Altered Stationary-Phase Response in a *Borrelia burgdorferi* *rpoS* Mutant

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The homolog of the chromosomally encoded stationary-phase sigma factor *RpoS* in *Borrelia burgdorferi* was inactivated using *gyrBr* as a selectable marker. Two-dimensional nonequilibrium pH gradient electrophoresis of stationary-phase cell lysates identified at least 11 differences between the protein profiles of the *rpoS* mutant and wild-type organisms. Wild-type *B. burgdorferi* had a growth phase-dependent resistance to 1 N NaCl, similar to the stationary-phase response reported for other bacteria. The *B. burgdorferi rpoS* mutant strain was less resistant to osmotic stress in stationary phase than the isogenic *rpoS* wild-type organism. The results indicate that the *B. burgdorferi rpoS* homolog influences protein composition and participates in stationary-phase-dependent osmotic resistance. This *rpoS* mutant will be useful for studying regulation of gene expression in response to changing environmental conditions.

Lyme disease is the most common arthropod-borne disorder in the United States, with a variable spectrum of clinical manifestations ranging from localized infection to systemic disease (1, 11, 54, 55). *Borrelia burgdorferi*, the spirochetal agent of Lyme disease, is normally maintained in an enzootic cycle between wild mammals and *Ixodes* ticks (30). In ticks and in mammals, the bacteria are present at low numbers throughout most of the infectious cycle. However, periods of rapid growth occur in ticks after blood engorgement (13, 43). Although little is known about the growth kinetics of *B. burgdorferi* within the mammalian host, spirochetes appear to be present in considerably lower densities in chronically infected mice than in acutely infected animals (4). *B. burgdorferi*, similar to other bacterial pathogens, undoubtedly has a repertoire of adaptive molecular responses to environmental signals to assist survival within and successful transmission between its hosts (16, 35, 36). For example, in vivo, differential synthesis of membrane proteins occurs during tick transmission (3, 52) and mammalian infection (19, 41). Temperature, pH, and growth phase also have been shown to modify the protein profile of cultivated *B. burgdorferi* (7, 8, 12, 23, 37, 44, 52, 53, 56; J. G. Frye, B. K. Kremer, T. R. Hoover, and F. C. Gherardini, Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999, abstr. D/B-259, p. 259).

Despite this evidence for the presence of adaptive responses in *B. burgdorferi*, the molecular mechanisms responsible for regulation of gene expression in response to environmental changes are unknown. This lack is due to the difficulty in studying the physiology of a fastidious organism and the limited availability of genetic tools to manipulate *B. burgdorferi*.

An important mechanism involved in regulation of bacterial gene expression in response to environmental signals is the use of alternative sigma factors to alter RNA polymerase specificity (59). Three genes encoding sigma factor homologs are present in the genome of *B. burgdorferi*: *rpoS* (σ\(^5\)), *ntrA* (σ\(^2\)), and *rpoD* (σ\(^0\)). In *Escherichia coli*, RpoS controls a regulon of more than 30 genes positively or negatively regulated in response to starvation and transition to stationary phase (15, 31). In the natural environment, *B. burgdorferi* and other bacteria often encounter nutrient limitations, resulting in periods of negligible or absent growth. *E. coli* and other bacteria respond to nutrient starvation by entering a metabolic state referred to as stationary phase (25, 26, 42, 58), allowing them to survive environmental stresses such as oxidative stress, heat, high salt, and near-UV radiation. The stationary-phase response depends in part on the expression of the sigma factor *rpoS* (22, 31). Several bacterial pathogens, including *Salmonella* (14, 29), *Yersinia enterocolitica* (24), * Vibrio cholerae* (60), and *Legionella pneumophila* (20), have RpoS homologs with various roles. For example, in *Salmonella enterica* serovar Typhimurium, RpoS regulates chromosomal and plasmid-encoded virulence genes, and the 50% lethal dose in mice is 1,000-fold higher for a *Salmonella* serovar Typhimurium *rpoS* mutant than for the wild-type strain (14, 29).

