose expression is increased during infection, the downregulation of loci involved in the tick phase of the enzootic cycle is believed to be an equally important aspect of the spirochete's pathogenic strategy (Brooks *et al.*, 2003; Pal *et al.*, 2004; Yang *et al.*, 2004; Caimano *et al.*, 2005; Strother *et al.*, 2007).

Utilization of alternative sigma factors to redirect transcription in response to environmental stimuli has emerged as an important mechanism for stress adaptation in many bacteria, including pathogens (Molofsky and Swanson, 2004; Kazmierczak *et al.*, 2005; Rodrigue *et al.*, 2006). One of the best-studied examples of a global stress response is the RpoS regulon in *Escherichia coli*, which co-ordinates the expression of genes involved in adaptation to nutrient limitation, acid shock, reactive oxygen species and UV irradiation (Hengge-Aronis, 2002). The annotated genomic sequence of *Bb* type strain B31-MI revealed three sigma factors, the housekeeping sigma factor σ^{70} (RpoD, BB0712), and two alternative sigma factors, RpoS (BB0771) and RpoN (BB0450) (Fraser *et al.*, 1997). In a series of seminal studies, Norgard and co-workers (Hubner *et al.*, 2001; Yang *et al.*, 2003; Smith *et al.*, 2007) demonstrated that RpoN acts in concert with the response regulator protein Rrp2, to induce the expression of RpoS, which in turn is required for the transcription of *ospC* and *dbpBA*. In *E. coli*, RpoS levels are regulated by a complex network of transcriptional, post-transcriptional and translational mechanisms as cells enter stationary phase (Hengge-Aronis, 2002). In *Bb*, RpoS appears to be controlled primarily at the transcriptional level and can be induced in exponentially growing cultures by temperature-shift (Caimano *et al.*, 2004); efficient translation of *rpoS* mRNA during early logarithmic growth also requires the small RNA, DsrA (Lybecker and Samuels, 2007). While no study, thus far, has examined expression of *rpoS* in *Bb*-infected ticks or mice, several RpoS-dependent loci (i.e. *ospC*, *dbpBA*, *ospF*) are known to be induced during the nymphal blood meal and continue to be expressed during infection (Schwan and Piesman, 2000; Gilmore *et al.*, 2001; Hefty *et al.*, 2001; McDowell *et al.*, 2001; Ohnishi *et al.*, 2001; Hodzic *et al.*, 2002; Liang *et al.*, 2002; Tokarz *et al.*, 2004). Previously, we demonstrated that RpoS also is required for the downregulation of at least two tick phase-associated lipoproteins, OspA and Lp6.6, in response to mammalian host signals (Caimano *et al.*, 2005). The loss of RpoS does not significantly impair the ability of *Bb* to survive diverse environmental stressors, including growth within dialysis membrane chambers (DMCs), but it does render spirochetes avirulent in mice (Caimano *et al.*, 2004). Together, these findings led us to postulate that, rather than serving as a global stress response regulator, RpoS in *Bb* orchestrates the expression of a subset of genes associated with the mammalian host-adaptation process (Caimano *et al.*, 2004; 2005).

Microarrays have proven to be a valuable tool for delineating the RpoS regulons of other microorganisms (Lacour and Landini, 2004; Patten *et al.*, 2004; Schuster *et al.*, 2004; Yildiz *et al.*, 2004; Weber *et al.*, 2005; Nunez *et al.*, 2006; Rahman *et al.*, 2006). While microarray technology has been used to examine differential gene expression in *Bb* cultivated under different environmental conditions (Liang *et al.*, 2002; Revel *et al.*, 2002; Brooks *et al.*, 2003; Ojaimi *et al.*, 2003; Tokarz *et al.*, 2004; Fisher *et al.*, 2005; Hyde *et al.*, 2006), only one study (Fisher *et al.*, 2005) has examined the contribution of alternative sigma factors to gene expression in *Bb* on a genome-wide scale, and then, only under standard *in vitro* growth conditions. The extent to which the manipulation of *in vitro* growth conditions can be used to reproduce the full spectrum of mammalian host signals that trigger differential gene expression in *Bb* during tick transmission remains uncertain (Stevenson *et al.*, 2006). Studies by us (Akins

et al., 1995; 1998; Lahdenne *et al.*, 1997; Hefty *et al.*, 2001; Parveen *et al.*, 2003; Caimano *et al.*, 2004; 2005) and others (Revel *et al.*, 2002; Brooks *et al.*, 2003; Tokarz *et al.*, 2004) indicate that the RpoS regulon could differ substantially during growth within the mammalian host and collectively underscore the importance of examining the RpoS regulon under conditions that approximate the environmental stimuli encountered by *Bb* during natural infection. Indeed, comparative microarray analyses of RpoS-dependent gene expression within spirochetes grown *in vitro* following temperature-shift and within DMCs performed as part of this study identified a large group of genes that are uniquely up- and downregulated by RpoS in response to mammalian host signals. Expression profiling of a subset of RpoS-dependent genes by quantitative reverse transcription polymerase chain reaction (qRT-PCR) revealed a level of complexity to gene regulation by this alternative sigma factor beyond that observed by microarray, most notably, the identification of genes whose expression is only partially dependent on RpoS, particularly *in vivo*. Based on our analysis of *rpoS* expression in *Bb*-infected *Ixodes scapularis* ticks, we have formulated a temporal framework for RpoS function and RpoS-dependent gene regulation during the spirochete's enzootic cycle.

Results

Importance of mammalian host-derived signals for RpoS-dependent differential gene expression in *Bb*

In order to define the experimental conditions for our microarray analysis of the RpoS regulon, we began by examining the extent to which the manipulation of *in vitro* conditions influences the expression of proteins known to be under the control of this alternative sigma factor. *Bb* strain 297 wild-type clone c162 was temperature-shifted from 23°C to 37°C into BSK-H medium at standard or reduced pH (7.5 versus 6.8 respectively) and grown to late-logarithmic phase for comparison with lysates of this same isolate cultivated within DMCs (Fig. 1A). In agreement with previous studies (Schwan *et al.*, 1995; Carroll *et al.*, 1999; 2000; Yang *et al.*, 2000; Hubner *et al.*, 2001; Revel *et al.*, 2002; Caimano *et al.*, 2004; 2005), expression of OspC and DbpA was induced by temperature-shift and enhanced further by a combination of increased temperature and reduced pH. Cultivation of *Bb* c162 following temperature-shift at reduced pH, on the other hand, did not noticeably diminish expression of either OspA or Lp6.6 as has been previously reported (Yang *et al.*, 2000; Revel *et al.*, 2002). Because spirochetes within DMCs replicate under conditions in which physiologic pH is strictly maintained by homeostatic mechanisms (Caimano *et al.*, 2004), the results shown in Fig. 1A demonstrate unequivocally that a decrease in pH is not a prerequisite for the downregulation of OspA and Lp6.6. Temperature-shift, with or without reduced pH, also failed to reproduce changes in the low molecular mass region of the spirochete's polypeptide profile (~20 kDa) that are characteristic of DMC-cultivated organisms (Akins *et al.*, 1998; Caimano *et al.*, 2005). Gilbert *et al.* (2007) recently developed a *Bb* strain 297 isolate, RpoSi, in which expression of *rpoS* is tightly controlled by a synthetic *flgB* promoter modified to include the *lac* operator. Using this isolate, we examined the effect of RpoS expression on the polypeptide profiles of *Bb* subjected to otherwise identical *in vitro* growth conditions. As shown in Fig. 1A, induction of *rpoS* resulted in the increased expression of OspC and DbpA without any noticeable downregulation of OspA and Lp6.6.

Schwan and Piesman (2000) reported that expression of OspC diminishes when spirochetes are serially passaged at 37°C. This finding, reproduced in the present study (Fig. 1B), indicates that spirochetes become desensitized to the ability of a thermal stimulus to induce RpoS-dependent changes in gene expression. We next used continuous cultivation of *Bb* within DMCs to model the prolonged exposure to mammalian host-derived signals that would occur during natural infection. In contrast to their *in vitro* passaged counterparts, *Bb* serially passaged within DMCs continuously expressed high levels of OspC as well as diminished levels of OspA

(Fig. 1B); the host-adapted phenotype was maintained for nine serial passages extending over approximately a 4-month time period (data not shown).

RpoS regulon in *Bb* during cultivation within DMCs

Microarray methodology has been used to delineate the RpoS regulon in *Bb* strain B31-MI under standard *in vitro* growth conditions (34°C in BSK-H) (Fisher *et al.*, 2005). Based on the studies described above, we postulated that the analysis of *in vitro* cultivated *Bb* would yield only a partial representation of the RpoS regulon as it exists within the mammalian host. For this reason, we performed microarray analyses of wild-type and RpoS-deficient *Bb* cultivated within DMCs. In order to gain a better understanding of how the composition of the RpoS regulon differs in response to mammalian host-specific signals, we extended these studies to include parallel microarray analyses of strain 297 wild-type and *rpoS* mutant *Bb* following temperature-shift *in vitro*.

DNA: DNA hybridizations confirmed the suitability of B31-MI-based microarrays for analysis of the RpoS regulon in 297—

For the gene expression analyses described below, we employed a 70-mer oligonucleotide microarray that contains gene targets for 1741 putative open reading frames (ORFs) (Terekhova *et al.*, 2006) based on the genomic sequence of *Bb* strain B31-MI (Fraser *et al.*, 1997). We first confirmed the suitability of these microarrays for use with our extensively characterized strain 297 wild-type and *rpoS* mutant isolates (Caimano *et al.*, 2004; 2005; Eggers *et al.*, 2004; 2006) by performing comparative hybridizations using genomic DNAs from both B31-MI and 297 wild-type isolates. Consistent with our earlier report demonstrating high genetic similarity between B31-MI and 297 (Terekhova *et al.*, 2006), only 101 of the borrelial gene targets present on the microarrays gave significantly lower hybridization signals for 297 genomic DNA compared with B31-MI (mean log₂ ratio ≤ -1.5) (Table S1). The single non-hybridizing chromosomal gene, *bb0845.1*, is annotated as a non-functional pseudogene. Two-thirds (66/100) of the B31-MI plasmid-borne genes that gave lower hybridization signals for 297 are found on plasmids known to be either truncated (i.e. lp36) or absent (i.e. lp38 and lp56) in the latter strain (Eggers *et al.*, 2002; Iyer *et al.*, 2003; Terekhova *et al.*, 2006). The absence of a hybridization signal for *ospC* was expected given the low sequence identity (73%) between the *bbb19/ospC* 70-mer oligo and the corresponding region of the strain 297 *ospC* orthologue. For the same reason, we were unable to detect any of the strain B31 *ospF* alleles (*bbm38*, *bbo39*, *bbr42* and *bbs41*); however, the RpoS dependence of the *ospF* paralogues *in vitro* and within DMCs already has been established (Caimano *et al.*, 2004; Eggers *et al.*, 2006).

General features of the *in vivo* RpoS regulon—Using the statistical criteria outlined in *Experimental procedures*, a total of 137 genes were identified as being differentially regulated by RpoS during cultivation of *Bb* within DMCs. One hundred and three genes were expressed at significantly lower levels in the *rpoS* mutant, implying that RpoS contributes, either directly or indirectly, to their transcription (Table 1), while 34 genes were downregulated in the wild type, implying that RpoS is required for their repression (Table 2). Consistent with previous microarray studies (Revel *et al.*, 2002; Brooks *et al.*, 2003; Ojaimi *et al.*, 2003; Tokarz *et al.*, 2004), the majority of differentially expressed genes were localized to plasmid replicons (81% of upregulated genes and 73% of RpoS-repressed genes) (Fig. 2).

