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RpoS Is Not Central to the General Stress Response in *Borrelia burgdorferi* but Does Control Expression of One or More Essential Virulence Determinants

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Borrelia burgdorferi*, the Lyme disease spirochete, undergoes dramatic changes in antigenic composition as it cycles between its arthropod and mammalian hosts. A growing body of evidence suggests that these changes reflect, at least in part, the need for spirochetes to adapt to the physiological stresses imposed by abrupt changes in environmental conditions and nutrient availability. In many microorganisms, global responses are mediated by master regulators such as alternative sigma factors, with *Escherichia coli* RpoS (σ^S) serving as a prototype. The importance of this transcriptional activator in other bacteria, coupled with the report by Hübner et al. (A. Hübner, X. Yang, D. M. Nolen, T. G. Popova, F. C. Cabello, and M. V. Norgard, Proc. Natl. Acad. Sci. USA 98:12724-12729, 2001) demonstrating that the borrelial RpoS ortholog controls expression of OspC and decorin-binding protein A (DbpA), prompted us to examine more closely the roles of RpoS-dependent and -independent differential gene expression in physiological adaptation by the Lyme disease spirochete. We observed that *B. burgdorferi rpoS* (*rpoS*$_{Bb}$) was induced following temperature shift and transcript levels were further enhanced by reduced pH (pH 6.8). Using quantitative real-time reverse transcription-PCR (RT-PCR), we demonstrated that, in contrast to its ortholog (*rpoS*$_{Ec}$) in *Escherichia coli*, *rpoS*$_{Bb}$ was expressed at significant levels in *B. burgdorferi* throughout all phases of growth following temperature shift. By comparing a *B. burgdorferi* strain 297 *rpoS*$_{Bb}$ mutant to its wild-type counterpart, we determined that RpoS$_{Bb}$ was not required for survival following exposure to a wide range of environmental stresses (i.e., temperature shift, serum starvation, increased osmolality, reactive oxygen intermediates, and increased or reduced oxygen tension), although the mutant was more sensitive to extremes of pH. While *B. burgdorferi* strains lacking RpoS were able to survive within intraperitoneal dialysis membrane chambers at a level equivalent to that of the wild type, they were avirulent in mice. Lastly, RT-PCR analysis of the *ospE-ospF-elp* paralogous lipoprotein families complements earlier findings that many temperature-inducible borrelial loci are controlled in an RpoS$_{Bb}$-independent manner. Together, these data point to fundamental differences between the role(s) of RpoS in *B. burgdorferi* and that in *E. coli*. Rather than functioning as a master regulator, RpoS$_{Bb}$ appears to serve as a stress-responsive activator of a subset of virulence determinants that, together with the RpoS-independent, differentially expressed regulon, encompass the spirochete's genetic programs required for mammalian host adaptation.

Borrelia burgdorferi, the Lyme disease spirochete, is transmitted via the bite of an *Ixodes* tick and is maintained within nature by small mammalian reservoir hosts, typically wild rodents. To be sustained within this enzootic cycle, *B. burgdorferi* must be able to adapt rapidly to these two strikingly different host environments. The ability to host adapt, therefore, is presumed to be central to the spirochete's pathogenic programs and likely involves the expression (or repression) of genes encoding factors that prepare the bacterium for growth within the new milieu. Indeed, it has now been well established that tick feeding initiates extensive changes in borrelial transcriptome and protein composition that continue throughout the infectious process (2, 4, 14, 18, 21, 31, 38, 41, 51, 63, 70, 73, 78, 85, 89, 91). The best-studied example of borrelial differential gene expression involves the reciprocal synthesis of outer surface protein A (OspA) and OspC during tick feeding, first described in a pioneering study by Schwan et al. (74), and subsequently confirmed by others (22, 32, 57, 94). Numerous other borrelial proteins, most of which are plasmid encoded, have been studied in the context of arthropod feeding and mammalian host adaptation and, for this reason, are presumed to be relevant to spirochetal virulence and disease pathogenesis (2–4, 6, 14, 15, 21, 22, 27, 29–31, 35, 38, 41, 55, 56, 60, 63, 67, 68, 72, 74, 81–83, 86, 91, 97). Although a number of in vitro studies have demonstrated that temperature and pH are key environmental signals influencing borrelial differential gene expression (2, 4, 7, 14, 16, 17, 25, 41, 51, 61, 66, 69, 79, 86, 89, 90), we and others have obtained compelling evidence that as-yet-undefined environmental cues are also essential for triggering the genetic program(s) underlying mammalian host adaptation (2, 4, 14, 27, 41, 51, 63, 67, 69, 70, 86, 89, 91).

Many microorganisms have evolved master regulators such as alternative sigma factors to coordinate the expression of multiple loci required for adaptation to environmental and/or physiological stress (1, 9, 42, 54, 64, 84, 88, 95). One of the

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best-studied examples of a global stress response is the induction of the RpoS-dependent regulon in *Escherichia coli* (44). The studies described herein point to several novel features that distinguish RpoS expression and function in *B. burgdorferi* from those of its *E. coli* counterpart. Our data suggest that RpoS is subject to a significant degree of transcriptional control in response to a number of environmental stimuli. In contrast to *E. coli*, both *B. burgdorferi rpoS* ($rpoS_{Bb}$) and $RpoS_{Bb}$-dependent loci were expressed early during exponential growth in rich medium. Although associated with environmental stress adaptation in a number of bacterial systems, including *E. coli* (45), the loss of RpoS in *B. burgdorferi* did not affect the spirochete's ability to adapt to the majority of stress agents tested, including growth within dialysis membrane chambers (DMCs). The inability of *B. burgdorferi* $rpoS_{Bb}$ mutant isolates to survive in either C3H/HeJ or SCID mice, however, points to a central role for $rpoS_{Bb}$ in pathogenesis, likely beginning early in the infectious cycle (e.g., tick feeding). The environmental stimuli driving expression of $RpoS_{Bb}$-dependent loci also appear to influence differential expression of $RpoS_{Bb}$-independent loci, which may be of equal importance to host adaptation during the enzootic cycle. Rather than serving as a master regulator of a global environmental and/or physiological stress response or adaptation to stationary phase, our data have led us to postulate that $RpoS_{Bb}$ functions as a stress-responsive activator of a critical, but most likely limited, subset of virulence determinants that, together with the RpoS-independent, differentially expressed regulon, encompass the genetic programs required for mammalian host adaptation and virulence expression by the Lyme disease spirochete.

METHODS AND MATERIALS

Bacterial strains and culture conditions. Virulent wild-type *B. burgdorferi* strain 297, originally isolated from the cerebrospinal fluid of a Lyme disease patient (77), and strain CE162 (a clonal derivative of wild-type strain 297) were cultivated in BSK-H medium (Sigma-Aldrich Chemical Co., St. Louis, Mo.) supplemented with 6% rabbit serum (Pel-Freeze Biologicals, Rogers, Alaska) and passaged no more than three times before experimental manipulations were performed. The mutants used in these studies, AH200 (297 *rpoS*::*ermC*) (47), generously provided by Michael Norgard (University of Texas Southwestern Medical Center, Dallas, Tex.), CE174 (CE162 *rpoS::ermC*) (see Table 1; also described below), and the complemented $rpoS_{Bb}$ mutant (CE467) were maintained under selection in BSK-H medium containing either erythromycin (0.06 $\mu g\ ml^{-1}$; AH200 and CE174) or both erythromycin (0.06 $\mu g\ ml^{-1}$) and kanamycin (400 $\mu g\ ml^{-1}$; CE467). The plasmid content of all strains was monitored as described previously (23). For standard growth experiments, cultures were maintained at 33°C. For 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), and 3,000 spirochetes per ml were then transferred to BSK-H medium prewarmed to 37°C; cultures maintained at 23°C were then allowed to continue until late logarithmic phase ($\sim 7 \times 10^7$ to 1×10^8 spirochetes per ml) prior to being harvested. For growth curves, cultures were temperature shifted from 23 to 37°C (in triplicate) as described above into BSK-H medium at pH 7.5 or adjusted to pH 6.8. Cells were enumerated over 12 to 16 days by dark-field microscopy with a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, Pa.). Calculations of growth curves were performed for each strain under each condition in triplicate in at least two independent trials. To obtain organisms in a host-adapted state, spirochetes were cultivated in DMCs (Spectra-Por, 8,000 molecular weight cutoff) at a starting dilution of 3,000 spirochetes per ml in BSK-H medium and implanted into the peritoneal cavities of either rats (2) or rabbits (75), as previously described.