Here, we begin a study to elucidate the role of RpoS in *B. burgdorferi* biology. As a first step, we inactivated the *rpoS* locus by allelic exchange with *gyrBr*, a mutated form of the B subunit of DNA gyrase, as a selectable marker. Although this technique has been used previously to inactivate several *B. burgdorferi* genes located on a 26-kb circular plasmid (cp26) (5, 57), *rpoS* is the first chromosomal gene to be inactivated in *B. burgdorferi*. Stationary-phase cells of the isogenic *B. burgdorferi* *rpoS* mutant have an altered protein composition compared with the *rpoS* wild-type organism and are more sensitive to

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>rpoS</em> locus</th>
<th><em>gyrBr</em> locus</th>
<th>Coumermycin A(\text{a}) phenotype(^*)</th>
<th>Reference</th>
</tr>
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<tr>
<td>B31-A</td>
<td>wt(^a)</td>
<td>wt</td>
<td>Sensitive</td>
<td>45</td>
</tr>
<tr>
<td>B31-4A</td>
<td>wt</td>
<td>wt</td>
<td>Sensitive</td>
<td>10</td>
</tr>
<tr>
<td>B31-NGR</td>
<td>wt</td>
<td><em>gyrBr</em></td>
<td>Resistant</td>
<td>45</td>
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<tr>
<td>B31-A59</td>
<td>wt</td>
<td><em>gyrBr</em></td>
<td>Resistant</td>
<td>This study</td>
</tr>
<tr>
<td>B31-A74</td>
<td><em>rpoS</em>::<em>gyrBr</em></td>
<td>wt</td>
<td>Resistant</td>
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<td>wt</td>
<td>Resistant</td>
<td>This study</td>
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</table>

\(^*\) Coumermycin resistance was defined as the ability to grow in the presence of \(\geq 0.5 \mu g\) of coumermycin A\(\text{a}\) per ml in liquid or solid BSK.

\(^\text{a}\) wt, wild type.
rpoS in pOK12, which has ends compatible with EcoRI. Infectious clone derived from B31. Coumermycin A1-resistant clones B31-A59 attenuated, noninfectious B31. B31 clone 4A (B31-4A) is a low-passage (P3) (45, 46). B31 (ATCC 35210) was originally isolated from a tick collected on Mo.) supplemented with 6% rabbit serum (Sigma) (2) or in solid BSK medium.

Therefore, the rpoS gene cannot be introduced into agents used to treat Lyme disease. Hence, this drug resistance phenotype cannot

Construction of the rpoS mutant. Recombinant plasmid pAE30 was constructed to inactivate the B. burgdorferi rpoS gene. A 4.2-kb fragment encompassing the rpoS locus of B31-4A was amplified from chromosomal DNA by PCR (38, 49) with primers 2 and 9 (Table 2 and Fig. 1A). The PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.), which contains an ampicillin resistance gene. Ampicillin is a beta-lactam antibiotic and is related to antimicrobial agents used to treat Lyme disease. Hence, this drug resistance phenotype cannot be introduced into B. burgdorferi. Therefore, the rpoS-containing fragment was recloned into pGK12, a 2.1-kb low-copy-number plasmid that contains a kanamycin resistance gene. The gfrB gene and its putative promoter were amplified by PCR from B31-NGR chromosomal DNA (Table 1) with primers 11 and 15 (Fig. 1B and Table 2) and cloned into the single BstI site of pGK12, which has ends compatible with EcoRI. The resulting plasmid (pAE30) was confirmed to contain the rpoS:gfrB construct by DNA sequencing with a 373A instrument (Applied Biosystems, Foster City, Calif.) (Fig. 1B). Plasmid DNAs were purified from E. coli with Qiagen purification kits (Qiagen, Chatsworth, Calif.). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, Mass.). The sequence of the B. burgdorferi rpoS locus was obtained from the web page of the Institute for Genomic Research at http://www.tigr.org, where rpoS has accession number BB771.

Transformation of B. burgdorferi. Transformation of B31-A with pAE30 by electroporation was performed as previously described (47).

TABLE 2. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Designation</th>
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<td>ptxK (BB768)</td>
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<tr>
<td>2</td>
<td>rpo8fa-F2</td>
<td>GTTGTGATGTTGAGCTCC</td>
<td>gcr (BB769)</td>
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<td>3</td>
<td>BB770-F1</td>
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<tr>
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<td>GACAGAACAATAACAGAGGCC</td>
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</tr>
<tr>
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<td>rpo8fa-B1</td>
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<td>BB770</td>
</tr>
<tr>
<td>11</td>
<td>U178+BgII</td>
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<td>BB770</td>
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<tr>
<td>12</td>
<td>246F</td>
<td>CTTCTTATGAAATCCGTTAGG</td>
<td>gfrB</td>
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<tr>
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<td>1580R</td>
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<td>gfrB</td>
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<td>14</td>
<td>1069F</td>
<td>CTTTACAGATATAAAGGGCTTGGG</td>
<td>gfrB</td>
</tr>
<tr>
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<td>1005R+BeII</td>
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<td>17</td>
<td>F17</td>
<td>GCATTCTCATTTTTAGACAGGATGAC</td>
<td>flaB</td>
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* The numbers refer to the relative positions and orientations of primers in the rpoS flanking region and gfrB given in Fig. 1.

osmotic stress. These results indicate that RpoS participates in the stationary-phase-related adaptive response.