***Bb* genes upregulated by RpoS within DMCs (Table 1)—**The established expression profiles of known RpoS-dependent genes such as *ospC*, *dbpA* and *ospF* suggest that many genes within the upregulated portion of the DMC RpoS regulon are induced during the tick-to-mammal transition and/or early infection. Indeed, the upregulation of *OspC* in response to the blood meal within a *Bb*-infected tick is critical for the establishment of infection (Ohnishi *et al.*, 2001; Grimm *et al.*, 2004; Stewart *et al.*, 2006; Tilly *et al.*, 2006). While the

overwhelming majority (78/103) of genes upregulated by RpoS within DMCs encode hypothetical proteins and conserved hypothetical proteins, a survey of the functionally annotated genes upregulated by RpoS revealed several additional genes whose products may contribute to establishing infection or perform virulence-related functions. Key among these are four genes related to chemotaxis: *bb0565* encodes the purine-binding chemotaxis protein CheW-2, while *bb0578*, *bb0680* and *bb0681* encode the methyl-accepting chemotaxis proteins Mcp1, Mcp4 and Mcp5 respectively. Although annotated as hypothetical proteins, *bba36* and *bbi42* have been shown to encode surface-exposed lipoproteins (Brooks *et al.*, 2006), and therefore may function at the pathogen–host interface.

In *E. coli*, many of the genes controlled by RpoS encode proteins with annotated functions related to metabolism, nutrient transport and/or stress adaptation (Schellhorn *et al.*, 1998; Patten *et al.*, 2004; Weber *et al.*, 2005; Rahman *et al.*, 2006). In contrast, only a handful of borrelial genes (*bb0116*, *bb0447*, *bb0548*, *bb0728*, *bb0812* and *bba34*) with predicted roles in spirochete metabolism and/or physiology were upregulated by RpoS within DMCs. *bb0116/malX* encodes a putative maltose/glucose-specific EIICB transporter component of the carbohydrate phosphotransferase system (PTS). *bb0447*, annotated as a Na⁺/H⁺ antiporter, also contains a putative fructose/mannitol-specific IIA PTS subunit domain involved in sugar transport and/or phosphorylation. Two genes, *bb0812* and *bb0728*, upregulated by RpoS within DMCs encode enzymes involved either in the synthesis or utilization of coenzyme A. *bb0812* encodes a putative DNA/pantothenate flavoprotein involved in the synthesis of coenzyme A (Kupke *et al.*, 2000), while *bb0728* encodes a NAD-dependent coenzyme A disulphide reductase (Cdr) thought to be involved in maintaining intracellular redox potential, as well as adaptation to oxidative stress (Boylan *et al.*, 2006). *bb0728* is predicted by TIGR to be part of a bicistronic operon with *bb0729*, encoding a glutamate transporter (GltP); we confirmed the co-transcription of these genes by RT-PCR using primers (Table S3) that amplify across the *bb0729-bb0728* junction (data not shown). Examination of the raw microarray data for *bb0729* revealed that this gene also was expressed at greater levels in the wild type compared with the *rpoS* mutant (4.57-fold, *P* = 0.0057) but fell just below the threshold required for statistical significance and was excluded from the DMC data set (see *Experimental procedures*). In addition to providing *Bb* with an essential nutrient, GltP also may provide a means of maintaining the cell's turgor in response to high osmolality by increasing the intracellular concentration of glutamate (Csonka, 1989). *bb0548/poIA* encodes DNA polymerase I, the enzyme responsible for removing the RNA primer during DNA synthesis (Kornberg and Baker, 1992); the increased expression of this gene also was observed following the addition of human blood to the growth medium *in vitro* (Tokarz *et al.*, 2004). Interestingly, only one metabolic gene upregulated by RpoS within DMCs is plasmid-borne. *bba34*, located on lp54, encodes OppA5, one of five periplasmic oligopeptide (Opp)-binding proteins in *Bb* (Bono *et al.*, 1998; Wang *et al.*, 2002; Medrano *et al.*, 2007). Medrano *et al.* (2007) recently confirmed the RpoS dependence of *oppA5* *in vitro*.

The majority of the 83 plasmid-borne genes upregulated by RpoS within DMCs localized to lp54, lp28-2 and members of the cp32 plasmid family (Fig. 2). Twenty of the 83 are distributed along the length of lp54. Although lp54 is known to serve as a depot for differentially expressed genes (Revel *et al.*, 2002; Brooks *et al.*, 2003; Ojaimi *et al.*, 2003; Tokarz *et al.*, 2004; Fisher *et al.*, 2005), this report is the first to recognize the RpoS dependence of many of these. In addition to *bba24/dbpA*, *bba25/dbpB* and *bba34/oppA5*, RpoS controls the expression of *bba04* and *bba05*, encoding the S2 and S1 lipoprotein serodiagnostic antigens respectively (Feng *et al.*, 1996). A cluster of six (*bba64*, *bba65*, *bba66*, *bba71*, *bba72* and *bba73*) genes, all exhibiting > 150-fold induction by RpoS (Table 1), is located at the right end of lp54 (Fig. 3). Recently, these genes have garnered considerable interest due to their differential expression in response to temperature, pH, and *in vivo* signals, and their potential role in virulence (Anguita *et al.*, 2000; Carroll *et al.*, 2000; Liang *et al.*, 2002; Brooks *et al.*, 2003; Ojaimi *et al.*, 2003; Tokarz *et*

et al., 2004; Clifton *et al.*, 2006; Gilmore *et al.*, 2007). With the exception of *bba71*, each is predicted to encode a lipoprotein. Once considered to be members of a single paralogous gene family (*pgf54*), all but two (*bba71* and *bba72*) are now designated by TIGR as being non-paralogous. While their similar expression profiles and close proximity on *lp54* might suggest that these genes are co-transcribed, Northern blot analyses identified individual mRNA bands corresponding to *bba64*, *bba65*, *bba66* and *bba73* (Carroll *et al.*, 2000); the RpoS dependence of one of these, *BBA66*, recently has been confirmed at the protein level (Clifton *et al.*, 2006). *lp28-2*, a plasmid not previously recognized as a repository for differentially expressed genes, harbours 17 of the 83 plasmid-borne genes upregulated by RpoS within DMCs (Fig. 2), all of which encode hypothetical proteins (Table 1). An examination of the intergenic spacing between each of the RpoS-dependent *lp28-2*-borne genes suggests that at least some of these may be co-transcribed (e.g. *bbg13-12*, *bbg21-bbg16* and *bbg24-23*). Twenty-three genes upregulated by RpoS are distributed among the members of the *cp32* family of circular plasmids (Fig. 2). The majority of these *cp32*-borne genes encode proteins of unknown function, with half belonging to just two paralogous families, *gbb* fam_PF06381 and *gbb* fam_PF02989, located within the putative late phage operon of the *cp32*-prophage ϕ BB-1 (Eggers *et al.*, 2000; Zhang and Marconi, 2005).

Bb genes subject to RpoS-mediated repression within DMCs (Table 2)—Thirty-four genes were identified as being downregulated by RpoS within DMCs. Based on the established expression patterns of *OspA* and *Lp6.6* (Schwan *et al.*, 1995; Lahdenne *et al.*, 1997; Akins *et al.*, 1998; Schwan and Piesman, 2000; Ohnishi *et al.*, 2001), we hypothesize that many of these genes are induced following acquisition by naïve larvae and then expressed constitutively within flat nymphs until the next blood meal. As with the RpoS upregulated genes, the majority (21/34) of the genes subject to RpoS-mediated repression encode hypothetical proteins or conserved hypothetical proteins. Ten genes (*bb0365*, *bba62*, *bba69*, *bbf20*, *bbg01*, *bbi29*, *bbi36*, *bbi38*, *bbi39*, *bbk01* and *bbk19*) downregulated by RpoS within DMCs, in addition to *ospA* and *ospB*, are known or predicted to encode lipoproteins. Four of the genes downregulated by RpoS within DMCs are involved in glycerol utilization. The first three, *bb0240*, *bb0241* and *bb0243*, are part of an operon (Ojaimi *et al.*, 2003) encoding a putative glycerol uptake facilitator (*bb0240/glpF*), a glycerol kinase (*bb0241/glpK*) and an anaerobic glycerol 3-phosphate dehydrogenase (*bb0243/glpA*) respectively; *bb0242*, which was also downregulated by RpoS, encodes a protein of unknown function. The co-ordinated downregulation of these four genes is consistent with a previous study noting the increased expression of *bb0240-b0243* at 23°C (Ojaimi *et al.*, 2003). The fourth gene related to glycerol utilization, *bb0368*, encodes a putative NAD(P)-dependent glycerol 3-phosphate dehydrogenase. *bb0582*, encoding a putative D-alanyl, D-alanine carboxypeptidase, may be involved in recycling of peptidoglycan and/or maintaining cell shape (Young, 2003). *bbk17*, the only plasmid-borne metabolic gene downregulated by RpoS, encodes an adenine deaminase (AdeC) (Jewett *et al.*, 2007) that may be involved in either salvaging adenine for purine nucleotide biosynthesis or providing sources of nitrogen and pentose sugars (Endo *et al.*, 1983; Nygaard *et al.*, 1996; Tozzi *et al.*, 2006).

As with the upregulated portion of the RpoS regulon, a high percentage (71%) of the plasmid-borne genes downregulated by RpoS localized to a small number of replicons (Fig. 2). More than half (13/34) are located on *lp36*, all but two of which encode proteins of unknown function (Table 2). An examination of the transcriptional orientation and intergenic spacing between the *lp36*-borne genes suggests at least some may be co-transcribed (e.g. *bbk23-21* and *bbk33-35*); for these, downregulation by RpoS would likely be mediated via repression at the upstream promoter for the first gene in each putative operon. *lp54*, in addition to harbouring *ospA*, *ospB* and *lp6.6*, contains two other downregulated genes, *bba74* and *bba69* (Fig. 3). The protein product of *bba74*, annotated as an outer membrane-associated porin (Oms28) (Skare *et al.*, 1996; 1997; Fraser *et al.*, 1997), has recently been shown to lack the β -barrel structure

typical of outer membrane porins and localized to the periplasm (Mulay, 2007; Mulay *et al.*, 2007). *bba69* is one of five putative lipoprotein genes downregulated by RpoS belonging to the gbb pep₃₅ paralogous protein family. Three of the remaining downregulated gbb pep₃₅ genes (*bbi36*, *bbi38* and *bbi39*) are located on lp28-4, while the location of the fifth, *bbj41*, in strain 297 is uncertain as this isolate lacks lp38 (Iyer *et al.*, 2003; Terekhova *et al.*, 2006). Although originally annotated as belonging to paralogous family 54 (*pfg54*) (Fraser *et al.*, 1997; Casjens *et al.*, 2000), all five of these genes have been placed by TIGR into pep₃₅ based on phylogenetic analyses (http://cmr.tigr.org/tigrscripts/CMR/ParalogDescription.cgi?align_id=147991&align_name=gbb%20fam_b_burgdorferi_b31.pep_35&ori_db=gbb). In contrast to the large number of RpoS-upregulated genes on lp28-2, only one gene on this replicon, *bbg01*, was downregulated by RpoS within DMCs.