Generation of *B. burgdorferi* $rpoS_{Bb}$ mutant. To generate a second *B. burgdorferi rpoS* mutant in addition to AH200, a 3-kb region of chromosomal DNA containing the *rpoS*::*ermC* locus and flanking regions was amplified from AH200 with PCR primers upsRpoS-5′ (5′-AACTTATCTTGGAGGAAATTGATG-3′) and dwnRpoS-3′ (5′-CTTGCAAATGCCTGAGTTATTGCA-3′) and used directly in an electrotransformation reaction mixture. Ten identical 50-μl reaction mixtures, with reactions carried out with TaKaRa ExTaq (Fisher Scientific) high-fidelity polymerase, were combined, purified with the Eppendorf Gel Clean-Up kit (Fisher Scientific), concentrated by ethanol precipitation, and used to electrotransform the virulent strain 297 clone according to the method described by Samuels (71). Transformants were recovered in 4 ml of BSK-H at 33°C overnight, plated in two 96-well plates each in a total of 40 ml of BSK-H containing the appropriate antibiotic, and monitored over a 5-week period for a color change in the medium that would be indicative of growth. Erythromycin-resistant spirochetes were passaged into larger culture volumes and then assessed for the insertion within $rpoS_{Bb}$ by PCR amplification with primers PrpoS-5′ (5′-GACTGCAGAACAAATCTTAAAAATAAAGAGGG-3′) and rpoS-3 (5′-CTTGCAAATGCCTGAGTTATTGCA3′). The isolate CE174 retained a full-strain 297 plasmid complement and was chosen for further analyses. The $RpoS_{Bb}$ mutant phenotype was also confirmed by analysis of whole-cell lysates by silver staining and Western blotting for the absence of OspC and decorin-binding protein A (DbpA), respectively (47).

Complementation of $rpoS_{Bb}$ mutation in *B. burgdorferi* strain 297. To generate a complementation construct, a 1.8-kb region of chromosomal DNA containing the $rpoS_{Bb}$ locus and upstream flanking region containing the promoter was amplified from wild-type strain 297 genomic DNA in a 50-μl reaction mixture with ExTaq and primers rpoS+prom(extd)-5′SphI (5′-GAGCATGCAACAAA TCTTAAAAATAAAGAGGG-3′) and rpoS-3′XbaI (5′-ACTCTAGATTAA TTTATTTCTTCTTTTAATTTTTA-3′), containing the indicated restriction enzyme sites (underlined). The resulting PCR amplicon was first cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions, then subsequently digested with SphI and XbaI (New England Biolabs, Beverly, Mass.), and subcloned into similarly digested cp32-based shuttle vector pCE320 (23). Purified recombinant $rpoS_{Bb}$/pCE320 plasmid DNA (10 to 20 μg) was then used to transform the strain 297 $rpoS_{Bb}$ mutants AH200 and CE174, as described above. After multiple attempts, no transformants were obtained with AH200. One erythromycin- and kanamycin-resistant CE174 transformant designated CE467, containing the complementing plasmid and all other strain 297 plasmids, was chosen for these studies. $RpoS_{Bb}$ complementation in CE467 was demonstrated by the restoration of OspC and DbpA expression in whole-cell lysates following temperature shift.

Nucleotide sequencing and computer analyses. Nucleotide sequencing was performed by the University of Connecticut Health Center Molecular Core Facility with a model 373A automated DNA sequencer and PRISM ready reaction DyeDeoxy Terminator cycle sequencing kits, according to the manufacturer's instructions (Applied Biosystems, Inc., Foster City, Calif.). Routine and comparative sequence analyses were performed with MacVector version 7.2 software (Accelrys Bioinformatics, San Diego, Calif.).

SDS-PAGE and Western blot analyses. *B. burgdorferi* whole-cell lysates were prepared from spirochetes cultivated in BSK-H medium at 23 or 37°C following a temperature shift from 23°C unless otherwise indicated. Cultures were harvested by centrifugation at $8,500 \times g$ for 20 min, and the resulting pellets were washed twice with phosphate-buffered saline. Equivalent amounts of cells were resuspended, boiled in reducing Laemmli sample buffer (Bio-Rad, Hercules, Calif.), and separated through 12.5% separating polyacrylamide minigels. Separated proteins were visualized by silver staining according to the method described by Morrissey (58). Levels of OspC present in samples run on silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) minigels were calculated with the densitometry functions available in the Chemi-Imager 4400 (Alpha Innotech Corp., software version 5.5). For immunoblotting, proteins were transferred to nylon-supported nitrocellulose membranes (Micro Separations, Inc., Westborough, Mass.) and incubated with 1:1,000 to 1:5,000 dilutions of previously described rat polyclonal antiserum directed against strains carrying DbpA (37), $OspC_{cN40}$, OspE (2), and OspF (4). Blots were assessed for loading and electrotransfer uniformity by immunoblotting with a monoclonal antibody (1H6-33) directed against FlaB (4). Blots were then probed with a 1:40,000 to 1:60,000 dilution of horseradish peroxidase-conjugated goat anti-rat or goat anti-mouse antiserum (Southern Biotechnology Associates, Birmingham, Ala.) and developed with the SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, Ill.).

Environmental stress assays. Serum starvation assays were performed essentially as previously described by Alban et al. (5). Briefly, cultures grown in BSK-H medium at 33°C to late logarithmic phase ($\sim 5 \times 10^7$ to 8×10^7 spirochetes per ml) were centrifuged at $9,000 \times g$ for 10 min at 4°C, then resuspended to a density of 1.5×10^7 spirochetes per ml in RPMI 1640 medium (Invitrogen) with or without the addition of supplemental rabbit serum (final concentration, 6%), and incubated at 37°C. Aliquots were removed daily for 7 days and assayed for spirochete viability as described below. For oxygen tension assays, wild-type

strain 297 and AH200 were diluted to 3,000 spirochetes per ml in BSK-H medium and incubated in standard, increased, and decreased oxygen environments. For standard (microaerophilic) conditions, 15-ml aliquots of diluted culture were placed in tightly capped 15-ml screw-cap tubes and placed in an ambient air incubator. For increased oxygen, 15-ml aliquots of diluted culture were transferred to loosely capped 50-ml conical tubes and placed at an ~30° angle in a 20% O_2–5% CO_2–75% N_2 incubator; this growth environment has been shown to result in an approximately twofold-higher concentration of dissolved oxygen than that under standard growth conditions (87). For the anaerobic condition, 15-ml aliquots of diluted culture were transferred to loosely capped 15-ml screw-cap tubes and placed in an anaerobe jar treated with BBL GasPak Plus anaerobic system envelopes with palladium catalysts. Cultures grown under standard, increased oxygen, and anaerobic conditions were incubated for 6 days, and endpoint densities were determined by the enumeration of triplicate cultures with a Petroff-Hausser counting chamber. For high-osmolarity and oxidative stress assays, wild-type 297 and AH200 were grown at 33°C to late logarithmic density in BSK-H medium. For high osmolarity, assays were performed as previously described (26). Briefly, cultures of each strain were divided into 1-ml aliquots containing 3.5×10^7 spirochetes per ml. To increase osmolarity, 0.25 ml of sterile 5 M NaCl was added to one aliquot (final concentration, 1 M), while sterile Milli-Q water was added to a corresponding control. Cultures were returned to 33°C, and aliquots were removed for plating at 30, 60, and 120 min. For oxidative stress assays, cultures identical to those described for the high-osmolarity assays were exposed to 0.5 and 1 mM hydrogen peroxide (Sigma-Aldrich) for 30 min at 33°C, while a corresponding control was left untreated. For acid shock assays, cultures were grown at 33°C to late logarithmic phase in BSK-H medium and diluted to a density of 3.5×10^6 spirochetes per ml into BSK-H medium adjusted to a final pH value of 6.0. Cultures were incubated at 33°C for 2, 6, 12, and 24 h, and spirochete viability was determined as described below. Spirochete viability was assayed by growth endpoint determinations performed by first diluting bacterial samples 1:100 (except for acid shock assays, where a 1:10 dilution was used) into fresh BSK-H medium, corresponding to ~3.5×10^5 spirochetes per ml. Cultures were then serially diluted 1:2 with BSK-H medium in sterile 96-well microtiter plates. For each environmental condition, duplicate twofold serial dilutions were performed for each experimental sample, and the plates were incubated in a CO_2 incubator at 33°C for approximately 21 days. The number of spirochetes surviving exposure to environmental stress was calculated by using the endpoint dilution well containing viable spirochetes as determined by dark-field microscopy. Percent survival was calculated as the number of viable spirochetes after treatment divided by the number of live spirochetes in the untreated control for each assay condition. Wild-type and mutant isolates were compared in at least two independent assays for each experimental condition.

Statistics. To determine the statistical significance of observed differences, values were compared either with Prism, version 3.00 (GraphPad Software, San Diego, Calif.), or with an unpaired *t* test with two-tailed *P* values and a 95% confidence interval. In the data presented in this study, asterisks indicate a level of significance where *P* values are <0.05; error bars represent the standard error of the mean. In addition, the product limit (Kaplan-Meier) method was used to compare the overall rates of survival for wild-type and mutant isolates following exposure to environmental stress agents (JMP 5.0 software, SAS Institute, Cary, N.C.).