MATERIALS AND METHODS

Bacterial strains. The B. burgdorferi strains used in this study are listed in Table 1. Bacteria were grown at 35°C in liquid BSK-H medium (Sigma, St. Louis, Mo.) supplemented with 6% rabbit serum (Sigma) (2) or in solid BSK medium (45, 46). B31 (ATCC 35210) was originally isolated from a tick collected on Shelter Island, N.Y. (6). B31 clone A (B31-A) is a clone derived from culture-attenuated, noninfectious B31. B31 clone 4A (B31-4A) is a low-passage (P5) infectious clone derived from B31. Coumermycin A1-resistant clones B31-A59 (A59), B31-A74 (A74), and B31-A29 (A29) were derived from B31-A by transformation with the recombinant plasmid pAE30 (described below). This plasmid contains a copy of gfrB, the mutated B subunit of DNA gyrase from B31-NGR (45), conferring resistance to coumermycin A1, inserted in a 4.2-kb B. burgdorferi chromosomal DNA fragment.

Spirochetes were counted by dark-field microscopy with a Petroff-Hausser counting chamber. The number of spirochetes was determined once or twice daily. Stationary phase was defined as the period of reduced bacterial growth after exponential growth. The onset of stationary phase usually corresponds to a cell density of approximately 10^8 spirochetes/ml.

Protein analysis. Total borrelial RNA from stationary-phase cultures was grown in liquid BSK-H to stationary phase by PCR with primers 11 and 13 (Table 2 and Fig. 1B) and digested with SspI. The gfrB from B31-NGR contains one SspI site, which is due to a dimethylating change in codon 133 (45; D. S. Samuels, unpublished data) and is not present in gfrB of spontaneous mutants. The PCR products from 38 of the 42 colonies were cut by SspI, indicating that approximately 90% of the Cou+ colonies were transformed and 10% were spontaneous resistance mutants.

Transfer blot analysis. B. burgdorferi total genomic DNA was isolated, digested with restriction endonucleases, and separated by pulsed-field gel electrophoresis in a 0.8% (wt/vol) agarose gel under field inversion conditions (47) with a PPI-200 programmable power inverter (MU Research, Watertown, Mass.). Bidirectional transfer to Biotrans nylon membranes (ICN, Irvine, Calif.), DNA hybridization with radiolabeled probes, and visualization by autoradiography were performed as described previously (47).

Screening of B. burgdorferi transformants by PCR. Coumermycin-resistant colonies were screened for allicic exchange at the rpoS locus by PCR. Individual colonies picked with sterile toothpicks were added to tubes containing a 20-μl PCR mix, and oligonucleotides 5 and 8 (Table 2 and Fig. 1A) were used to amplify a fragment that spanned the rpoS gene. Reaction conditions were 94°C for 1 min and then 30 cycles with 94°C for 30 s, 55°C for 45 s, and 68°C for 3 min in a GeneAmp 9600 DNA Thermal Cycler (Perkin-Elmer, Norwalk, Conn.) with 96-well PCR plates. E. coli colonies that contained plasmid pAE30 or a pOK12 derivative with a 6-kb rpoS-spanning fragment were used as positive controls. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. To obtain clonal mutants, individual colonies were aspirated with a sterile Pasteur pipette and grown in 5 ml of liquid BSK containing coumermycin A1 (0.5 μg/ml). Appropriate dilutions of cultures were replated in solid BSK-H with coumermycin A1 (0.5 μg/ml), and individual colonies were picked, grown up, and subjected to PCR and Southern blot analysis to confirm insertion of gfrB within rpoS (see below).