We recently proposed that repression of *ospA* and *lp6.6* by RpoS is mediated by the binding of a repressor protein to the polyT tracts located just upstream of the -35 sequences for these σ^{70} -dependent promoters (Sohaskey *et al.*, 1999; Caimano *et al.*, 2005). Using the *ospA* and *lp6.6* promoters as guides, we examined the upstream regions of the other downregulated genes (Table 2) for T-rich regions to determine whether these genes are regulated by a common mechanism. Results from these analyses yielded five additional genes with polyT tracts in close proximity to their putative promoters, including *bb0240*, the first gene in the *glp* operon, and the adenine deaminase gene *bbk17* (Table 3). A T-rich region also was found flanking *bba69* but is noticeably further upstream than those of the other *in vivo* RpoS-repressed genes (Table 3). Analysis of the promoter regions for genes lacking polyT tracts revealed that two of the five downregulated gbb pep₃₅ paralogous genes, *bbi36* and *bbi38*, contain perfect 18 bp inverted repeats immediately upstream of their putative -35 sequences (Table 3). Of note, the *bbi36/bbi38* inverted repeats are dissimilar to the repeat motif recently identified upstream of *ospC* in strain B31 (Xu *et al.*, 2007).

The *in vitro* RpoS regulon differs substantially from its DMC counterpart

A comparison of the *in vitro*- and DMC-derived microarray data sets revealed that 44 of the genes upregulated by RpoS during DMC cultivation also were upregulated following temperature-shift *in vitro* and are therefore designated as 'core' RpoS-dependent genes. While a small number of core genes encode products with known or predicted functions (i.e. DbpA, DbpB, OppA5, Mcp4 and Mcp5), the majority encode hypothetical and conserved hypothetical proteins of unknown function, almost half of which are predicted to be lipoproteins (Table 1). The upregulated portion of the DMC data set contains 59 genes that were not present within the *in vitro* data set (Table 1). Although the vast majority (48/59) of these encode hypothetical proteins or conserved hypothetical proteins (Table 1), this group includes a small number of genes encoding functions related to chemotaxis and spirochete metabolism (Table 1). Interestingly, all 17 of the lp28-2-borne genes upregulated by RpoS within DMCs were present exclusively within this data set.

The *in vitro* data set contains 66 genes which were not present within the DMC data set (Table S2); all but two of these, *bbk32* and *bbq03*, exhibited low levels of induction. An examination of the raw data from the DMC-derived microarrays revealed that 21 of the 66 genes present exclusively within the *in vitro* data set also exhibited a low level of RpoS-dependent upregulation within DMCs (> 2.5 -fold, $P \leq 0.01$) but their mean $\log_2$ ratios fell below the threshold (≥ 4.59 -fold) used to screen the DMC microarray data (see *Experimental procedures*). This finding suggests that some of the genes present exclusively within the *in vitro* data set also are upregulated by RpoS within DMCs. Indeed, qRT-PCR analyses, presented below, confirmed that this was the case for at least one gene, *bb0670* (Fig. 5B). qRT-PCR analyses revealed that *bbk32* was expressed at substantially lower levels in the DMC-

cultivated wild-type *Bb* (0.005 copies per 100 *flaB*) used for the microarray hybridizations compared with their *in vitro* counterparts (20.79 copies per 100 *flaB*). These data, however, seemed to be at odds with the demonstrated expression of BBK32 during infection (Lahdenne *et al.*, 2006; Li *et al.*, 2006) and its potential role in virulence (Seshu *et al.*, 2006), prompting us to examine RNAs from additional DMC-cultivated c162 (wild type) and c174 (*rpoS* mutant). Results from these subsequent analyses yielded substantially higher *bbk32* transcript levels (11.13 copies per 100 *flaB*) in the wild-type isolate and demonstrated a level of RpoS dependence (46.5-fold, $P < 0.01$) comparable to that observed at 37°C *in vitro* (data not shown). One of the most striking observations to emerge from these comparative microarray analyses was that *no* genes were downregulated by RpoS in wild-type *Bb* following temperature-shift *in vitro*, providing further evidence that mammalian host-derived signals are essential for RpoS-mediated repression.

Expression profiling of selected genes identified by microarray using real-time qRT-PCR

Unlike previous microarray studies of the *Bb*, which used qRT-PCR to measure relative differences in transcripts (i.e., $\Delta\Delta C_T$) (Brooks *et al.*, 2003; Ojaimi *et al.*, 2003; Tokarz *et al.*, 2004; Fisher *et al.*, 2005; Hyde *et al.*, 2006), we opted to quantify transcript copy numbers for selected genes identified by the microarrays using the primer pairs described in Table S3. At the outset, we measured transcript levels for *rpoS* and two prototypical RpoS-dependent core genes, *ospC* and *dbpA* (Hubner *et al.*, 2001), in *Bb* cultivated *in vitro* following temperature-shift (37°C) and within DMCs. As expected, expression of *ospC* and *dbpA* was absolutely dependent on the presence of RpoS (Fig. 4). Interestingly, we observed only a modest, although significant ($P < 0.05$), difference between the levels of *ospC* in wild-type *Bb* grown at 37°C compared with DMCs despite the presence of markedly greater *rpoS* transcript levels within DMC-cultivated *Bb*. Transcript levels for *dbpA*, on the other hand, were noticeably lower in wild-type *Bb* during DMC cultivation compared with 37°C (Fig. 4). Using the comparative microarray data as a guide, we selected 21 additional genes, 15 upregulated and six downregulated, for analyses by qRT-PCR, with the majority of these encoding polypeptides with either predicted metabolic functions or potential roles in virulence. Of the five (*bb0680*, *bb0844*, *bba07*, *bba34* and *bbi42*) core genes examined, all but one (*bb0680*) was found to be absolutely dependent on RpoS, although their transcript copy numbers were markedly lower than those of *ospC* and *dbpA* (Fig. 4).

Seven genes were selected from those identified by microarray as being upregulated by RpoS exclusively within DMCs (Table 1). Consistent with their 'exclusively *in vivo*' designation, five of the genes within this group, *bb0548*, *bbg13*, *bbg17*, *bbg19* and *bbg24*, displayed significant RpoS dependence and greater absolute transcript levels within DMC-cultivated *Bb* compared with their 37°C counterparts (Fig. 5A). The expression profile for the sixth gene, *bb0728*, encoding Cdr, differed in that its expression was upregulated by RpoS at 37°C as well as within DMCs; upregulation of *bb0729*, co-transcribed with *bb0728*, also was RpoS-dependent under both growth conditions. We next selected three genes from those identified by microarray as being upregulated by RpoS exclusively *in vitro* (Table S2). Although qRT-PCR analyses of all three genes within *Bb* grown at 37°C were in agreement with the *in vitro*-derived microarray data, each displayed a distinct expression profile within DMCs. Expression of *bb0588* was induced 3.77-fold by RpoS at 37°C but only 1.33-fold within DMCs (Fig. 5B); the lower level of induction for *bb0588* observed within DMCs is primarily due to increased RpoS-independent expression. *bb0021* was expressed at 2.84-fold higher levels in wild-type compared with *rpoS* mutant *Bb* *in vitro*, but was not RpoS-dependent *in vivo* (Fig. 5B). Expression of the third gene, *bb0670*, encoding CheW-3, was significantly upregulated by RpoS both *in vitro* and within DMCs (Fig. 5B); however, the 3.18-fold induction observed in the DMC-derived microarrays (Table 3), and confirmed here by qRT-PCR, was below the threshold required for inclusion in the DMC data set.

Quantitative RT-PCR analyses of six genes (*bb0240*, *bb0242*, *bba15*, *bba62*, *bbk17* and *bba74*) selected from those subject to RpoS-mediated repression within DMCs (Table 2) confirmed that each was expressed at significantly lower levels ($P \leq 0.05$) in wild-type compared with *rpoS* mutant *Bb* within DMCs (Fig. 6). Interestingly, the *in vitro* expression profiles for these genes differed notably. Rather than being repressed, all six were expressed at high levels in wild-type *Bb* grown *in vitro*. Moreover, four of the six genes examined (*bb0242*, *bba62*, *bba74* and *bbk17*) were expressed at significantly lower levels in the *rpoS* mutant compared with wild-type *Bb* at 37°C (Fig. 6). The transcript differences observed between wild type and the *rpoS* mutant for *bba62*, however, did not result in an appreciable difference at the protein level (Fig. 1 and Caimano *et al.*, 2005). As a whole, these findings strike a cautionary note against relying on the use of *in vitro* conditions alone to predict a given gene's expression pattern during natural infection.

Identification of consensus promoter sequences upstream of RpoS-dependent genes in *Bb*

Previous studies in *Bb* have demonstrated that the sequence of the 'extended' -10 region is critical for promoter recognition by RpoS (Eggers *et al.*, 2004; 2006; Yang *et al.*, 2005). In an attempt to develop a consensus sequence for RpoS binding in *Bb*, we used MEME (<http://meme.sdsc.edu>) (Bailey *et al.*, 2006) to search for conserved motifs within the upstream promoter regions of two subsets of RpoS-dependent genes. The first consisted of the 400 bp regions found upstream of the translational start codons for the 44 core genes upregulated by RpoS both *in vitro* and within DMCs (Table 1). The second consisted of the upstream regions for 10 genes whose absolute RpoS dependence has been verified experimentally as part of this study (Fig. 4) or elsewhere (Hubner *et al.*, 2001; Caimano *et al.*, 2004; Clifton *et al.*, 2006; Eggers *et al.*, 2006), including four (*ospC*, *dbpBA*, *erpK* and *bba66*) with known transcriptional start sites (Marconi *et al.*, 1993; Hagman *et al.*, 1998; Babb *et al.*, 2004; Clifton *et al.*, 2006). In contrast to *E. coli*, for which MEME successfully identified an RpoS consensus sequence (Weber *et al.*, 2005), this program was unable to identify any conserved motifs in the -10 regions for either of the two RpoS-dependent *Bb* subsets, most likely because of the high A + T content (70%) of the borrelial genome (Fraser *et al.*, 1997). Visual inspection of upstream regions for the 10 absolutely RpoS-dependent borrelial genes, however, enabled us to identify TG(G/A)(G/A)ATA(T/A)ATT as a putative RpoS consensus extended -10 region sequence in *Bb* (Table 4). This consensus sequence is somewhat divergent from that of *E. coli* [TGN₀₋₂CTA(T/C)(A/G)CT] (Lacour and Landini, 2004; Weber *et al.*, 2005), most notably at the nucleotide corresponding to position -15. In *E. coli*, a C in this position strongly favours RpoS, while a G favours σ^{70} (Becker and Hengge-Aronis, 2001). The borrelial RpoS consensus sequence contains an A at this position. Interestingly, mutagenesis studies of the *ospC* promoter suggest that this position may be involved in promoter recognition by RpoS but does not appear to serve as a 'discriminator' nucleotide for sigma factor selectivity (Eggers *et al.*, 2004; 2006; Yang *et al.*, 2005). We next examined the upstream regions for 10 genes that were found by qRT-PCR to be recognized by both RpoS and at least one of *Bb*'s other sigma factors (RpoN or σ^{70}) (Fig. 5). We were unable to find putative promoter elements for five (*bb0670*, *bbg13*, *bbg17*, *bbg19* and *bbg24*) of the 10 genes examined, including four that exhibited enhanced RpoS-dependent expression within DMCs (Table 1). The putative extended -10 elements identified in the upstream regions for the remaining five (*bb0021*, *bb0548*, *bb0588*, *bb0680* and *bb0729-0728*), however, were highly similar to the consensus sequence derived from the absolutely RpoS-dependent genes. Putative -35 sequences were identified within the upstream regions for each of the absolutely RpoS-dependent genes, as well as for the five dually transcribed genes that had identifiable -10 regions (Table 4). None of the upstream regions for the dually transcribed genes contained the RpoN promoter consensus sequence described by Studholme *et al.* (2000) while only two (*bb0021* and *bb0588*) were scored as positive by the SEQSCAN RpoN promoter scoring matrix used by Fisher *et al.* (2005). Thus, σ^{70} is likely

responsible for the RpoS-independent expression component for the majority of dually transcribed genes.