Standard RT-PCR. Total RNA was isolated from wild-type and AH200 (*rpoS*$_{Bb}$ mutant) spirochetes cultivated in vitro to late logarithmic phase at 23°C and following temperature shift (37°C at either pH 7.5 or 6.8) in BSK-H medium and following implantation within DMCs with Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNAs were resuspended in RNase-free water and treated with RQ1 RNase-free DNase (Promega) to remove contaminating genomic DNA, followed by phenol-chloroform extraction and ethanol precipitation. Primers specific for *rpoS*$_{Bb}$ are as follows: rpoS-RTPCR 5′ (5′-CAGTAAGAGAACACAAGCTAATTACTCAC-3′) and rpoS-RTPCR 3′ (5′-ATCCTCGTATAGATTCAAGAGTGTTG-3′). Primer pairs specific for each of the strain 297 *ospE-ospF-elp* genes and *flaB* are the same as those described by Hefty et al. (41). Standard reverse transcription-PCRs (RT-PCRs) were performed with the Titan One-Step RT-PCR kit (Roche) with 50 to 100 ng of total RNA per 25-μl reaction mixture. Negative control reactions were performed by substituting Expand High Fidelity *Taq* polymerase (Roche) for the Titan RT enzyme mixture. Positive-control reactions were performed with Expand High Fidelity *Taq* polymerase and either wild-type or AH200 genomic DNA purified with the IsoQuick DNA purification kit (Orca Research, Bothell, Wash.). Reaction conditions for all reactions consisted of a single cycle at 50°C for 30 min and 94°C for 3 min; then 38 cycles of 94°C for 15 s, 54°C for 15 s, and 68°C for 1 min; followed by a final extension at 68°C for 5 min. Five microliters of each amplification reaction mixture was then subjected to agarose gel electrophoresis and subsequently stained with ethidium bromide to visualize amplicons.

Quantitative RT-PCR. Total RNA from wild-type and AH200 (*rpoS*$_{Bb}$ mutant) spirochetes were cultivated in vitro to late logarithmic phase at 23°C throughout all phases of growth following temperature shift (37°C at either pH 7.5 or 6.8); following implantation within DMCs, the RNA was isolated with Trizol reagent and subjected to DNase, as described above. Real-time RT-PCR was performed with a Roche LightCycler instrument and the LightCycler RNA Master SYBR Green I kit (Roche Applied Science, Indianapolis, Ind.) according to the manufacturer's instructions. Reactions containing either 50 ng (*rpoS*) or 0.5 ng (*flaB*) of total RNA in the reaction mixture were performed with primer pair rpoS-LC 5′ (5′-CAGGACAAATACAAAGAGGCAATG-3′) and rpoS-LC 3′ (5′-CGGG TCATATTTTTCAGCAGCTC-3′) and primer pair flaB-LC 5′ (5′-CTTCTCA AGGCGGAGTTAATTCTC-3′) and flaB-LC 3′ (5′-TTAGTTGTTGCTGCTA CAACCTCA-3′) under the following conditions: 1 cycle of 61°C for 20 min and 95°C for 30s, followed by 45 cycles of 95°C for 1 s, 60°C for 5 s, and 72°C for 10 s. RNAs (50 ng per reaction mixture) were assayed for contaminating DNA with the LightCycler FastStart DNA Master SYBR Green I kit (Roche) with the same primers and under the same reaction conditions as described above. For quantitation, *rpoS*-LC and *flaB*-LC amplicons were first cloned into the pCR2.1-TOPO cloning vector (Invitrogen), and then purified recombinant plasmid DNAs containing the amplicon of interest were diluted (10^7 to 10^2 copies μl^{-1}) and used to generate external standard curves according to the manufacturer's instructions. Each assay run included RT-PCRs containing relevant purified plasmid (10^6 copies μl^{-1}) DNA as a template to serve as an internal control for the calibration of external standard curves. Quantitative real-time RT-PCRs (qRT-PCRs) containing experimental samples, calibration standards, or negative controls were each performed in duplicate. Copies of *rpoS*$_{Bb}$ and *flaB* present in each sample were calculated with LightCycler data analysis software (version 3.5.3) based on their respective standard curves, subtracted for background present in the negative control reaction mixtures, and the copies of *flaB* were corrected for dilution. To determine increase, the ratio of copies of *rpoS*$_{Bb}$ to copies of *flaB* was calculated for each sample, and then the *rpoS*$_{Bb}$/*flaB* ratio of each sample was compared to that of the 23°C sample.

Analysis of fluorescent reporters. *E. coli* cultures were maintained in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) or LB agar (LB broth with 1.5% agar) supplemented with kanamycin (50 μg ml^{-1}). Shuttle vectors containing green fluorescent protein (GFP) reporters under the control of either borrelial (P_{ospC} and P_{ospE}) or *E. coli* (P_{osmY}) promoter regions transformed into *E. coli* MC4100 [F^- *araD139* Δ(*argF-lacZYA*) *U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*] (76) have been described previously (41). Cultures containing the various *gfp*-P_{Bb} shuttle vectors were grown overnight at 25°C, then diluted to an A_{600} of 0.01 in SOB (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 10 mM $MgCl_2$) supplemented with zeocin (50 μg ml^{-1}), and incubated at 37°C with shaking at 250 rpm. For each assay, the optical density at 600 nm was determined at hourly intervals; beginning 2 h after inoculation, 0.5 ml of sample was fixed in 1% paraformaldehyde (final concentration, 0.8%) in Difco FA buffer (Becton Dickinson, Palo Alto, Calif.) and analyzed by flow cytometry as described below. Assays were performed in triplicate for each construct. For flow cytometric analyses, samples containing $\geq 10^7$ paraformaldehyde-fixed cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson) with a 15-mW 488-nm air-cooled argon laser and an ≈635-nm red diode laser as previously described (23). Data were collected in duplicate for 50,000 events for each sample. Flow cytometry data were analyzed using either CELLQUEST version 3.3 (Becton Dickinson) or WinMDI, version 2.8 (http://facs.scripps.edu/software.html).

Animal infectivity studies. *B. burgdorferi* wild-type clone 297 (CE162), *rpoS*$_{Bb}$ mutant AH200 (47), CE174 (a transformable insertionally inactivated *rpoS*$_{Bb}$ mutant in the CE162 background; see above), and CE467 (CE174 complemented in *trans* with a shuttle vector containing a copy of *rpoS*$_{Bb}$ under the control of its native promoter; see above) were assessed for infectivity using 3- to 5-week-old female C3H/HeJ (Jackson Laboratories) and BALB/c SCID (T.V. Rajan, University of Connecticut Health Center) mice. Low-passage cultures to be used for infections were grown to mid-logarithmic density in BSK-H medium at 33°C, enumerated by dark-field microscopy, and diluted to a density of 2×10^5 spirochetes per ml. Mice (four to five per group, per strain) were inoculated intradermally with 0.05 ml of diluted culture for a dose of 10^4 organisms per mouse. Mice were assessed for infection at 2 to 4 weeks postinoculation by cultivation of ear punch biopsies in BSK-H medium containing Borrelia Antibiotic Cocktail (Sigma-Aldrich) grown at 33°C. Negative cultures were monitored weekly for at least 6 weeks by dark-field microscopy. Immunocompetent mice were subjected to tail bleeding at 2 and 4 weeks postinoculation, and the

collected sera were assayed by Western immunoblot analyses with preparative minigel electrotransfers of whole-cell lysates prepared from *B. burgdorferi* wild-type 297 grown at 37°C in BSK-H medium.

RESULTS

Transcription of $rpoS_{Bb}$ is induced following temperature shift and further enhanced by reduced pH. Previous studies have demonstrated that the *B. burgdorferi* RpoS ortholog (BB0771) is required for increased expression of two borrelial lipoproteins, DbpA and OspC, following a shift in growth temperature in vitro from 23 to 37°C (47). This finding, coupled with the importance of RpoS for stress adaptation in other bacterial systems, prompted us to more closely examine its role in mediating differential gene expression and/or physiological adaptation in *B. burgdorferi*. In contrast to *E. coli*, where levels of RpoS are controlled primarily through posttranscriptional mechanisms (44), expression of $rpoS_{Bb}$ in *B. burgdorferi* is under the control of a second alternate sigma factor, RpoN (47), which in turn requires an interaction with the response regulator Rrp2 for activity (92). This unique control mechanism suggested that expression of $RpoS_{Bb}$ could be partially or completely regulated at the transcriptional level. We therefore began our studies by assessing $rpoS_{Bb}$ levels following temperature shift. To do this, total RNAs prepared from wild-type *B. burgdorferi* strain 297 grown to late logarithmic phase (8×10^7 cells per ml) in BSK-H medium at either 23°C or after temperature shift to 37°C were used to perform qualitative RT-PCR for $rpoS_{Bb}$; control reactions for *flaB* were used to check RNA integrity and to normalize samples. Results from these studies demonstrated a substantial increase in $rpoS_{Bb}$ transcript following temperature shift (data not shown); using densitometry, we estimated at least a twofold increase in $rpoS_{Bb}$ transcript in samples after temperature shift at pH 7.5 (data not shown). Expression of $RpoS_{Bb}$ and $RpoS_{Bb}$-dependent genes (e.g., OspC) has been shown to be enhanced by a reduction in the pH of BSK-H medium from 7.5 to 6.8, in concert with temperature shift (47). To examine the contribution of pH to $rpoS_{Bb}$ expression, we performed RT-PCR on RNAs isolated from late-logarithmic-phase cultures of wild-type *B. burgdorferi* after temperature shift to 37°C in BSK-H medium at pH 6.8. Results from these studies demonstrated that the level of $rpoS_{Bb}$ present following temperature shift at pH 6.8 was further increased over that seen in similarly temperature-shifted samples at pH 7.5 (2.9-fold compared to that at 23°C) (data not shown). These results are consistent with immunoblot data demonstrating that, while undetectable when spirochetes were cultivated to late logarithmic phase in BSK-H medium at 23°C, $RpoS_{Bb}$ was readily detectable following temperature shift in standard BSK-H (37°C; pH 7.5) and further enhanced when the pH was adjusted to 6.8 (data not shown).