Spf digestion of the PCR-amplified gfrB locus from potential transformants. To determine the proportion of Cou+ colonies that were “true” transformants, i.e., that had a copy of the B31-NGR gfrB in their chromosome, the gfrB locus in 42 randomly chosen Cou colonies was amplified by PCR with primers 11 and 13 (Table 2 and Fig. 1B) and digested with SpfI. The gfrB from B31-NGR contains one SpfI site, which is due to a dimethylating change in codon 133 (45; D. S. Samuels, unpublished data) and is not present in gfrB of spontaneous mutants. The PCR products from 38 of the 42 colonies were cut by SpfI, indicating that approximately 90% of the Cou+ colonies were transformed and 10% were spontaneous resistance mutants.

Protein analysis. Total borrelial RNA from stationary-phase cultures was grown in liquid BSK-H was extracted with the ULTRASPECII RNA Isolation System (Biotec, Houston, Tex.) according to the manufacturer’s instructions. After denaturation with glyoxal and dimethyl sulfoxide, 10 μg of total RNA was electrophoresed in a 1% (wt/vol) agarose gel in 10 mM sodium phosphate buffer, pH 7.0 (50). RNA transfer to nylon membranes (MSI, Westboro, Mass.), hybridization with radiolabeled probes, and visualization by autoradiography were performed as described previously (47).
FIG. 1. Schematic diagram of the *B. burgdorferi* chromosomal rpoS gene and plasmid pAE30. (A) Location of rpoS in a 6-kb chromosomal region. (B) Construct pAE30 used for transformation of *B. burgdorferi*. The *gyrB* gene is inserted in the single *BbsI* site of the rpoS locus. Solid arrows indicate direction of gene transcription; open arrows represent primers used in this study, and their numbers refer to the primers listed in Table 2. R, *Eco*RI; B, *BbsI*; Ba, *BamHI*; X, *XbaI*. Thick lines represent the pOK12 vector, and thin lines represent the *B. burgdorferi* insert.
RESULTS

Construction of a B. burgdorferi rpoS mutant. The plasmid pAE30 used for disruption of the B. burgdorferi rpoS gene contains a 4-kb fragment of borrelial chromosomal DNA with gyrB inserted into the rpoS locus in the reverse orientation (Fig. 1B). This disrupts rpoS in the first quarter of the gene and leaves approximately 2 kb of flanking sequence on either side of gyrB to mediate allelic exchange. Clone B31-A was transformed with pAE30 DNA by electroporation. Seven mutants with an rpoS::gyrB genotype were identified by PCR screening of 576 Cour colonies with primers that flank the rpoS::gyrB insertion within rpoS (5 and 8, Table 2 and Fig. 2). An estimated 90% of the Cour colonies were transformants, as determined by SspI digestion of the gyrB gene (see Materials and Methods), whereas 10% resulted from spontaneous resistance mutations. Hence, an estimated 1.4% of the transformants were rpoS mutants.

The mutant colonies were picked from solid medium for growth in liquid BSK-H with coumermycin A₁ (0.5 μg/ml) and replated on solid BSK to ensure clonality. The homozygous rpoS::gyrB genotype of individual clones was confirmed by PCR with primers 5 and 8. The additional wild-type PCR product present in the initial screen (Fig. 2) was not present in cloned mutants. Two rpoS mutants (A74 and A29) and A59, a Cour¹ transformant with a wild-type rpoS locus in which allelic exchange had occurred at the gyrB locus, were chosen for subsequent analyses.

FIG. 2. Agarose gel of Cour¹ transformants screened by PCR for insertion of gyrB in the chromosomal rpoS gene. The rpoS gene from individual B. burgdorferi colonies was amplified by PCR with primers 5 and 8 (Table 2 and Fig. 1A). The asterisk indicates the position of the mutant PCR fragment containing gyrB. The additional wild-type PCR product was not present in cloned mutants (Fig. 3). Controls: 1 and 2, E. coli colonies containing plasmid pAE30 and a pOK12 derivative with an rpoS-flanking fragment, respectively; 3, blank spot on transformation plate; 4, reagent blank. The positions of DNA size standards are indicated on the left.

Confimation of the structure of the mutant rpoS gene. To confirm that the rpoS mutants were the result of allelic exchange at the chromosomal rpoS locus, we amplified chromosomal sequences flanking the cloned 4-kb fragment from pAE30 with primers 1 and 10 (Table 2 and Fig. 1A), in combination with internal gyrB primers 12 and 14 (Table 2 and Fig. 1B). The PCR results were only compatible with allelic exchange occurring at the rpoS locus (Fig. 3).