Examination of *rpoS* expression in *Bb*-infected ticks

No study, thus far, has examined the expression of *rpoS* within *Bb*-infected *I. scapularis* ticks. In order to gain a broader understanding of the RpoS regulon and RpoS function during the enzootic cycle, we used qRT-PCR to measure *rpoS* transcript levels in *Bb* strain 297-infected *I. scapularis* nymphs, prior to and during a blood meal on a naïve mouse, and in engorged naïve larvae following a blood meal on a *Bb*-infected mouse. As shown in Fig. 7, the *flaB*-normalized transcript levels for *rpoS* were dramatically increased in *Bb* within engorged nymphal ticks as compared with the levels observed in either flat nymphs or fed larvae; the marked disparity between the *rpoS* transcript levels in the two 'fed' states was particularly noteworthy. The normalized *rpoS* transcript levels observed in engorged *Bb*-infected nymphs (2.66 copies/100 *flaB*) were comparable to those observed in wild-type *Bb* cultivated within DMCs (3.02 copies per 100 *flaB*) (Figs 4 and 7).

Discussion

In *E. coli*, the alternative sigma factor RpoS acts as the master regulator of the general stress response as cells enter stationary phase growth and/or encounter adverse environmental conditions (Hengge-Aronis, 1996). In recent years, there has been a growing body of evidence that the RpoS regulon in *Bb* evolved to facilitate adaptation of the spirochete to environmental conditions quite unlike those encountered by free-living bacteria. Indeed, one of the most striking general observations made in the present study is that the *Bb* RpoS regulon contains a paucity of genes with annotated physiological or metabolic functions. Rather than functioning as a master regulator of a general stress response, as in *E. coli*, the borrelial RpoS appears to serve as an activator of genes acting at the tick-mammalian host interface during transmission and at least into early infection (Caimano *et al.*, 2004). Recently, we demonstrated that RpoS also is required for the downregulation of the tick phase-specific lipoproteins OspA and Lp6.6 in response to mammalian host-derived signals (Caimano *et al.*, 2005). Thus, RpoS appears to act as a 'gatekeeper' for the co-ordinated reciprocal regulation of borrelial loci involved in the mammalian and arthropod host phases of the enzootic cycle.

RpoS-dependent borrelial genes fall into two distinct classes

The qRT-PCR analyses of a representative subset of genes upregulated by RpoS, presented here, brought to light a degree of regulatory complexity that was not discernible from the relative expression levels provided by microarrays. Most notably, qRT-PCR enabled us to identify two distinct classes of RpoS-dependent genes in *Bb*. The first consisted of core genes whose expression was absolutely dependent on the presence of RpoS both *in vitro* and within DMCs. Genes within the second class, in contrast, were expressed at appreciable levels in the absence of RpoS, suggesting that these genes are dually transcribed by both σ^{70} and RpoS. A comparison of the normalized transcript copy numbers for all RpoS upregulated genes examined by qRT-PCR revealed that their expression levels varied remarkably. The consistently lower transcript levels for the dually transcribed compared to the core genes suggest that the 'hybrid' promoter elements for the dually transcribed genes are recognized less efficiently by RNA polymerase compared with those promoters which are recognized by RpoS exclusively. In *E. coli*, promoter strength and sigma factor selectivity are determined, in large part, by a combination of *cis*-acting sequence elements, including individual nucleotides both within and outside of the consensus -10 and -35 sites, the spacing between these two elements, DNA supercoiling and the presence of upstream motifs such as UP elements (Kusano *et al.*, 1996; Gourse *et al.*, 2000; Alverson *et al.*, 2003; Bordes *et al.*, 2003; Typas and Hengge, 2005; 2006; Yang *et al.*, 2005; Typas *et al.*, 2007). A comparison of the promoter regions for

the core and dually transcribed borrelial genes enabled us to identify a putative RpoS consensus extended -10 motif binding site. The close similarity between the binding sites for RpoS and σ^{70} , combined with the presence of consensus -35 sequences upstream of both the absolutely dependent and dually transcribed genes, however, raises the enigmatic question of why some borrelial RpoS-dependent promoters are recognized by σ^{70} while others are not. Numerous studies in *E. coli* have demonstrated that *trans*-acting factors, such as IHF, CRP, Fis, Lrp and H-NS, also play a crucial role in modulating promoter recognition either by interfering with or by enhancing the binding of one sigma factor over another (Typas *et al.*, 2007). The *Bb* genome contains at least two DNA-binding proteins, Hbb (BB0232) and Gac (Knight *et al.*, 2000), either of which could contribute to promoter recognition and/or RpoS-mediated transcription.

Promoter recognition by RpoS is modulated by mammalian host signals

Almost two-thirds of the 103 genes significantly upregulated by RpoS within DMCs were absent from the *in vitro*-derived data set. qRT-PCR analyses of a subset of these '*in vivo* only' genes revealed that each belonged to the dually transcribed class of RpoS-dependent genes. We postulate that the enhanced RpoS-dependent expression of these genes observed during DMC cultivation is due, in part, to increased promoter recognition by RpoS in response to mammalian host signals. The notion that RpoS-dependent promoter recognition can vary in response to specific environmental stimuli is not without precedent. Weber *et al.* (2005) demonstrated that the composition of the RpoS regulon in *E. coli* was modular and dependent on the environmental stress to which the bacteria were exposed. What is the potential biological significance of dual promoter recognition? One possibility is that RpoS is required to augment transcription of some genes within selective niches, while the σ^{70} ensures a sufficient level of basal expression during points within the enzootic cycle in which RpoS is not expressed (see below). The continued expression of these dually transcribed genes in the absence of RpoS also provides a reasonable explanation for the ability of the *rpoS* mutant to grow normally within DMCs (Caimano *et al.*, 2004).

Genes subject to RpoS-mediated repression within the mammalian host are likely expressed during the arthropod phase of the enzootic cycle

The identification of a large number of genes that are subject to RpoS-mediated repression within DMCs represents a significant advance in our understanding of how *Bb* modulates its transcriptome between the arthropod and mammalian hosts. Almost one-third of the genes downregulated by RpoS within DMCs are known or predicted to encode lipoproteins. The products of two of these, OspA and OspB, interact with ligands present on the surface of cells within the tick midgut and are required to colonize ticks following acquisition (Pal *et al.*, 2000; 2001; 2004; Fikrig *et al.*, 2004; Yang *et al.*, 2004). Loss of *bb0365*, encoding the LA7 lipoprotein, resulted in markedly lower survival rates in ticks following the nymphal blood meal and post-molt but had no effect on virulence in mice or larval acquisition (U. Pal, pers. comm.). *Bb* lacking *lp28-4*, the plasmid location for three of the five downregulated *gbb pep_35* paralogous lipoproteins, exhibited a decreased ability to infect tick midguts (Strother *et al.*, 2005) and were unable to be tick-transmitted to naïve mice (Elias *et al.*, 2002). While little is known about the unique physiological requirements of spirochetes within ticks, the increased expression of *bb0240-0243* suggests that glycerol may be available as an alternative carbon source between the larval and nymphal blood meals. Another notable metabolic gene within this portion of the RpoS regulon is *bbk17*, encoding an adenine deaminase believed to be involved in purine salvage; enhanced expression of this gene within flat versus fed ticks, as would be predicted by qRT-PCR analyses of *Bb*-infected ticks (see below), may enable spirochetes to adjust to distinct differences in the purine precursors present within the flat tick midgut (Sonenshine *et al.*, 2003) compared with host blood (McCann and Katholi, 1990). Unlike the other downregulated genes examined here by qRT-PCR, *bbk17* was expressed at

low levels within DMC-cultivated wild-type *Bb*, a finding consistent with recent studies by Jewett *et al.* (2007) demonstrating a role for this gene during infection.

Previously, we proposed that a polyT tract upstream of the *ospA* and *lp6.6* promoters comprises part of the binding site for an as-yet-unidentified *in vivo* specific repressor (Margolis and Samuels, 1995; Caimano *et al.*, 2005). The finding of a similar motif within the upstream regions of eight additional downregulated genes provides further support for this notion. The presence of an 18 bp inverted repeat motif upstream of *bbi36* and *bbi38*, however, suggests that RpoS-mediated repression in *Bb* may be controlled by at least two distinct mechanisms. The absence of a conserved sequence motif within the upstream regions for the remaining downregulated genes, on the other hand, raises the possibility that the downregulation of these genes is mediated by sigma factor competition. This regulatory model, whereby alternative sigma factors compete with each other and σ^{70} for a limiting amount of RNA polymerase, has been proposed as a mechanism for the RpoS-mediated repression of numerous genes in *E. coli* (Farewell *et al.*, 1998) and *Pseudomonas* (Schuster *et al.*, 2004).

A temporal framework for RpoS function and RpoS-dependent gene regulation during the enzootic cycle

Microarray technology, in concert with the DMC model, has enabled us to define the genomic boundaries of the RpoS regulon during growth *in vivo*. In order to place these data within the context of the *Bb* enzootic cycle, it is also necessary to establish the temporal boundaries for RpoS function and RpoS-dependent gene regulation. Based on the signal transduction pathways that initiate expression of RpoS *in vitro* (Hubner *et al.*, 2001; Yang *et al.*, 2003) and studies demonstrating the regulation of RpoS-dependent genes/proteins within *Bb*-infected ticks (Schwan *et al.*, 1995; Schwan and Piesman, 2000; Ohnishi *et al.*, 2001; Hodzic *et al.*, 2002; Tokarz *et al.*, 2004), it has been widely presumed that the upregulation of RpoS-dependent genes and RpoS-mediated repression (i.e. the RpoS-ON state) commences during the nymphal blood meal. Here, we have provided the first definitive evidence that this is indeed the case. Because RpoS-deficient *Bb* are avirulent (Caimano *et al.*, 2004), it is not possible to confirm the RpoS dependence of individual genes expressed during infection. Nevertheless, one can surmise that the strong expression of *rpoS* and RpoS-dependent genes observed in DMC-cultivated organisms represents a reasonable facsimile of the RpoS regulon as it exists in spirochetes at the outset of infection. Consistent with this conjecture, Liang *et al.* (2002) detected transcripts for 18 RpoS-dependent genes within the skin of acutely infected mice. Less clear, however, is whether the RpoS-ON state persists throughout the duration of infection. Three lines of evidence argue for this being the case. First, at least six RpoS-dependent genes (*dbpA*, *dbpB*, *bb0384*, *bba64*, *bba65* and *bbi42*) detected in acute mouse tissues continued to be expressed up to 4 months post inoculation (Liang *et al.*, 2002). Second, with rare exceptions (Akin *et al.*, 1999; Liang *et al.*, 2004), repression of *OspA* and *OspB* is well maintained throughout infection (Montgomery *et al.*, 1996; Coleman *et al.*, 1997; Hodzic *et al.*, 2003; Crother *et al.*, 2004; Lederer *et al.*, 2005). Third, we showed here that spirochetes exhibit the classic mammalian host-adapted phenotype (i.e. *OspC*⁺/*OspA*⁻) for months within the immunoprivileged niche created by DMCs (Elias *et al.*, 2002; Purser *et al.*, 2003) (Fig. 1B). How, then, would *Bb* maintain the ON state of the regulon while contending with the development of humoral immune responses against surface-exposed RpoS-dependent gene products that are no longer required? As recently shown for *OspC* and BBA64, one means of resolving this dilemma would be to selectively downregulate individual RpoS-dependent genes (Gilmore *et al.*, 2007; Xu *et al.*, 2007). Regardless of regulon status during chronic infection, the markedly reduced levels of *rpoS* (Fig. 7), in concert with the re-initiation of *OspA* expression (de Silva *et al.*, 1997; Schwan and Piesman, 2000; Gilmore *et al.*, 2001), demonstrate that, by the time of larval acquisition, spirochetes have reverted to the RpoS-OFF state that persists until the next blood meal. Paradoxically, the mammalian host-derived stimuli

that are such powerful inducers of *rpoS* during the nymphal blood meal are superseded or overridden within feeding naïve larvae. The signal transduction apparatus and pathways that enable the spirochete to distinguish between these two ostensibly identical stimuli have yet to be discovered, although recent findings by Scheckelhoff *et al.* (2007) suggesting a role for host-derived catecholamines in promoting induction of OspA during the mammalian host-to-tick transition have provided an important clue.