Induction kinetics of RpoS and RpoS-dependent promoters differ in *B. burgdorferi* and *E. coli*. To more accurately assess the level of increase in $rpoS_{Bb}$ due to decreased pH and/or increased temperature, we performed qRT-PCR using primers specific for $rpoS_{Bb}$ on RNAs isolated from spirochetes following temperature shift at pHs 7.5 and 6.8 and compared these transcript levels to that found in spirochetes grown to late logarithmic phase in BSK-H (pH 7.5) at 23°C. To correlate the effect of growth phase with $rpoS_{Bb}$ transcript levels under these growth conditions, total RNA was isolated daily beginning with the mid-log phase of growth (the earliest time point at which spirochetes could be accurately enumerated and at which sufficient material for SDS-PAGE analysis could be obtained) until cultures had entered stationary phase (Fig. 1A). Results from qRT-PCR studies with primers specific for $rpoS_{Bb}$ indicate an average relative ratio of 0.0005 copies of $rpoS_{Bb}$ per copy of *flaB* at 23°C, whereas temperature-shifted cultures grown in BSK-H at pH 7.5 had significantly higher levels of $rpoS_{Bb}$ (6.30-fold increase in the $rpoS_{Bb}$/*flaB* ratio) at the earliest time point assayed (Fig. 1B, lane T1). Moreover, the levels of $rpoS_{Bb}$ in *B. burgdorferi* were essentially unchanged throughout all phases of logarithmic growth, although we did observe an increase (6.36-fold; pH 7.5) (Fig. 1B, lane T6 versus T1) during entry into stationary phase. This increase, however, may be due to the increased acidity of the culture medium as a consequence of spirochetal growth, as indicated by color change of the phenol red indicator dye present in BSK-H. This explanation is supported by the consistently higher levels of $rpoS_{Bb}$ observed when spirochetes were temperature shifted in BSK-H at pH 6.8 (Fig. 1B), where levels of $rpoS_{Bb}$ transcript at pH 6.8 were higher than those at pH 7.5 throughout all phases of growth. The enhancement observed during entry into stationary phase of pH 7.5 cultures was also observed in temperature-shifted cultures at pH 6.8 (Fig. 1B, lane T4). Analysis of total RNA isolated from DMC-cultivated spirochetes found that the level of $rpoS_{Bb}$ within the mammalian host is equivalent to the highest levels detected in vitro in BSK-H at pH 7.5 (Fig. 1B, lanes DMC and T6) and approached the highest levels observed at pH 6.8 (Fig. 1B). We next analyzed whole-cell lysates of wild-type strain 297 cultures grown to late logarithmic phase at 23°C and at sequential time points following temperature shift to determine whether sufficient levels of $RpoS_{Bb}$ were present to mediate the induction of $RpoS_{Bb}$-dependent genes. Not surprisingly, significant levels of OspC were observed during mid-logarithmic-phase growth, well before entry into stationary phase (Fig. 1C). Indeed, if one uses densitometry to correct for the unavoidable underloading at this sample point (Fig. 1C, lane 1), then the level of OspC expression during logarithmic growth is approximately 70% of levels achieved during stationary phase; expression of DbpA also showed similar induction kinetics (data not shown).

To determine whether these findings reflect a functional distinction between RpoS in *B. burgdorferi* and *E. coli* or, alternatively, a unique feature of the $RpoS_{Bb}$-dependent promoters elements themselves, we used a newly developed GFP reporter system for analyzing borrelial promoters in *E. coli* by flow cytometry (24). For these studies, we transformed both wild-type (ZK126) (19) and *rpoS* mutant (ZK1000) (12) *E. coli* with a P_{ospC}-*gfp* reporter contained on a cp32-based shuttle vector (pCE320) (23) and measured GFP expression over time by flow cytometry. As shown in Fig. 2, expression of GFP from the borrelial *ospC* promoter (P_{ospC}-*gfp*) in *E. coli* did not become readily detectable until cultures reached late logarithmic phase (6 h), with maximal expression not observed until cultures entered stationary phase (10 h). This expression pattern closely mirrored that of a reporter under the control of the promoter for *osmY* (P_{osmY}-*gfp*), a gene known to be induced during stationary phase in an RpoS-dependent manner in *E. coli* (52, 96) (Fig. 2). The low-level expression of the P_{ospC}-

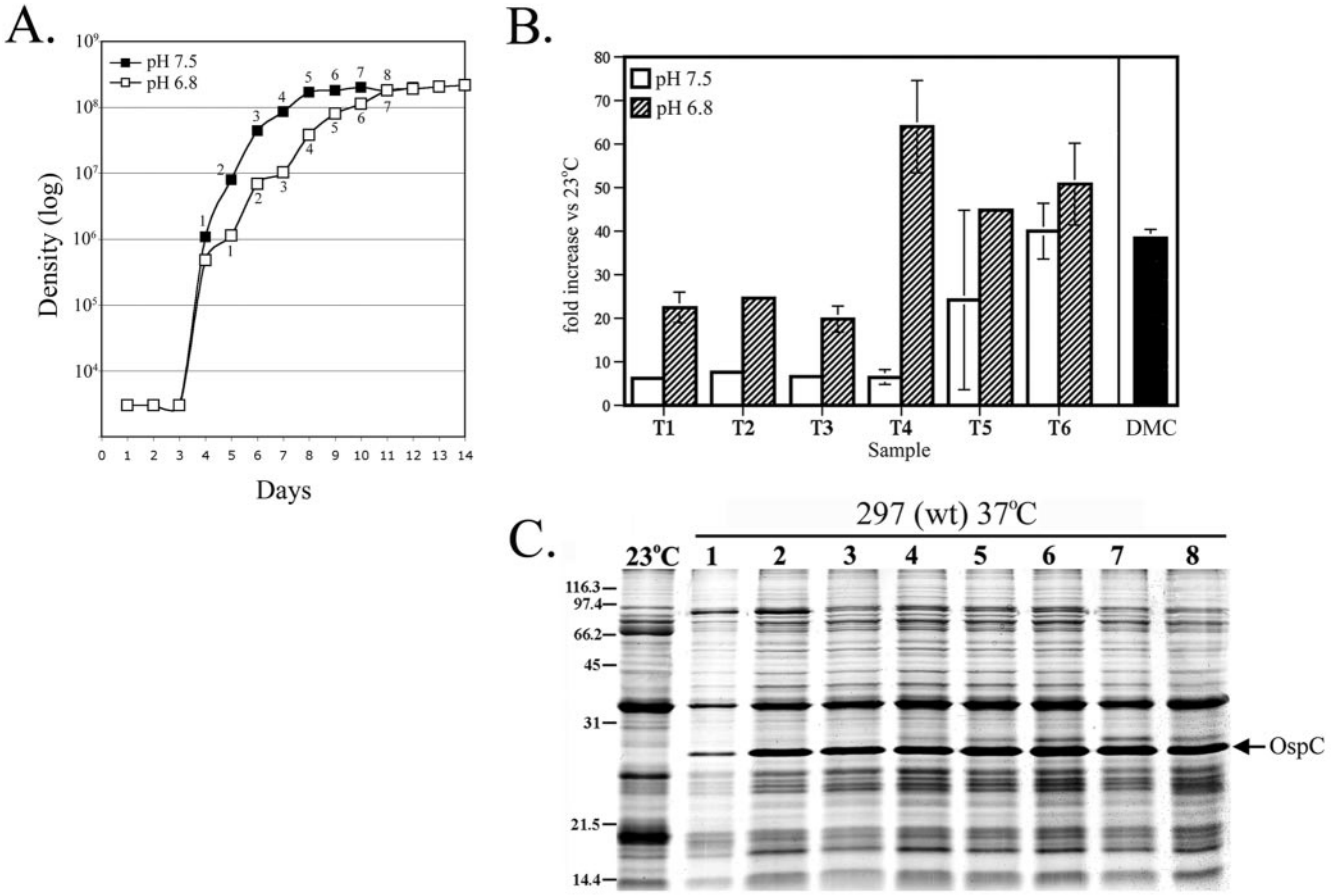

FIG. 1. Expression kinetics of $rpoS_{Bb}$ and RpoS-dependent loci in *B. burgdorferi*. (A) Growth curve indicating time points at which samples were taken from wild-type strain 297, following temperature shift from 23 to 37°C in BSK-H medium at either pH 7.5 (solid squares) or 6.8 (open squares), for subsequent RNA and protein analyses. (B) qRT-PCR analysis of $rpoS_{Bb}$. RNAs were isolated from spirochetes cultivated at 23°C and after temperature shift to 37°C in BSK-H medium at either pH 7.5 or 6.8 and after cultivation within DMCs. (C) Whole-cell lysates of wild-type *B. burgdorferi* strain 297 ($\sim 10^7$ per lane) cultivated at 23°C and following temperature shift to 37°C in BSK-H medium at pH 7.5 were separated by SDS-PAGE and then silver stained. Molecular mass markers (in kilodaltons) are indicated. Sample numbering in panels B and C corresponds to similarly numbered points in panel A. wt, wild type.