Southern blot analysis provided further evidence for the presence of gyrB within the rpoS locus (Fig. 4). Hybridization with a probe specific for rpoS resulted in restriction patterns consistent with the presence of a single rpoS gene and the integration of gyrB into the rpoS locus (Fig. 4A and C). Similarly, a probe specific for gyrB identified additional bands in...
FIG. 4. Southern blot analysis of Cour transformants. Total borrelial DNA of the Cour rpoS wild-type A59 and the rpoS mutant A74 was digested with restriction endonucleases BglII, XbaI, and BamHI. (A) Hybridization with an rpoS probe (generated with primers 6 and 7 [Table 2 and Fig. 1A]). (B) Hybridization with a probe specific for gyrB (generated with primers 11 and 13 [Table 2 and Fig. 1B]). A59, Cour rpoS wild-type strain; A74, rpoS::gyrBr. DNA source, restriction enzyme, and probe are indicated above the lanes. DNA size standards are indicated on the left. (C) Relevant BglII, XbaI, and BamHI restriction sites at the chromosomal loci for gyrB (upper line) and rpoS (lower line). The rpoS gene of rpoS mutant A74 was disrupted by the insertion of gyrBr into the BbsI site. A size bar (1.1 kb) is indicated. Hatch marks indicate discontinuity to distal restriction sites.
A specific rpoS transcript of approximately 1 kb was identified in B31-A and B31-4A, a size that matches the predicted length of a transcript encompassing only the rpoS gene (Fig. 5A). In contrast, no detectable rpoS transcript was observed in the rpoS mutant A74 (Fig. 5A). No distinct transcript for BB770 was identified in B31-A, B31-4A, and A74 with a probe specific for this gene (data not shown). The rpoS blots were reprobed with an internal flaB probe, and the results confirmed that equivalent amounts of RNA were analyzed for all three strains (Fig. 5B).

**Growth phase-dependent resistance of wild-type B. burgdorferi to osmotic stress.** RpoS participates in stationary-phase-mediated resistance to environmental stresses in many bacteria (17, 22, 34). Next, we tested whether growth phase affects the ability of wild-type B. burgdorferi to survive treatment with a high salt concentration. A representative experiment in which resistance to high salt was determined for clone B31-A with a wild-type rpoS allele is shown in Fig. 6. With an initial inoculum of 10^5 bacteria/ml, stationary phase was reached after 4 days and the number of viable bacteria remained constant through day 7. Log-phase and stationary-phase organisms were exposed to 1 N NaCl for 10, 40, and 100 min, and the number of viable spirochetes was determined. Greater than 90% of the log-phase bacteria were dead after the 10-min exposure, and no viable bacteria were detected by microscopic examination after 100 min (Fig. 6B). Between days 4 and 7, greater than 90% of the bacteria survived 1 N NaCl for 10 min, but only 30% were viable after 100 min (Fig. 6B). However, on day 7 the majority of spirochetes remained viable after 100 min of exposure to 1 N NaCl (Fig. 6B). The distinct patterns of osmotic resistance in log phase and early and late stationary phase were confirmed by repetition of the experiment five times. In all experiments less than 0.2% (0.005 to 0.14%) of log-phase spirochetes survived 70 to 100 min of exposure to 1 N NaCl. Survival of bacteria in late stationary phase varied between 74 and 105% after 10 to 100 min in 1 N NaCl.

To determine the ability of the LIVE/DEAD BacLight bacterial viability kit to accurately reflect cell viability, we compared data from this assay with the results of a growth endpoint determination obtained with a microdilution assay (Fig. 7). In the microdilution assay, the growth endpoint of B31-A log-phase bacteria was determined at a dilution corresponding to 1.3 spirochetes (Fig. 7B). After 40 min of exposure to 1 N NaCl, the growth endpoint occurred at a dilution corresponding to 87 spirochetes in the untreated control culture (Fig. 7B). Thus, exposure of a log-phase culture to 1 N NaCl for 40 min resulted in greater than 90% reduction in the number of viable organisms, as assessed by limiting dilution. This value is comparable to the results obtained with the LIVE/DEAD BacLight bacterial viability kit (Fig. 6 and 8). These results also demonstrate a good correlation between the LIVE/DEAD BacLight bacterial viability kit and the growth endpoint determination by limiting dilution with respect to absolute numbers of viable bacteria.