Experimental procedures

Bacterial strains and culture conditions

Virulent wild-type *Bb* isolates c162 [a clonal derivative of strain 297 originally isolated from the cerebrospinal fluid of a Lyme disease patient (Steere *et al.*, 1984)] and c174 [(c162 *rpoS::erm*) (Caimano *et al.*, 2004; Eggers *et al.*, 2004)] were cultivated in BSK-H medium (Sigma-Aldrich Chemical, St Louis, MO) supplemented with 6% rabbit serum (Pel-Freeze Biologicals, Rogers, AK); c174 was maintained under selection using erythromycin (0.06 $\mu\text{g ml}^{-1}$). The plasmid content of all isolates was monitored as described previously (Eggers *et al.*, 2002) and passaged no more than twice prior to use in experiments. Infectivity of c162 and avirulence of c174 was confirmed in mice (Caimano *et al.*, 2004). Spirochetes were enumerated by darkfield microscopy using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA). For standard temperature-shift experiments, organisms were cultivated at 23°C to mid-logarithmic phase ($\sim 1 \times 10^7$ to 3×10^7 spirochetes per ml) then transferred to BSK-H medium at a starting density of 3000 spirochetes per ml and grown at 37°C until late-logarithmic phase ($\sim 7 \times 10^7$ to 1×10^8 spirochetes per ml) prior to being harvested; 23°C cultures were then allowed to continue until late-logarithmic phase. The pH of culture medium was measured at the time of harvest to ensure that the growth medium remained in the neutral range (pH ≥ 7.0). For reduced acid pH studies, cultures were temperature-shifted from 23°C to 37°C into BSK-H medium adjusted to pH 6.8 prior to inoculation. To obtain organisms in a host-adapted state, spirochetes were cultivated in DMCs (Spectra-Por, 8000 molecular weight cut-off) in BSK-H medium at a starting dilution of 3000 spirochetes per ml and implanted into the peritoneal cavities of either rats or rabbits as previously described (Akins *et al.*, 1998; Sellati *et al.*, 1999). For RpoS-induction experiments, *Bb* RpoSi, a strain 297 isolate in which expression of *rpoS* is controlled by an isopropyl β -D-1-thiogalactopyranoside (IPTG)-inducible hybrid *flac* promoter (Gilbert *et al.*, 2007), was temperature-shifted, in triplicate, in BSK-H medium in the absence or presence of IPTG (5 mM) and kanamycin (400 $\mu\text{g ml}^{-1}$; to select for transformants). For serial passage experiments, spirochetes grown at 23°C were either temperature-shifted to 37°C or implanted within DMCs as described above, then at late-logarithmic phase, spirochetes were immediately used to inoculate fresh BSK-H medium at a starting density of 3000 spirochetes per ml and allowed to reach late-logarithmic phase before being passaged again under the same growth condition for a total of five passages. All *E. coli* strains were maintained in Luria-Bertani broth (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl) with the appropriate antibiotic. Solid-phase selection was performed on LB agar plates (LB with 1.5% agar) supplemented with the appropriate antibiotic. Routine molecular cloning and plasmid propagation were performed with *E. coli* strain Top10 (Invitrogen, Carlsbad, CA).

SDS-PAGE and Western blot analyses

Whole-cell lysates were prepared from spirochetes cultivated in BSK-H at 23°C, following temperature-shift to 37°C, or within DMCs. Cultures were harvested by centrifugation at 8500 g for 20 min, and the resulting pellets washed twice with phosphate-buffered saline (PBS). Equivalent amounts of cells ($\sim 1 \times 10^7$ total spirochetes) were re-suspended and boiled in reducing Laemmli sample buffer (Bio-Rad, Hercules, CA) and separated through 12.5% separating polyacrylamide mini-gels. Separated proteins were visualized by silver stain according to the method described by Morrissey (1981). For immunoblotting, proteins were

transferred to nylon-supported nitrocellulose membrane (Micro Separations, Westborough, MA) and incubated with rat polyclonal antiserum directed against Lp6.6 (Lahdenne *et al.*, 1997), DbpA (Hagman *et al.*, 1998) or OspE (Akins *et al.*, 1998) followed by horseradish peroxidase-conjugated goat anti-rat (Southern Biotechnology Associates, Birmingham, AL) secondary antibody. Rat polyclonal antiserum directed against FlaB (Caimano *et al.*, 2005) was used to assess loading and electrotransfer uniformity. Membranes were developed using the SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL).

DNA: DNA hybridizations

Total genomic DNA was isolated from 50 ml of *Bb* cultures (1×10^8 spirochetes per ml) using Isoquick nucleic acid extraction Kit (ORCA Research, Bothell, WA) according to the manufacturer's instructions and quantified spectrophotometrically. DNA was digested with HindIII for 2 h at 37°C to generate fragments < 2 kb. Two micrograms of digested 297 DNA (test DNA) was labelled with cy3-dUTP and 2 µg of B31-MI (reference) DNA was labelled with cy5-dUTP. Equal amounts of cy3- and cy5-labelled DNAs were hybridized as described previously (Terekhova *et al.*, 2006). Raw spot intensities were generated using GenePix Pro v6 software (Axon instruments). Background subtraction, data normalization and mean log₂ (cy3/cy5) ratio calculations were all performed using GeneTraffic software Version 3.11 (Iobion, La Jolla, CA).

Generation of samples for microarray analyses

Wild-type (c162) and *rpoS* mutant (c174) *Bb* were cultivated in BSK-H following temperature-shift as described above. Culture densities were assessed daily to ensure that each sample was taken during late-logarithmic phase (8×10^7 to 1×10^8 spirochetes per ml). Following harvest, the pH of spent growth medium was measured to ensure that each culture remained within a neutral range (pH 7.2–7.5). SDS-PAGE (Fig. S1) and immunoblot using sera directed against Lp6.6 and DbpA (data not shown) were used to confirm that each c162 and c174 experimental sample exhibited the expected polypeptide profile prior to being used in microarray experiments. cDNAs generated from c162- and c174-derived RNAs were analysed by qRT-PCR to ensure that transcripts for *rpoS* and *ospC* were present only in the wild-type isolate and induced by temperature-shift and growth within DMCs.

RNA labelling and hybridization

Total RNA was isolated from organisms cultivated either *in vitro* following temperature-shift or within DMCs using TRizol reagent (Invitrogen) according to manufacturer's instructions. Contaminating genomic DNA was removed using Turbo DNAfree (Ambion, Austin, TX). The absence of contaminating DNA was confirmed by testing each treated RNA in amplification reactions containing primers specific for *flaB* as previously described (Caimano *et al.*, 2004). For microarray experiments, 12 µg each of DNase-treated RNA from three independently implanted DMCs and five independent temperature-shifted (37°C) cultures of wild type (c162) and *rpoS* mutant (c174) was converted to cDNA using the PowerScript fluorescent labelling kit (Clontech, Mountain View, CA) in reactions containing random hexamer (Promega, Madison, WI) and random 18-mer (IDT, Skokie, IL). The resulting cDNA was then split into two equal aliquots for labelling with cy3 or cy5 (Amersham, Piscataway, NJ) according to the manufacturer's instructions; unincorporated dye was removed by ethanol precipitation. Precipitated cy3- and cy5-labelled probes generated from wild-type *Bb* RNAs were then mixed with their corresponding cy5- and cy3-labelled *rpoS* mutant counterparts (i.e. dye-swap) and the combined probes were further purified using QiaQuick PCR purification columns (Qiagen, Valencia, CA), dried in a speed vac for 45–60 min and re-suspended in 25 µl of nuclease-free water. Re-suspended cy3/cy5 probes were mixed with 18 µl of DNase, RNase-free formamide (Acros Organics, Belgium) and 17 µl of 4× Hybridization buffer (Amersham Biosciences,

Piscataway, NJ) in a final volume of 60 μ l. The mixture was denatured by heating at 95°C for 5 min, cooled on ice for 1 min and applied to previously described *Bb* glass slide microarrays (Terekhova *et al.*, 2006). Prior to use, slides were pre-hybridized in a solution of 30% formamide, 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulphate (SDS) and 0.1% bovine serum albumin (Sigma) for 45–60 min at 42°C. Following prehybridization, slides were washed twice in distilled water, dipped in isopropanol and dried immediately by centrifugation at 1400 g for 3 min. After application of the labelled probes, slides were covered with lifter slips (Fisher Scientific) and placed in humidified hybridization chamber. The chambers were placed in water bath and incubated overnight at 42°C. After overnight hybridization slides were washed twice with 2 $\times$ SSC/0.1% SDS for 10 min at 42°C, twice with 0.1 $\times$ SSC/0.1% SDS for 10 min at room temperature and four times with 0.1 $\times$ SSC for 1 min at room temperature. Washed slides were immediately dried by centrifugation at 2700 r.p.m. for 3 min.

Microarray scanning and data analysis

The hybridized microarray slides were scanned on a GenePix 4000B microarray scanner (Axon Instruments, Union City, CA) at 10 μ m resolution. The resulting dual-colour images were recorded in 16-bit multi-image TIFF files which were analysed by GenePix Pro software (Axon Instruments). GenePix data were then input to Microsoft Excel for background correction, normalization and filtering. Statistical analyses were performed in Microsoft Excel using data derived from the GAL files. Briefly, pixel intensity values were corrected for background by subtracting the mean value for 'blank' spots (for individual slides) from the spot intensities for each ORF. The spot intensities were then normalized by first summing the intensity values for all ORFs for each slide, setting wild type as 'reference', and then dividing the sum of intensities for each slide by the sum of intensities for the reference slide; the resulting normalization factor was used to multiply all background-corrected spot intensities. The normalized values were employed for two statistical tests. First, the mean intensity values were calculated for each ORF in both wild type and mutant; the ratios (wild type/mutant) of the mean intensities were determined and log₂-transformed. The mean and standard deviation (SD) of the log₂ values for all ORFs was calculated. Those individual ORFs with a log₂ ratio exceeding ± 2 SD of the mean log₂ ratio for all ORFs were considered to have significant differences in expression between wild type and mutant. For DMC-derived microarrays, only those genes that exhibited wild type/mutant ratios ≥ 4.50 or ≤ -2.41 were considered to be expressed at significantly different levels in the wild type. For *in vitro* based microarrays, only those genes that exhibited a wild type/mutant ratio ≥ 2.53 or ≤ -1.60 were considered to be expressed at significantly different levels in the wild type. In addition, a two-tailed *t*-test was performed for each ORF to determine if pixel intensity values for the two conditions were different. ORFs with $P < 0.01$ were considered to represent significantly different expression levels. Except where indicated, all ORFs included in the DMC and *in vitro* microarray data sets passed both statistical tests.