gfp reporter observed in the $rpoS_{Ec}$ mutant background is likely due to the unmasking of σ^{70} promoter recognition sequences in the absence of RpoS or, alternatively, could reflect differences in promoter recognition by the corresponding transcriptional factors orthologs in *B. burgdorferi* and *E. coli* (24). Taken together, these findings further support the existence of differences between the kinetics of RpoS expression and RpoS-dependent genes in *B. burgdorferi* and *E. coli*.

Survival of *B. burgdorferi* wild-type and RpoS mutant AH200 following exposure to environmental stress. The temperature-inducible expression of $rpoS_{Bb}$ in *B. burgdorferi* suggests that spirochetes may be experiencing some level of stress under this condition. This notion is supported by in vitro studies by Hubalek et al. (46) demonstrating that 37°C approaches the upper limit of permissible growth temperatures (22 to 39°C) for *B. burgdorferi* sensu stricto isolates. We therefore examined whether loss of $rpoS_{Bb}$ rendered spirochetes more sensitive to increased temperatures. At the outset, we performed routine temperature shift assays to reproduce the phenotype associated with AH200 (a strain 297 $rpoS_{Bb}$ mutant) observed by Hübner et al. (47); as expected, AH200 failed to express either OspC or DbpA following a shift in growth temperature from 23 to 37°C as assessed by both silver staining (OspC) and Western blot (DbpA) analysis (see below). To assess whether the loss of $RpoS_{Bb}$ results in altered growth following temperature shift, we performed growth curve assays comparing wild-type strain 297 to the $rpoS_{Bb}$ mutant AH200 following temperature shift from 23 to 37°C in BSK-H at pHs 7.5 and 6.8. As shown in Fig. 3, wild-type strain 297 and AH200 grew with nearly identical kinetics under both in vitro growth conditions. Thus, although $rpoS_{Bb}$ and RpoS-dependent genes are significantly induced under these conditions, $RpoS_{Bb}$ itself does not appear to be required for adaptation to increased temperature at either pH 7.5 or 6.8.

We next assessed whether $RpoS_{Bb}$ was required for survival following exposure to conditions that have a measurably detrimental effect on growth of wild-type virulent strain 297. For this, we chose a number of well-established classical environmental stresses that have been associated with the induction of an RpoS-dependent general stress response in other organisms: (i) nutrient deprivation (growth in RPMI medium with or without the addition of serum) (Fig. 4A), (ii) increased or

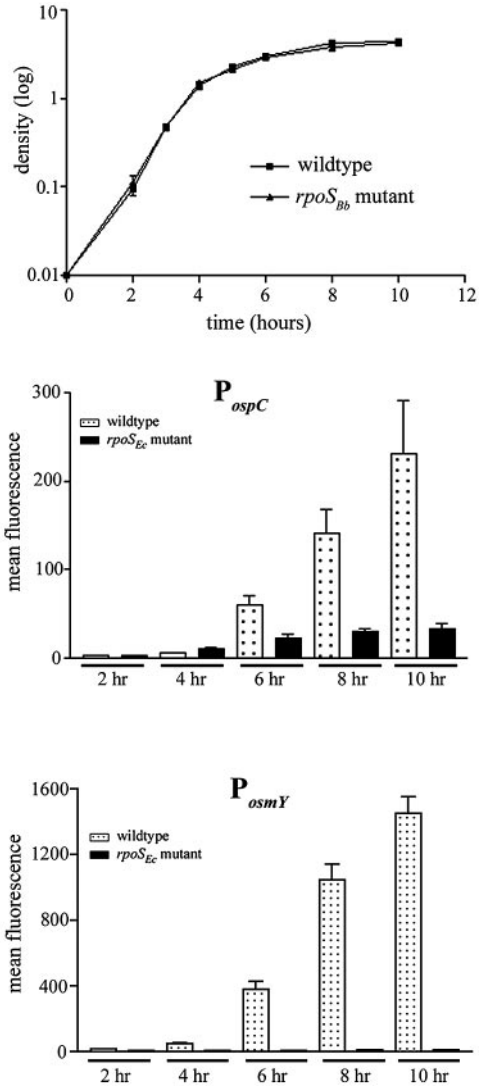

FIG. 2. Induction kinetics of RpoS-dependent promoters differ in *B. burgdorferi* and *E. coli*. Analysis of RpoS-dependent *B. burgdorferi* (P_{ospC}) and *E. coli* (P_{osmY}) promoters with a GFP reporter system in *E. coli*. Samples from wild-type (ZK126; squares) and $rpoS_{Ec}$ mutant (ZK1000; triangles) *E. coli* transformed with the indicated reporter constructs analyzed by flow cytometry. The top panel depicts a representative growth curve for both wild-type and $rpoS_{Ec}$ mutant isolates. Mean fluorescence intensities for P_{ospC}-*gfp* (middle panel) and P_{osmY}-*gfp* (bottom panel) reporter constructs were plotted against time on the representative graphs. Each graph represents the average of three trials.

decreased dissolved oxygen (Fig. 4B), (iii) high osmolarity (30, 60, and 120 min of exposure to 1 M NaCl) (Fig. 4C), (iv) exogenous peroxide (30 min of exposure to 0.5 or 1 mM H_2O_2) (Fig. 4D), and (v) acidic pH (pH 6.0) (Fig. 4E). In the first four cases, the $rpoS_{Bb}$ mutant AH200 survived exposure to the stress agent to the same extent as the wild-type isolate. In contrast, the loss of $RpoS_{Bb}$ resulted in increased sensitivity to acidic pH (Fig. 4E); Kaplan-Meier analysis revealed that survival of the $rpoS_{Bb}$ mutant was diminished in comparison to the wild type following exposure to acidic pH (log rank $\chi^2 = 3.809$; $P = 0.051$). Exposure to pH values below 6.0 (pHs 5.0 and 4.0) resulted in no survival of either wild-type or $rpoS_{Bb}$ mutant isolates at any time point (data not shown).

RpoS mutants grow within DMCs but are avirulent in mice. The above in vitro studies do not preclude the possibility that $RpoS_{Bb}$ controls the expression of one or more loci essential for adaptation to growth within the mammalian host. To examine this possibility, we grew wild-type and $rpoS_{Bb}$ mutant isolates within DMCs implanted into the peritoneal cavities of rats and rabbits. In addition to reflecting the differential gene

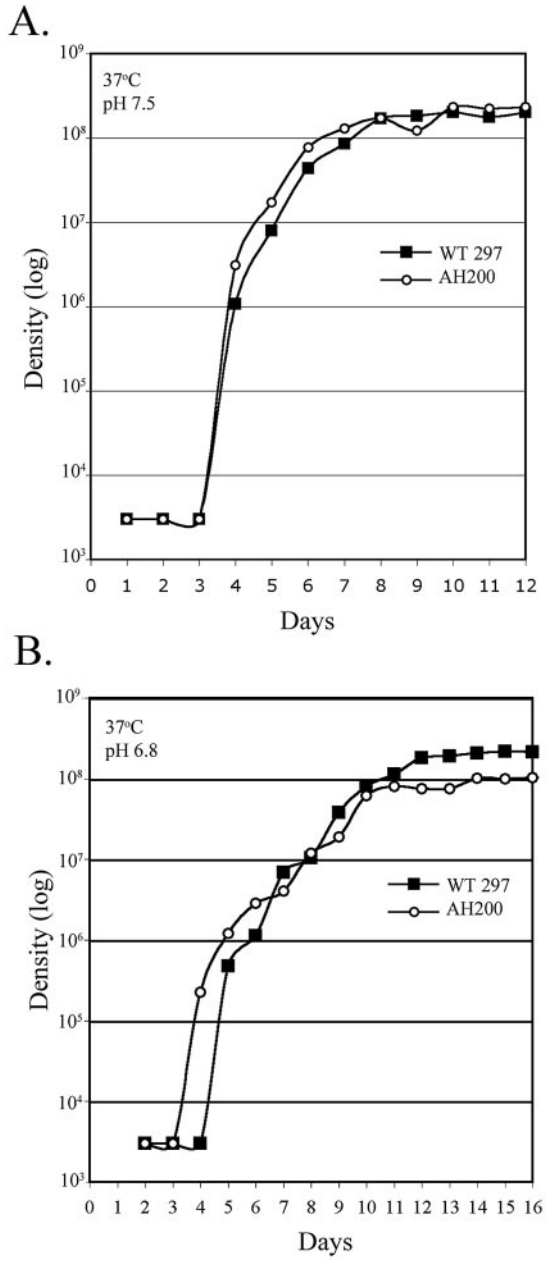

FIG. 3. Growth phenotypes of wild-type and $rpoS_{Bb}$ mutant bacteria at increased temperatures and reduced pH. Growth curve analyses of wild-type (WT; solid squares) and $rpoS_{Bb}$ mutant (AH200; open circles) *B. burgdorferi* strain 297 cultivated in BSK-H medium following temperature shift to 37°C at pH 7.5 (A) and 6.8 (B). The graph depicting the growth of the wild-type isolates is the same as that shown in Fig. 1A.

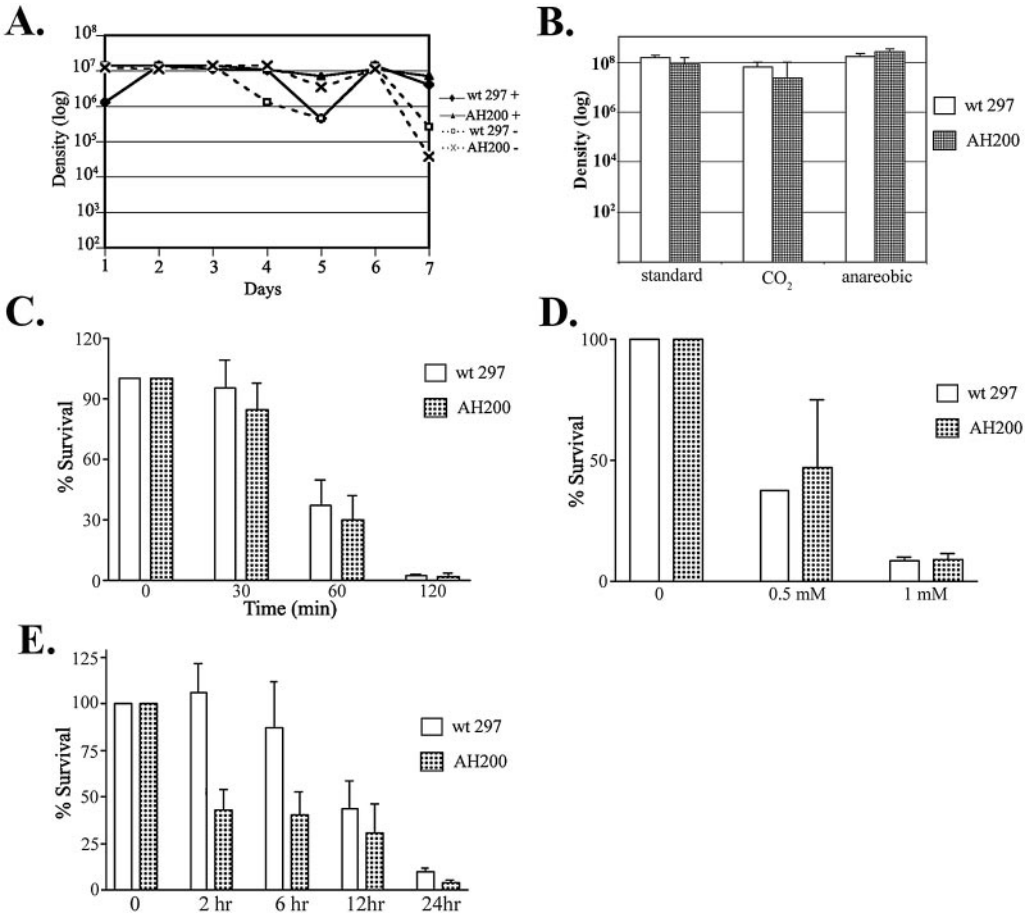

FIG. 4. RpoS is not required for survival following exposure to environmental stress. Survival of *B. burgdorferi* wild-type strain 297 and *rpoS*$_{Bb}$ mutant AH200 following cultivation in RPMI with (+) or without (−) 6% normal rabbit serum (A), under various oxygen tensions (standard microaerophilic, increased oxygen [CO_2], and anaerobic) (B), or following exposure to high osmolarity (1 M NaCl) (C), exogenous peroxide (0.5 or 1 mM H_2O_2) (D), or acidic pH (pH 6.0) (E). Percent survival was calculated based on no-exposure controls for each sample. Percent survival values represent the averages of two independent trials. The log rank and *P* values for the results shown in panel E, determined by the Kaplan-Meier method, were $\chi^2 = 3.809$ and $P = 0.051$. wt, wild type.

expression patterns observed during mammalian host adaptation (2, 14, 41, 63, 70), this animal model is presumably representative of the physiological and nutritional stresses to which spirochetes must adapt in vivo (27, 67). The use of low starting numbers of inocula (10^3 spirochetes/ml) in these studies ensures that spirochetes harvested following cultivation within DMCs have undergone numerous rounds of replication in vivo. Following explantation, both the wild-type and *rpoS*$_{Bb}$ mutants consistently grew to the same approximate density (6×10^6 to 2×10^7 spirochetes per ml) within DMCs (data not shown). It has previously been demonstrated by us and others that expression of OspC and DbpA increases during growth in DMCs (2, 14, 69). Consistent with these earlier studies, qRT-PCR performed on total RNAs isolated from wild-type strain 297 cultivated within DMCs demonstrated that *rpoS*$_{Bb}$ was expressed at higher levels during DMC cultivation than after temperature shift alone (Fig. 1B). Thus, while *rpoS*$_{Bb}$ and RpoS$_{Bb}$-dependent genes were strongly induced during DMC cultivation, the corresponding RpoS-dependent regulon does not appear to be required for the physiological adaptation(s) mediated by this growth environment.

By design, cultivation within DMCs sequesters spirochetes from the external cellular milieu; as a consequence, this methodology does not allow for identification of phenotypes associated with virulence-related functions such as dissemination, cell adhesion, and/or immune evasion. Growth of *rpoS*$_{Bb}$ mutants within DMCs, therefore, does not rule out a requirement for one or more RpoS$_{Bb}$-dependent loci in colonization of various mammalian tissues and/or infectivity. To further examine the role of RpoS$_{Bb}$ in vivo, we performed virulence studies with C3H/HeJ mice with wild-type strain 297 and the *rpoS*$_{Bb}$ mutant AH200. Prior to infection, isolates were tested to ensure that each possessed a full complement of strain 297 plasmids, thereby ruling out the likelihood that any loss of virulence was due to the absence of the virulence-associated plasmids lp25 and lp28-1 (27, 55, 68). Results from these studies are summarized in Table 1. With the combined results from two independent infection studies, we found that the *rpoS*$_{Bb}$ mutant AH200 was avirulent (0 of 9 mutant strain samples were avirulent versus 9 of 9 of the wild type). To confirm that this phenotype was due to loss of *rpoS*$_{Bb}$ and not a secondary mutation, we constructed a complementation plasmid encoding a wild-type copy of *rpoS*$_{Bb}$ under the control of its native promoter contained on pCE320, a cp32-based shuttle vector