Nucleotide sequence and computer analyses

All nucleotide sequencing was performed by the UCHC Molecular Core Facility using an Applied Biosystems model 373A-automated DNA sequencer and PRISM™ ready reaction DyeDeoxy™ Terminator cycle sequencing kits according to manufacturer's instructions (Applied Biosystems, Foster City, CA). Unless otherwise indicated, primers used in qRT-PCR studies were designed using MacVector version 8.1 software (Accelrys Bioinformatics, San Diego, CA). Routine and comparative sequence analyses were performed using MacVector (version 9.5.1, MacVector, Cary, NC). MEME (<http://meme.sdsc.edu>) was used to search for conserved sequence motifs within the 400 bp regions upstream of the translation start sites for genes upregulated by RpoS. Lipoprotein predictions were based on SPLIP (Setubal *et al.*, 2006), Lipop (Juncker *et al.*, 2003), TMHMM (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) and PSORT (Gardy *et al.*, 2005).

Real-time qRT-PCR

Wild-type and mutant DNase-treated RNAs (4 µg) from *in vitro* and DMC-cultivated spirochetes used in the microarray studies described above were converted to cDNA using SuperScript First-Strand Synthesis for RT-PCR (Invitrogen) in the presence and absence of reverse transcriptase (RT) according to the manufacturer's instructions. Individual cDNA were assessed first using primers specific for *flaB* and *ospC* (Table S3) and then pooled and assayed by real-time PCR (in quadruplicate) using iQ SYBR Green Supermix (Bio-Rad). Reaction conditions for each gene-specific primer pair (Table S3) were optimized for temperature and Mg²⁺ concentration. Control reactions were performed using pooled 'No RT' cDNA samples. For quantification, amplicons corresponding to each gene of interest were cloned into the pCR2.1-TOPO cloning vector (Invitrogen), then purified recombinant plasmid DNAs for each amplicon were diluted (10⁷–10² copies µl⁻¹) to generate a standard curve. Transcript copy numbers for each gene of interest were calculated using the iCycler post-run analysis software based on internal standard curves then normalized against copies of *flaB* present in the same cDNA. To determine the statistical significance of observed differences, normalized copy number values were compared within Prism v5.00 (GraphPad Software, San Diego, CA) using an unpaired *t*-test with two-tailed *P*-values and a 95% confidence interval.

qRT-PCR analysis of *Bb*-infected *I. scapularis* ticks

Pathogen-free *I. scapularis* larvae were obtained from a colony maintained at Oklahoma State University. To obtain *Bb*-infected nymphs, 3- to 5-week-old C3H/HeJ mice were syringe-inoculated intradermally with a dose of 10⁴ spirochetes; infection was confirmed by serology and/or ear punch biopsy at 2 weeks. At 3–5 weeks post inoculation, naïve larvae (200–300 per mouse) were placed on anaesthetized *Bb*-infected mice, allowed to feed to repletion and then collected over water as previously described (Hagman *et al.*, 2000). Engorged larvae were then allowed to molt into flat nymphs. To obtain fed nymphs, flat *Bb*-infected nymphs (10–12 per mouse) were placed in polypropylene capsules secured to the shaved backs of naïve C3H/HeJ mice as previously described (Hagman *et al.*, 2000), allowed to feed until near or fully engorged (~48–60 h post attachment) and then removed using forceps. Individual fed nymphs were immediately placed on dry ice and then stored in TRizol (Invitrogen) until all ticks were collected. To obtain fed *Bb*-infected larvae, naïve pathogen-free larvae were placed on infected C3H/HeJ mice, allowed to feed to repletion, returned to a humidified chamber and then harvested at 6–8 days post repletion. To isolate RNA from infected ticks, ~200 fed larvae and flat nymphs, or 75 fed nymphs were crushed in 1 ml of TRizol using a glass homogenizer then centrifuged at 4000 g for 1 min to remove tick cuticle material. DNA-free RNA and cDNA were generated as described above. Due to an extraneous (non-specific) *flaB* PCR amplicon observed in the SYBR green melting curve assays with *flaB* primers, obtained only with tick-derived *Bb* cDNAs, the previously described *flaB* primer/FAM-490-probe-based assay (Fisher *et al.*, 2005) was used to normalize *rpoS* expression in *Bb*-infected ticks.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A. *Borrelia burgdorferi* wild-type (WT) c162 was cultivated at 23°C, following a temperature-shift to 37°C at pH 7.5 and 6.8, and within DMCs. RpoSi was cultivated *in vitro* following temperature-shift to 37°C in the absence (–) or presence (+) of 5 mM IPTG. Whole-cell lysates were separated by SDS-PAGE and then either silver-stained or immunoblotted using polyclonal sera directed against FlaB, DbpA and Lp6.6. B. Wild-type strain 297 *Bb*, cultivated initially at 23°C, were serially passaged either *in vitro* in BSK-H medium at 37°C or within DMCs for a total of five passages (P1–P5). Whole-cell lysates were separated by SDS-PAGE and then silver-stained or immunoblotted using polyclonal sera directed against OspE. Molecular mass markers (kDa) are indicated.

Fig. 2.

Summary of genes upregulated or downregulated by RpoS within DMCs by replicon. Numbers represent the total number of genes identified for each replicon for each category. Plasmids lp36, lp38 and lp56 are either divergent or missing from strain 297; pending the availability of complete plasmid sequences for *Bb* 297, B31 plasmid locations are used for the corresponding strain 297 orthologues. No genes on lp25 were induced by RpoS. No genes on cp26, cp32s, lp21, lp25, lp28-3 or lp56 were found to be downregulated by RpoS during DMC cultivation.

Fig. 3.

Genes encoded on lp54 found to be differentially regulated by RpoS. The locations of differentially regulated lp54-borne genes are indicated by their position on the replicon. For upregulated genes, transcriptional orientation is indicated by open (+ strand) or shaded (– strand) bars. Arrows are used to indicate transcriptional orientation of downregulated genes. The wild type/*rpoS* mutant relative expression levels are indicated on the left using a logarithmic scale.

Fig. 4.

qRT-PCR analysis of representative core genes upregulated by RpoS both *in vitro* and within DMCs. Values represent the average transcript copy number for each gene normalized per 100 copies of *flaB*. Bars indicate the standard error of the mean. Normalized transcript copy numbers between wild-type and *rpoS* mutant *Bb* under each condition were compared using an unpaired *t*-test and are significantly different ($P \leq 0.05$).

Fig. 5.

A. qRT-PCR analysis of genes up-regulated by RpoS exclusively within DMCs. *bb0729* (*gltP*), though not included in the DMC dataset, was examined here based on semi-quantitative RT-PCR data demonstrating its co-transcription with *bb0728* (see text). Values represent the average transcript copy number of each gene normalized per 100 copies of *flaB*. Bars indicate the standard error of the mean. Normalized transcript copy numbers between wild-type and *rpoS* mutant *Bb* under each condition were compared using an unpaired *t*-test and are significantly different ($P \leq 0.05$) except where indicated; ns, not significant.

B. qRT-PCR analysis of genes up-regulated by RpoS exclusively *in vitro*. Values represent the average transcript copy number of each gene normalized per 100 copies of *flaB*. Bars indicate the standard error of the mean. Normalized transcript copy numbers between wild-type and *rpoS* mutant *Bb* under each condition were compared using an unpaired *t*-test and are significantly different ($P \leq 0.05$).

Fig. 6. qRT-PCR analysis of genes downregulated by RpoS. Values represent the average copy number of each gene normalized per 100 copies of *flaB*. Bars indicate the standard error of the mean. Normalized transcript copy numbers between wild-type and *rpoS* mutant *Bb* under each condition were compared using an unpaired *t*-test and are significantly different ($P \leq 0.05$) except where indicated. ns, not significant.

Fig. 7.

qRT-PCR analysis of *rpoS* in *Bb* strain 297-infected *I. scapularis* ticks. *Bb*-infected ticks were assayed for expression of *rpoS* as nymphs (Flat Nymph), during nymphal engorgement (Fed Nymphs) and immediately following larval acquisition (Fed Larvae), then normalized per 100 copies of *flaB*. Bars indicate the standard error of the mean.

Table 1

Borrelia burgdorferi strain 297 genes upregulated by RpoS within DMCs.

Gene ID	Description ^a	Replicon ^e	Gbb family ^b	Functional category ^a	Lipo ^c	DMC WT/mutant ^d	P-value	In vitro WT/mutant ^{d,e}	P-value
<i>bba64</i>	P35 antigen	lp54		CE	Y	1612.82	1.93E-09	18.29	4.06E-04
<i>bb0844</i>	Hypothetical protein	Chromosome		U	Y	1282.96	1.99E-20	70.40	3.56E-07
<i>bba73</i>	P35 antigen, putative	lp54		CE	Y	900.75	1.10E-20	53.84	1.25E-09
<i>bba65</i>	Hypothetical protein	lp54		U	Y	812.35	9.17E-04	35.13	9.75E-08
<i>bba66</i>	Hypothetical protein	lp54		U	Y	409.06	3.82E-04	19.34	5.41E-08
<i>bba25</i>	Decorin-binding protein B (<i>dbpB</i>)	lp54	fam_PF02352	CE	Y	369.86	3.22E-15	58.42	1.27E-15
<i>bba72</i>	Hypothetical protein	lp54	pep_35	U	Y	269.42	1.98E-19	61.07	2.50E-09
<i>bba37</i>	Hypothetical protein	lp54		U		209.15	3.78E-04	10.35	1.01E-05
<i>bba71</i>	Hypothetical protein	lp54	pep_35	U		150.64	3.69E-06	30.10	1.08E-10
<i>bba04</i>	S2 antigen	lp54	pep_45	CE	Y	140.30	7.84E-10	5.32	5.06E-06
<i>bba07</i>	ChpAI protein, putative	lp54		GM	Y	138.97	2.85E-15	7.10	1.79E-05
<i>bba36</i>	Lipoprotein	lp54		CE	Y	73.90	2.23E-06	29.59	3.85E-15
<i>bbj24</i>	Hypothetical protein	lp38		U		67.35	1.11E-02	6.92	9.79E-05
<i>bba24</i>	Decorin-binding protein A (<i>dbpA</i>)	p54	fam_PF02352	CE	Y	42.22	3.23E-14	72.94	1.09E-13
<i>bba34</i>	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein (<i>opp</i>)	lp54	fam_PF00496	TP	Y	32.41	3.36E-12	20.78	3.23E-05
<i>bbj23</i>	Hypothetical protein	lp38		U		30.76	1.26E-06	11.15	5.19E-10
<i>bba33</i>	Hypothetical protein	lp54	pep_55	U	Y	29.00	2.30E-09	8.17	1.15E-06
<i>bb0771</i>	RNA polymerase sigma factor (<i>rpoS</i>)	Chromosome	fam_PF04542	TR		27.96	1.67E-03	5.75	6.74E-04
<i>bbi42</i>	Outer membrane lipoprotein	lp28-4	pep_37	CE	Y	22.17	7.38E-11	15.39	1.94E-11
<i>bba26</i>	Hypothetical protein	lp54		U		17.68	1.08E-04	6.70	5.94E-05
<i>bbd19</i>	Hypothetical protein	lp17		U		17.46	1.18E-06	3.73	5.39E-05
<i>bbj28</i>	Hypothetical protein	lp38		U		17.12	2.05E-09	5.98	6.40E-08
<i>bbq06</i>	Conserved hypothetical protein	lp56	fam_PF05628	HX		15.69	1.39E-07	5.99	1.40E-06
<i>bba05</i>	S1 antigen	lp54		CE	Y	13.74	1.00E-03	19.96	7.69E-08
<i>bbq64</i>	Hypothetical protein	lp56		U		10.63	2.69E-07	2.84	3.60E-02
<i>bb0680</i>	Methyl-accepting chemotaxis protein (<i>mcp4</i>)	Chromosome	fam PF00015	CH		10.35	8.71E-06	3.62	2.14E-04
<i>bbp27</i>	Rev protein (<i>rev</i>)	cp32-1	fam_PF04404	HX	Y	9.61	1.77E-04	4.08	8.76E-03