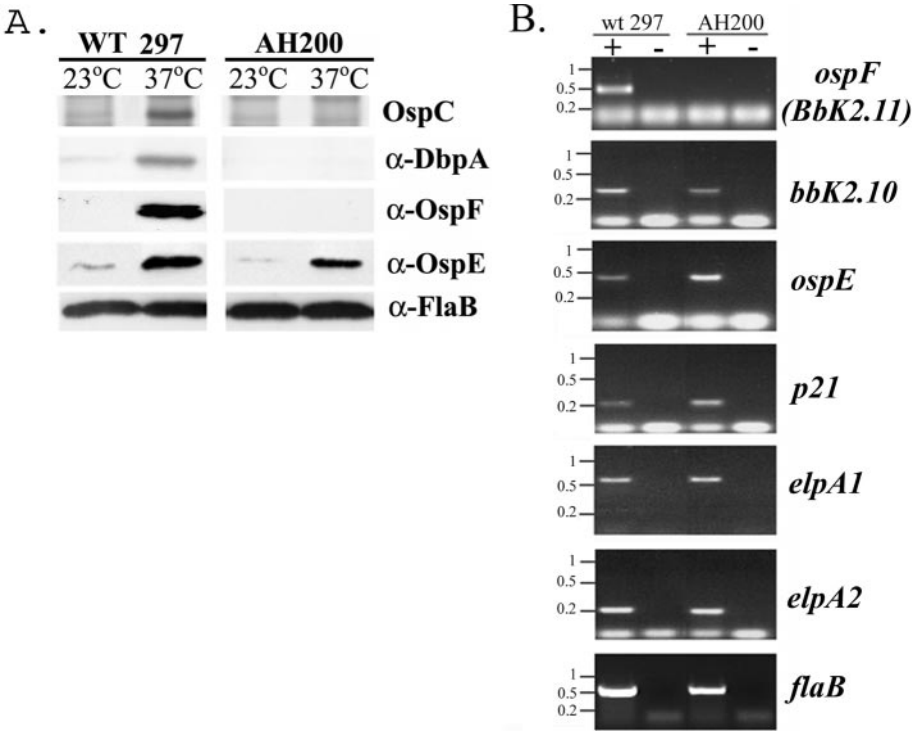

FIG. 5. *ospE-ospF-elp* paralogous loci utilize predominantly RpoS-independent pathways. (A) Analysis of wild-type (WT) and $rpoS_{Bb}$ mutant (AH200) *B. burgdorferi* strain 297 samples cultivated at 23°C and following temperature shift to 37°C in BSK-H medium at pH 7.5 or 6.8. Whole-cell lysates ($\sim 10^7$ lysates per lane) were separated by SDS-PAGE and either silver stained (OspC) or immunoblotted with sera directed against DbpA, OspF, OspE, or FlaB. (B) RT-PCR of wild-type (wt) strain 297 and AH200 following temperature shift to 37°C with primers specific for individual *ospE-ospF-elp* loci and *flaB* with (+) or without (−) reverse transcriptase. DNA controls were also performed with 297 genomic DNA (not shown). Molecular weight markers in kilobases are indicated to the left.

(23). Unfortunately, we were unable to electrotransform AH200 with this or other vectors. To overcome this technical hurdle, we constructed a second strain 297 *rpoS* mutant, CE174, in the wild-type transformable strain 297 clone CE162. Combined data from two independent mouse infection studies using this second $rpoS_{Bb}$ mutant yielded results identical to those obtained with AH200 (0 of 10 samples were avirulent for CE174 versus 10 of 10 samples for CE162), while studies performed using CE467, the CE174-derived $rpoS_{Bb}$ mutant transformed with $rpoS_{Bb}$/pCE320, demonstrated that infectivity could be restored (5 of 5 samples) when $RpoS_{Bb}$ was supplied in *trans* (Table 1). Essentially identical results were obtained with these same isolates with SCID mice (Table 1), a background that should allow for the growth of spirochetes defective in RpoS-dependent loci potentially associated with immune evasion functions. Ear punch biopsies taken from SCID mice infected with either CE162 (8 of 8 mice) or CE467 (7 of 8 mice) were culture positive 2 weeks postinfection, while all mice infected with CE174 were culture negative (0 of 8 mice) (Table 1). To our knowledge, this represents the first report of complementation of an $rpoS_{Bb}$ mutant with a wild-type copy of $rpoS_{Bb}$.

TABLE 1. $RpoS_{Bb}$ is required for virulence in *B. burgdorferi*

Strain	Description	Virulence in:			Reference or source
		C3H/HeJ[a]	SCID[b]	DMC[c]	
297	Uncloned wild type	9/9	ND	Y	77
AH200	297 *rpoS*::*erm*^{r}	0/9	ND	Y	47
CE162	Wild-type 297 clone	10/10	8/8	Y	This work
CE174	162 *rpoS*::*erm*^{r}	0/10	0/8	Y	This work
CE467	CE164 + *rpoS*/pCE320	5/5	7/8	ND	This work

[a] Infectivity determined by immunoblot assay and cultivation of tissue biopsies.
[b] Infectivity determined by cultivation of tissue biopsies. ND, not determined.
[c] Survival of spirochetes within DMCs assessed by enumeration following explantation. ND, not determined.

Analysis of *ospE-ospF-elp* expression in wild-type and $rpoS_{Bb}$ mutant *B. burgdorferi*. Recent studies have demonstrated that a number of borrelial cp32-encoded loci (Bdr and Mlp paralogous gene families) that have previously been shown to exhibit increased expression following temperature shift and during host adaptation (2, 65, 89, 91, 98) are expressed in an $RpoS_{Bb}$-independent manner (70, 93). Here, we extended these studies by examining the $RpoS_{Bb}$ dependence of the cp32-encoded *ospE-ospF-elp* lipoprotein genes (2–4, 15, 40, 41, 41) following temperature shift in vitro. Immunoblot analyses revealed that expression of OspF, but not OspE, was eliminated in AH200 (Fig. 5A); identical results were obtained with CE174 (24). Although the high degree of amino acid similarity makes it difficult to track expression of the more closely related alleles by immunoblotting, we were able to perform RT-PCR with primers specific for each of the remaining *ospE-ospF-elp* genes in wild-type and AH200 isolates following temperature shift. As shown in Fig. 5B, only *ospF* and its nearly identical paralog *bbk2.11* are expressed in an $RpoS_{Bb}$-dependent manner. In

contrast, the third *ospF* allele in strain 297, *bbk2.10*, was expressed in an $RpoS_{Bb}$-independent manner. This result is interesting and also not completely unexpected, given that the mature Bbk2.10 polypeptide is quite distantly related to OspF/Bbk2.11 (3, 15) and therefore potentially serves a separate functional role during infection.

DISCUSSION

During growth in rich medium, *E. coli* RpoS is primarily responsible for directly or indirectly coordinating the expression of at least 100 genes during entry into stationary phase (45, 48, 53). During logarithmic growth, RpoS is also required for survival following exposure to diverse environmental stresses, including acidic pH, UV irradiation, various forms of starvation, high osmolality, and reactive oxygen species (44). Studies by Hübner et al. (47) demonstrating that expression of at least two borrelial lipoprotein-encoding loci (OspC and DbpA) is controlled by the putative borrelial RpoS ortholog in response to increased temperature and decreased pH, combined with the importance of this alternative sigma factor in other bacterial systems, prompted us to undertake a more detailed examination of $RpoS_{Bb}$ to determine its role in mediating differential gene expression and physiological adaptation in *B. burgdorferi*. We began our studies by more closely examining the effect of temperature, a key environmental signal involved in borrelial differential gene expression (2, 4, 8, 13, 14, 25, 41, 47, 61, 65, 69, 79, 86, 89, 91), on $rpoS_{Bb}$ expression. Using qRT-PCR, we demonstrated that $rpoS_{Bb}$ is induced at least sixfold following a temperature shift from 23 to 37°C in vitro beginning early in mid-logarithmic-phase growth. While expression of $rpoS_{Bb}$ was further increased (~3- to 4-fold) in response to reduced pH in combination with increased temperature, pH does not appear to exert its effects independent of temperature, since growth at 23°C at reduced pH does not induce expression of $RpoS_{Bb}$-dependent genes (data not shown). Our findings are discordant, however, with two previously published microarray studies which failed to detect a significant increase in $rpoS_{Bb}$ (61, 69); Ojaimi et al. (61) did observe a 1.6-fold increase in $rpoS_{Bb}$ following a temperature shift to 35°C, but this change did not exceed their statistical threshold. The disparity between these studies is most likely due to an underestimation of induction ratios as a result of the relatively limited dynamic range of microarrays compared to qRT-PCR (20). Our qRT-PCR data were also supported by similar increases in $RpoS_{Bb}$ protein levels. While results from the RT-PCR studies presented herein suggest that reduced pH positively influences $rpoS_{Bb}$ expression in vitro, there is some cause to question whether reduced pH is necessary for host adaptation, since DMC-cultivated organisms express increased $rpoS_{Bb}$ (~38-fold higher in DMCs versus conditions at 23°C) and exhibit an antigenic profile characteristic of mammalian infection (2) within a physiological pH range; the average measured pH of DMC fluid following explantation is ~7.6 (data not shown). Although recent studies suggest that some degree of posttranscriptional control may be mediated by DsrA, a small regulatory RNA (M. C. Lybecker and D. S. Samuels, personal communication), $RpoS_{Bb}$ appears to be unequivocally regulated at the level of transcription.