Gene ID	Description ^a	Replicon ^a	Gbb family ^b	Functional category ^a	Lipid ^c	DMC WT/mutant ^d	P-value	In vitro WT/mutant ^{d,e}	P-value
<i>bbi03</i>	Hypothetical protein	cp32-8	fam_PF02989	U		8.81	6.65E-07	2.59	2.53E-02
<i>bb0681</i>	Methyl-accepting chemotaxis protein (<i>mcp5</i>)	Chromosome	fam_PF00015	CH		8.58	3.48E-22	3.50	2.32E-08
<i>bbm01</i>	Hypothetical protein	cp32-9	fam_PF06381	U		8.52	1.50E-03	2.85	1.81E-02
<i>bbk52.1</i>	Conserved hypothetical protein, pseudogene	lp36		HX		8.46	1.09E-02	3.58	1.36E-06
<i>bb0566</i>	Hypothetical protein	Chromosome		U		8.34	2.39E-09	2.94	7.07E-06
<i>bbm01</i>	Hypothetical protein	cp32-6	fam_PF06381	U		7.91	3.91E-10	3.05	4.86E-05
<i>bbk57</i>	Hypothetical protein	lp54		U	Y	7.82	6.67E-05	3.92	3.28E-03
<i>bbs01</i>	Hypothetical protein	cp32-3	fam_PF06381	U		7.80	9.38E-13	3.14	1.25E-03
<i>bbk03</i>	Hypothetical protein	cp32-7	fam_PF02989	U		7.41	3.33E-08	2.59	2.99E-02
<i>bbk04</i>	Hypothetical protein	cp32-7	fam_PF02989	U		6.92	5.31E-18	2.85	6.21E-03
<i>bbi01</i>	Hypothetical protein	cp32-8	fam_PF06381	U		6.27	2.36E-06	3.53	1.30E-03
<i>bbk02</i>	Hypothetical protein, authentic frameshift	cp32-4		U		5.69	1.17E-09	3.31	4.60E-04
<i>bbq02</i>	Hypothetical protein	lp56		U		5.56	8.78E-03	2.73	1.88E-03
<i>bbk32</i>	Hypothetical protein	lp54		U	Y	5.55	1.14E-06	4.44	3.56E-06
<i>bbi36</i>	Conserved hypothetical protein (<i>bppA</i>)	cp32-8	fam_PF03112	HX		5.30	1.55E-09	5.70	2.52E-06
<i>bbq44</i>	Conserved hypothetical protein	lp56	fam_PF03196	HX		5.28	3.15E-07	4.22	1.41E-07
<i>bbq43</i>	Conserved hypothetical protein	lp56	fam_PF03112	HX		5.04	2.25E-12	4.50	7.76E-10
<i>bbg24</i>	Hypothetical protein	lp28-2		U		698.31	9.79E-07		
<i>bbg23</i>	Hypothetical protein	lp28-2		U		478.64	3.75E-07		
<i>bbg13</i>	Hypothetical protein	lp28-2		U		403.77	1.90E-05		
<i>bbg21</i>	Hypothetical protein	lp28-2	fam_TIGR01547	U		388.20	6.34E-08		
<i>bbg19</i>	Hypothetical protein	lp28-2		U		303.35	4.76E-09		
<i>bbg12</i>	Hypothetical protein	lp28-2		U		254.93	2.57E-05		
<i>bbg10</i>	Hypothetical protein	lp28-2	fam_PF00015	U		169.38	2.03E-06		
<i>bbg17</i>	Hypothetical protein	lp28-2		U		135.99	1.17E-06		
<i>bbg16</i>	Hypothetical protein	lp28-2		U		128.61	6.42E-04		
<i>bbg20</i>	Hypothetical protein	lp28-2		U		122.53	1.85E-07		
<i>bbg14</i>	Hypothetical protein	lp28-2		U		116.13	8.93E-08		
<i>bbj27</i>	Hypothetical protein	lp38	fam_PF02687	U		53.12	1.97E-04		
<i>bbg18</i>	Hypothetical protein	lp28-2		U		51.45	1.84E-08		

Gene ID	Description ^a	Replicon ^a	Gbb family ^b	Functional category ^a	Lipid ^c	DMC WT/mutant ^d	P-value	In vitro WT/mutant ^{d,e}	P-value
<i>bb104</i>	Hypothetical protein	cp32-8	fam_PF02989	U		47.52	6.50E-03		
<i>bbg03</i>	Conserved hypothetical protein, authentic frameshift	lp28-2		HX		46.08	3.90E-03		
<i>bbq65</i>	Conserved hypothetical protein, pseudogene	lp56		HX		33.11	1.62E-04		
<i>bbq01</i>	Conserved hypothetical protein	lp54	fam_PF05628	HX		24.35	5.30E-17		
<i>bbg11</i>	Hypothetical protein	lp28-2		U		23.57	6.13E-05		
<i>bbg30</i>	Hypothetical protein	lp28-2		U		17.29	5.35E-03		
<i>bbb09</i>	Hypothetical protein	cp26		U		16.76	9.63E-03		
<i>bb129</i>	Conserved hypothetical protein	cp32-8	fam_PF04404	HX		16.03	6.96E-09		
<i>bb0400</i>	Hypothetical protein	Chromosome	pep_29	U		14.16	1.66E-05		
<i>bbg26</i>	Hypothetical protein	lp28-2		U		12.73	4.04E-03		
<i>bbi43</i>	Conserved hypothetical protein	lp28-4	pep_31	HX		11.39	1.28E-03		
<i>bb0447</i>	Na ⁺ /H ⁺ antiporter	Chromosome		TP		10.56	3.60E-03		
bb0548	DNA polymerase I (polA)	Chromosome		R		10.51	1.04E-06		
<i>bbq58</i>	Hypothetical protein	lp56		U		10.05	5.35E-04		
<i>bb0116</i>	PTS system, maltose and glucose-specific IICB component (<i>malX</i>)	Chromosome	pep_96	TP		9.59	1.09E-06		
<i>bb0384</i>	Basic membrane protein C (<i>bmpC</i>)	Chromosome	fam PF02608	CE		9.52	4.13E-09		
<i>bb0565</i>	Purine-binding chemotaxis protein (<i>cheW-2</i>)	Chromosome	fam_PF01584	CH		9.37	9.54E-05		
<i>bb0633</i>	Exodeoxyribonuclease V, beta chain (<i>recB</i>)	Chromosome	pep_85	R		8.72	1.71E-05		
<i>bbd001</i>	Conserved hypothetical protein	lp17	pep_36	HX		8.69	2.29E-03		
bb0728	Coenzyme A disulphide reductase (<i>cdr</i>)^f	Chromosome	fam PF0007	IM		8.57	1.60E-09		
<i>bb102</i>	Hypothetical protein	cp32-8	fam_PF07094	U		8.15	4.03E-05		
<i>bbu09</i>	Conserved hypothetical protein	lp21	pep_31	HX		7.71	4.29E-09		
<i>bbm02</i>	Hypothetical protein	cp32-6	fam_PF07094	U		7.43	6.86E-12		
<i>bbh10</i>	Hypothetical protein	lp28-4		U		7.35	2.85E-06		
<i>bbi31</i>	Conserved hypothetical protein	lp28-4	fam_PF05628	HX		7.22	3.63E-06		
<i>bb0812</i>	Pantothenate metabolism flavoprotein (<i>dfp</i>)	Chromosome		B		6.91	4.75E-03		
<i>bbb14</i>	Hypothetical protein	cp26		U		6.52	3.50E-04		

Gene ID	Description ^a	Replicon ^a	Gbb family ^b	Functional category ^a	Lipo ^c	DMC WT/mutant ^d	P-value	In vitro WT/mutant ^{d,e}	P-value
<i>bbp29</i>	Conserved hypothetical protein	cp32-1	fam_PF04404	HX		6.39	8.36E-05		
<i>bba09</i>	Conserved hypothetical protein	lp54	fam_PF03434	HX		6.16	3.83E-03		
<i>bbs03</i>	Hypothetical protein	cp32-3	fam_PF02989	U		5.95	6.71E-07		
<i>bbo01</i>	Hypothetical protein	cp32-7	fam_PF06381	U		5.72	1.50E-07		
<i>bbk54</i>	Conserved hypothetical protein	lp36	pep_31	HX		5.65	5.35E-03		
<i>bbo02</i>	Hypothetical protein	cp32-7	fam_PF07094	U		5.60	1.88E-14		
<i>bbo06</i>	Conserved hypothetical protein	cp32-7	pep_97	HX		5.53	4.68E-09		
<i>bbg05</i>	Transposase-like protein, authentic frameshift	lp28-2		X		5.49	9.10E-08		
<i>bb0689</i>	Hypothetical protein	Chromosome		U		5.47	7.17E-06		
<i>bbk02</i>	Hypothetical protein	lp36	pep_41	U		5.42	8.87E-03		
<i>bb0563</i>	Hypothetical protein	Chromosome	pep_2	U		5.39	4.22E-16		
<i>bbo26</i>	Conserved hypothetical protein	cp32-7	fam_PF02999	HX		5.08	3.28E-09		
<i>bbo25</i>	Conserved hypothetical protein	cp32-7	fam_PF06238	HX		5.02	8.14E-10		
<i>bb0363</i>	Periplasmic protein	Chromosome	pep_40	X		4.93	4.77E-05		
<i>bb01</i>	Hypothetical protein	cp32-4	fam_PF06381	U		4.88	9.89E-04		
<i>bb0578</i>	Methyl-accepting chemotaxis protein (<i>mcpI</i>)	Chromosome	fam_PF00015	CH		4.76	1.30E-07		
<i>bbp01</i>	Hypothetical protein	cp32-1	fam_PF06381	U		4.74	7.27E-11		
<i>bb0467</i>	Conserved hypothetical protein	Chromosome		HX		4.73	1.64E-05		
<i>bb0834</i>	ATP-dependent Clp protease, subunit C (<i>clpC</i>)	Chromosome	fam_PF07724	PD		4.60	6.16E-03		

^aDescriptions, replicon locations and functional categories are based on *B. burgdorferi* strain B31-MI.

^bParalogous family designations are based on current TIGR *B. burgdorferi*B31-MI genome database and may differ from original genome annotation (Fraser *et al.*, 1997).

^cY, known or predicted to encode lipoproteins (see *Experimental procedures*).

^dValues indicate the fold increase in wild-type compared with *rpoS* mutant *Bb*.

^eNo values are given for genes where the mean log2 ratio and/or P-values did not meet the statistical threshold for inclusion (see text).

^f*bb0728* is co-transcribed by with *bb0729*. The raw microarray data for *bb0729* fell below the threshold for inclusion in the DMC data set but also appear to be upregulated by RpoS (see text). Genes selected for analysis by qRT-PCR are bolded.

Table 2

Borrelia burgdorferi 297 genes subject to RpoS-mediated repression within DMCs.

Gene ID	Description ^a	Replicon	Gbb family ^b	Functional category	Lipo ^c	DMC WT/mutant ^d	P-value
<i>bbg01</i>	Hypothetical protein	lp28-2	pep_67	U		-100.58	2.24E-03
<i>bbk23</i>	Hypothetical protein	lp36	fam_Pf02414	U		-87.70	2.48E-06
<i>bbk35</i>	Hypothetical protein	lp36		U		-63.63	6.07E-03
<i>bb0616</i>	Conserved hypothetical integral membrane protein	Chromosome		HX		-29.22	3.16E-03
<i>bbk22</i>	Conserved hypothetical protein	lp36	fam_Pf02890	HX		-18.45	1.58E-08
<i>bbf20</i>	Conserved hypothetical protein	lp28-1	pep_39	HX	Y	-17.00	9.67E-09
<i>bbk24</i>	Conserved hypothetical protein	lp36	fam_Pf01672	HX		-16.12	3.81E-08
<i>bbk21</i>	Plasmid partition protein, putative (<i>parA</i>)	lp36	fam_Pf01656	HX		-14.60	1.24E-07
<i>bbk18</i>	Conserved hypothetical protein	lp36		HX		-13.60	1.21E-11
<i>bb0242</i>	Hypothetical protein	Chromosome		U		-11.63	8.99E-03
<i>bba69</i>	Hypothetical protein	lp54	pep_35	U	Y	-10.77	1.03E-02 ^e
<i>bbk17</i>	Adenine deaminase (<i>adeC</i>)	p36	fam_Pf01979	NM		-10.38	4.81E-03
<i>bbk34</i>	Hypothetical protein	lp36U		U		-9.62	2.69E-04
<i>bbk01</i>	Hypothetical protein	lp36	pep_67	U	Y	-9.58	2.45E-04
<i>bbk13</i>	Conserved hypothetical protein	lp36	pep_19	HX		-8.37	2.49E-13
<i>bbd18</i>	Hypothetical protein	lp17U		U		-7.54	8.18E-05
<i>bb0240</i>	Glycerol uptake facilitator (<i>glpF</i>)	Chromosome		TP		-6.85	1.65E-07
<i>bbi36</i>	Hypothetical protein	lp28-4	pep_35	U	Y	-6.77	1.11E-02 ^e
<i>bba74</i>	Outer membrane-associated protein (<i>oms28</i>)	p54		CE		-6.36	1.27E-09
<i>bbk33</i>	Hypothetical protein	lp36U		U		-5.83	4.21E-03
<i>bb0241</i>	Glycerol kinase (<i>glpK</i>)	Chromosome	fam_Pf00370	IM		-5.56	1.22E-05
<i>bbi29</i>	Hypothetical protein	lp28-4	fam_Pf05714	U		-5.56	4.70E-05
<i>bba15</i>	Outer surface protein A (<i>ospA</i>)	p54	fam_Pf00820	CE	Y	-5.40	1.44E-10
<i>bbk19</i>	Hypothetical protein	lp36	pep_22	U	Y	-5.26	5.84E-11
<i>bb0365</i>	LA7 lipoprotein	Chromosome		CE	Y	-5.12	1.01E-07
<i>bbj41</i>	P35 antigen, putative	lp38	pep_35	CE		-4.94	4.79E-03
<i>bb0368</i>	Glycerol 3-phosphate dehydrogenase, NAD(P)+ (<i>gpdA</i>)	Chromosome	fam_Pf01210	FM		-4.68	3.19E-03
<i>bbi38</i>	Hypothetical protein	lp28-4	pep_35	U	Y	-4.48	2.79E-03

Gene ID	Description ^a	Replicon	Gbb family ^b	Functional category	Lipo ^c	DMC WT/mutant ^d	P-value
<i>bba16</i>	Outer surface protein B (<i>ospB</i>)	p54	fam_Pf00820	CE	Y	-4.12	1.35E-11
<i>bb0582</i>	D-alanyl, D-alanine carboxypeptidase, putative	Chromosome		CE		-3.82	9.80E-04
<i>bba62</i>	Lipoprotein	lp54		CE	Y	-3.71	7.03E-08
<i>bb0243</i>	Glycerol 3-phosphate dehydrogenase, anaerobic (<i>glpA</i>)	Chromosome	fam_Pf01266	IM		-2.99	2.29E-06
<i>bbi39</i>	Hypothetical protein	lp28-4	pep_35	U	Y	-2.60	2.54E-03
<i>bb0785</i>	Stage V sporulation protein G	Chromosome		X		-2.48	9.33E-05

^aDescriptions, replicon locations and functional categories are based on *B. burgdorferi* strain B31-MI.

^bParalogous family designations are based on current TIGR *B. burgdorferi* B31-MI genome database and may differ from original genome annotation (Fraser *et al.*, 1997).

^cY, known or predicted to encode lipoproteins (see *Experimental procedures*).

^dValues indicate the fold increase in wild-type compared with *rhoS* mutant *Bb*.

^eP-value below statistical significance threshold of $P < 0.01$. Genes selected for analysis by qRT-PCR are bolded.

Table 3
Identification of putative upstream regulatory motifs for genes subject to RpoS-mediated repression within DMCs.

Promoter	Upstream motif	-35	-10	RBS	Start ^a
<i>bba15/ospA</i>	TTTTATTTT	-11- TTGTTA	-16- TATAAT	-30- AGGAGA	-7- ATG
<i>bba62/lp6.6</i>	TTTTTTTATTTA	-19- TTGATT	-20- TATAAT	-14- AGGAGA	-7- ATG
<i>bba74^b</i>	TTATATATTTTATTT	-9- TTGATT	-17- TAAAT	-22- AGGAGA	-7- ATG
<i>bba18</i>	TATTTTTTTTATTTAT	-12- TTGATA	-22- TATTAT	-21- AGGAGA	-5- ATG
<i>bb0240</i>	TTAAATTTTTTT	-35- TTGCAA	-14- TTTAAT	-77- AGGAGA	-7- ATG
<i>bba69</i>	TTCTCTTTTTTT	-63- TTGACA	-10- TATAAT	-49- AGGAGA	-5- ATG
<i>bbg01</i>	TTTTTATTTATATTT	-33- TTAAAGA	-12- TATAAT	-21- AGGAGA	-7- ATG
<i>bbk01</i>	TATTTTATTT	-24- TTGATA	-22- TATTAT	-27- AGGAGA	-8- ATG
<i>bbk13</i>	TTTCTTTTAT	-6- TTGTCT	-18- TATGAT	-76- AGGAG	-7- ATG
<i>bbk17</i>	TTTTATTTTCT	-8- TTGCAA	-23- TATAAT	-49- AGGAGA	-9- ATG
<i>bbi36/bbi38^c</i>	TAATATTACAATTGATA-N ₄ -TATCAAAATTGTAATATATTA	-5- TTGAAC	-20- TCAAAT	-7- AGGAGA	-8- ATG

^aTranslation start site.

^bPromoter for *bba74* was confirmed by primer extension (Mulay, 2007).

^cThe upstream sequences and spacing for *bbi36* and *bbi38* are identical within the areas of interest. RBS, putative ribosome binding site.

Table 4

Comparison of RpoS-dependent *Bb* promoters.

Gene	-35 Region										Extended -10 Region										RBS	Reference ^d	
<i>E. coli</i> consensus	T	T	G	A	C	A																	Lacour and Landini (2004); Weber <i>et al.</i> (2005)
Absolutely RpoS-dependent							T	G	N	N	-16	A	C	T	A	t/c	a/g	C	T				
<i>bbb19/ospC</i>	T	T	G	A	A	A	-11-	T	G	G	A	C	T	A	A	T	A	A	A	-12-	AGGAG		Marconi <i>et al.</i> (1993); Eggers <i>et al.</i> (2004); Yang <i>et al.</i> (2005)
<i>dlpBA</i>	T	T	T	A	T	T	-10-	T	G	G	G	C	T	A	A	A	A	A	T	-24-	GAGG		Hagman <i>et al.</i> (1998)
<i>bba66</i>	T	T	G	A	T	T	-11-	T	G	A	A	C	T	A	A	G	T	T	T	-68-	GAGG		Clifton <i>et al.</i> (2006)
<i>blm38/erpK</i>	T	T	G	T	A	A	-11-	T	G	C	G	T	T	A	G	A	A	T	T	-10-	GGAG ^b		Eggers <i>et al.</i> (2004)
<i>ospF</i>	T	T	G	T	A	T	-10-	T	G	C	G	T	T	A	G	A	C	T	T	-10-	AGGAG		
<i>bba07</i>	T	T	T	A	T	T	-11-	T	G	A	G	A	T	A	T	A	T	A	T	-75-	GGAG ^b		
<i>bbi42</i>	T	T	G	A	G	A	-11-	T	G	G	T	A	T	A	T	A	G	T	T	-108-	AGGA		
<i>bbk32</i>	T	T	A	T	T	A	-10-	T	G	T	A	A	T	A	T	A	T	T	T	-77-	GGAG		
<i>bb0844</i>	T	T	G	T	A	T	-11-	A	G	A	A	A	T	A	T	A	T	A	T	-38-	GGAG		
<i>bba34/oppA5</i>	T	T	G	A	A	A	-12-	G	G	A	T	A	T	A	T	A	T	A	T	-258-	GGAG ^b		
Bb RpoS consensus	T	T	G	A	<i>a/t</i>	<i>t/a</i>		T	G	g/a	g/a	A	A	T	A	t/a	A	T	T				
Dually transcribed																							
<i>bb0588</i>	T	T	G	A	A	C	-13-	T	G	A	G	G	T	A	T	G	T	T	T	-186-	AGGA		
<i>bb0680</i>	T	T	G	A	G	A	-13-	T	G	C	T	G	T	A	A	A	A	T	T	-56-	GAGG		
<i>bb0729-bb0728</i>	T	T	A	A	G	T	-11-	T	G	C	A	C	T	A	A	G	C	T	T	-7-	GAGG		
<i>bb0021</i>	T	T	G	A	T	G	-8-	A	G	A	A	A	T	A	T	T	T	T	T	-154-	GAGG		
<i>bb0548</i>	T	T	G	G	A	T	-7-	A	T	T	A	C	T	T	A	A	A	T	T	-246-	GGAG ^b		

^aReference used to define transcriptional start site.^bTranslation start site is based on presence of RBS consensus sequence and differs from the TIGR annotation Nucleotide positions matching the proposed *Bb* RpoS consensus -35 and extended -10 regions are highlighted. No promoters were found for *bb0670*, *bbg13*, *bbg17*, *bbg19*, and *bbg24*.