In many bacterial systems, including *E. coli* (43) and *Pseudomonas aeruginosa* (49), the role of RpoS is twofold. During growth in rich medium, RpoS directly (or indirectly) controls the expression of loci involved in growth adaptation during stationary phase (45). This alternate sigma factor also controls the expression of loci involved in survival following exposure to environmental stresses during logarithmic-phase growth (10, 11, 28, 33, 43, 49, 80, 95). The studies described in this report, however, provide compelling evidence to suggest that RpoS in *B. burgdorferi* is functionally distinct on both accounts from its orthologs in other bacteria, most notably that of *E. coli*. First, the substantial levels of $rpoS_{Bb}$ and RpoS-dependent gene expression observed in exponentially growing borrelial cultures clearly distinguish *B. burgdorferi* from *E. coli*. Although a modest increase was observed upon entry of spirochetes into stationary phase, more than ample levels of $RpoS_{Bb}$ were present during mid-logarithmic growth to achieve near maximal expression levels of at least two $RpoS_{Bb}$-dependent loci, *ospC* and *dbpA*. These expression kinetics are fully consistent with studies demonstrating that OspC is expressed early during arthropod feeding (36, 62, 72) and further point to an early, perhaps pivotal, role for RpoS in host adaptation, a process that presumably initiates with tick feeding. The second notable difference between $RpoS_{Bb}$ and its orthologs in many other bacteria is exemplified by in vitro environmental stress assays demonstrating that loss of $RpoS_{Bb}$ did not significantly alter the ability of spirochetes to survive exposure to a wide range of stresses, acidic pH adaptation notwithstanding. These findings are not entirely consistent with an earlier report by Elias et al. (26) which found that a B31 mutant (A74 *rpoS*::*gyrB*^{r}) was more sensitive to high-salt conditions than its wild-type parent when cultures were examined late in stationary phase. This discrepancy may be due to strain differences rather than RpoS itself; the parental isolate used to make the B31 mutant (B31-A74) is an avirulent high-passage isolate that may contain secondary mutations in addition to $rpoS_{Bb}$. Results from our nutrient deprivation assay, on the other hand, are consistent with studies by Murgia et al. (59) demonstrating that an $rpoS_{Bb}$ mutant *B. burgdorferi* formed nonmotile cysts under unfavorable growth conditions (i.e., serum starvation and exposure to antibiotics) at a rate equal to that of wild-type isolates. The ability of *B. burgdorferi* $rpoS_{Bb}$ mutants to grow to wild-type levels within DMCs provides further compelling evidence that $RpoS_{Bb}$ is not central to the physiological (i.e., nutritional) adaptation associated with growth within the mammalian host.

At first glance, our findings were surprising in light of the role of RpoS in stress adaptation in other bacteria, most notably *E. coli*, *Salmonella* (33), and *P. aeruginosa* (49), but these organisms have evolved to adapt to truly diverse growth environments. *B. burgdorferi*, an organism with only limited biosynthetic capabilities (34), does not exist in nature as a free-living organism and therefore is confined to a narrow host range with only limited exposure to environmental stresses. Accordingly, BLAST searches of the borrelial genome yielded, at best, putative orthologs for only 22 of the 118 loci believed to be controlled by RpoS in *E. coli* and *Salmonella* (45, 48, 53). Our finding that loss of $RpoS_{Bb}$ resulted in increased sensitivity to acidic pH, on the other hand, is consistent with a more typical role for RpoS in stress adaptation (33), but this phenotype was only observed in vitro at a pH well below that to which spirochetes would be presumably exposed to during the enzootic

cycle. Indeed, the acid tolerance of *B. burgdorferi* appears to be within a very narrow range with little to no survival observed after exposure to pH values below 6.0 (data not shown), in contrast to the ability of other organisms, such as *E. coli*, to tolerate a much wider range of pH levels (33). Taken together, results from our qRT-PCR and acid exposure studies are suggestive of a role for $RpoS_{Bb}$ in differential gene expression in response to acidic pH and/or during entry into stationary phase; a better understanding of the $RpoS_{Bb}$-dependent regulon will be necessary to delineate those loci responsive to reduced pH versus those sensitive to growth phase-mediated $RpoS_{Bb}$-dependent regulation.

While $RpoS_{Bb}$ appears to serve only a limited role in environmental stress adaptation, the failure of $rpoS_{Bb}$ mutants to infect mice implies that this transcriptional activator controls the expression of one or more essential virulence determinants. Although the ability of $rpoS_{Bb}$ mutants to grow within DMC but not infect mice might seem contradictory, we believe that this phenotypic distinction actually provides considerable insight into the role of the $RpoS_{Bb}$-dependent regulon in vivo by helping to distinguish between the physiological and nonphysiological demands placed on spirochetes during growth within the mammalian host. The failure of $rpoS_{Bb}$ mutants to establish infection in both C3H/HeJ and SCID mice strongly suggests that the function(s) of $RpoS_{Bb}$-dependent virulence-associated loci is not involved exclusively in immunoevasion and/or antigenic variation and instead suggests that $RpoS_{Bb}$-dependent loci are involved in other virulence-related functions such as adhesion, dissemination, and/or survival within mammalian tissues. Although there is still some debate as to the precise role of OspC during the enzootic cycle (36, 62), recent studies by Grimm et al. (36) demonstrated that insertional inactivation of *ospC* results in a loss of virulence. We, therefore, cannot rule out the possibility that the avirulence of $rpoS_{Bb}$ mutants is due to the loss of *ospC* alone. One way to test this hypothesis would be to compare the infectivity of $RpoS_{Bb}$- and OspC-deficient backgrounds supplied with *ospC* in *trans*; the requirement for use of a heterologous $RpoS_{Bb}$-independent promoter element (e.g., P_{flaB}) to drive expression of *ospC* in an $rpoS_{Bb}$ mutant, however, might in fact confound the interpretation of results from such studies by producing constitutively high levels of OspC and/or inappropriate expression kinetics. The use of a temperature-inducible $RpoS_{Bb}$-independent promoter such as P_{ospE} with an expression pattern that more closely mirrors that of P_{ospC} (24) should help to minimize the likelihood that one of these scenarios influences the results of *ospC* complementation studies. More importantly, such studies will enable us to examine whether any of the other known (e.g., DbpA-, DbpB-, or OspF-dependent) or as-yet-unidentified $RpoS_{Bb}$-dependent loci contribute to either spirochetal growth in vivo or immune evasion. A more detailed characterization of the $RpoS_{Bb}$-dependent regulon expressed in vivo combined with targeted mutagenesis should help to address this question. The requirement for $RpoS_{Bb}$-dependent loci during infection is consistent with a well-established role for RpoS in virulence of a number of pathogens, including *Salmonella* (28, 50), *Legionella pneumophila* (9, 39), and *P. aeruginosa* (80); in most of these organisms, however, RpoS also contributes to environmental stress adaptation. While our finding that $RpoS_{Bb}$ was required for spirochetal virulence but not environmental stress adaptation was somewhat unexpected, it is not without precedent. In *L. pneumophila*, RpoS is required for survival within both macrophages (9) and protozoan hosts (39) but does not appear to be required for growth phase-dependent responses to low pH and peroxide (39).

We also extended our analysis of the role of $RpoS_{Bb}$ in differential gene expression by examining the $RpoS_{Bb}$ dependence of the strain 297 *ospE-ospF-elp* loci whose expression parallels that of *ospC* and *dbpA*. Previous studies have demonstrated that expression of these lipoprotein loci is temperature inducible (2, 4, 41, 61, 78, 79) and further enhanced by mammalian host signals (2, 4, 41). The high degree of sequence identity within the respective *ospE-ospF-elp* promoter regions, combined with their similar expression patterns, suggests that these loci should be regulated by a common mechanism. The RT-PCR studies presented here, however, demonstrated that these loci are, in fact, regulated by both $RpoS_{Bb}$-dependent and $RpoS_{Bb}$-independent pathways. These results have also been confirmed with a *gfp* reporter system in both *B. burgdorferi* and *E. coli* (24). Our observation that the majority of *ospE-ospF-elp* loci are expressed in an $RpoS_{Bb}$-independent manner is consistent with reports examining the expression of two other borrelial paralogous gene families. Yang et al. (93) demonstrated that although the expression of class I and II Mlp family members is influenced by temperature, pH, and cell density through regulatory pathways involving RpoN/RpoS, no members exhibited the strict dependence on $RpoS_{Bb}$ observed with OspC, DbpA, and OspF/Bbk2.11. An analysis of the Bdr family similarly revealed that while expression of several members of this large paralogous family was increased in response to many of the same signals as $RpoS_{Bb}$-dependent loci, including cultivation within DMCs, no detectable differences in their expression were observed with an $rpoS_{Bb}$ mutant (70).

In summary, these studies have enabled us to refine our understanding of the role of the alternate sigma factor RpoS in *B. burgdorferi*. While expression of $rpoS_{Bb}$ and $RpoS_{Bb}$-dependent genes is responsive to multiple environmental stimuli, $RpoS_{Bb}$ itself does not appear to function as a master regulator of physiological and/or environmental stress adaptation in vitro or in vivo. $RpoS_{Bb}$ does, however, control the expression of one or more essential virulence determinants that, together with the $RpoS_{Bb}$-independent differentially expressed loci, encompass the spirochetal regulon responsible for host adaptation. Analysis of differential gene expression within DMC-cultivated spirochetes and *B. burgdorferi*-infected ticks and tissues by genome-wide methodologies, such as microarrays, should provide a meaningful framework for in vitro experiments aimed at defining the degree to which these two regulatory pathways are influenced by environmental signals and, ultimately, provide rational targets for future mutagenesis studies.

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