**Survival of the isogenic rpoS mutant under high-salt conditions.** Our results indicate that osmotic resistance in wild-type B31-A is growth phase dependent and increases during stationary phase. To assess the role of RpoS in resistance to osmotic stress, we compared the survival of B31-A and the rpoS mutant A74 after addition of 1 N NaCl to the medium. Wild-type and mutant bacteria in log phase died shortly after NaCl was added (Fig. 8, day 3). However, in early stationary phase, the rpoS mutant was more susceptible to 1 N NaCl than wild-type B31-A (Fig. 8, day 5). Although both the wild-type and mutant organisms were more resistant to prolonged osmotic shock in late stationary phase, survival was decreased by more than 50% in the rpoS mutant relative to B31-A (Fig. 8, day 7). These results indicate that RpoS participates in growth phase-dependent osmotic resistance in B. burgdorferi.

Due to the limited set of genetic methods for B. burgdorferi, we were not able to use genetic complementation to confirm that the observed phenotype of A74 was due to inactivation of rpoS. However, we tested another rpoS mutant (A29, Table 1) for osmotic resistance and obtained comparable results (data not shown). The Coulter rpoS wild-type A59 (Table 1) showed a growth phase-dependent survival in high-salt medium similar to that of B31-A (data not shown). Therefore, it is unlikely that the presence of the altered B subunit of the DNA gyrase in rpoS mutants A74 and A29 accounted for their increased susceptibility to osmotic stress.

**Protein analysis of the rpoS wild-type and mutant strains.** 2D-NEPHGE (9, 39) was used to assess differences in protein
composition between rpoS mutant A74 and isogenic rpoS wild-type A59. Representative silver-stained 2D gels containing protein lysates from 10^8 spirochetes grown to stationary phase are shown in Fig. 9. Two independent cultures of each clone were analyzed, and 11 protein spots with three- to eightfold differences in abundance, as determined by densitometry, were repeatedly detected (Fig. 9). The lysates from rpoS wild-type strain A59 had five protein spots that were either absent or markedly decreased in the lysates from rpoS mutant strain A74 (Fig. 9, spot numbers 7, 8, 9, 10, and 11). In contrast, six protein spots were present in the lysates from rpoS mutant A74 that were either absent from or markedly decreased in lysates from rpoS wild-type A59 (Fig. 9, spot numbers 1, 2, 3, 4, 5, and 6). The levels of OspA, OspD (39), and flagellin (FlaB) were not significantly different in the isogenic strains (Fig. 9).

**DISCUSSION**

*Borrelia burgdorferi* survives within and is transmitted between two very different host environments, the tick vector and the mammalian host. In many bacteria, alternative sigma factors regulate gene expression in response to environmental conditions. The genome of *B. burgdorferi* contains two genes encoding homologs of alternative sigma factors, rpoS and ntrA, but nothing is known about their function. Here we describe the site-directed inactivation of rpoS in *B. burgdorferi* and the characterization of the rpoS mutant.

By using 2D gel electrophoresis of stationary-phase spirochetes, we identified 11 proteins that differed at least threefold in abundance in the rpoS mutant compared with the rpoS wild-type strain. Interestingly, 6 of the 11 proteins were made in higher amounts by the rpoS mutant than by the isogenic wild-type organism. In *E. coli*, RpoS mainly acts as a positive regulator of a group of genes expressed in stationary-phase cells (21, 22), and only a small number of genes have been reported to be negatively regulated (15). In contrast, our results suggest that the borrelial RpoS participates in both up- and downregulation of gene expression. The natural environment and life cycle of *B. burgdorferi* differ greatly from those of members of the *Enterobacteria*, for which the role of RpoS has been studied most extensively (21, 22, 28, 31). Therefore, we anticipate that many genes regulated by RpoS in *B. burgdorferi* will have functions and expression patterns distinct from those of *E. coli* and related enteric organisms. Synthesis of most proteins, including OspA, OspD (39), and flagellin (FlaB), was not significantly altered in the rpoS mutant relative to the rpoS wild type, suggesting that either σ^70 or σ^54 is responsible for transcription of the respective genes.

The designation of BB771 as an RpoS homolog is based on sequence similarity with RpoS of other bacteria. The most closely related RpoS homolog is found in *Pseudomonas aeruginosa* (34% identity and 58% similarity using the BLASTP program, National Center for Biotechnology Information) (18). Preliminary results indicate that the *B. burgdorferi* rpoS gene partially complements a *Shigella flexneri* rpoS mutant in an acid resistance assay (data not shown). Similar to *E. coli*, *B. burgdorferi* wild-type organisms had an increased osmotic resistance in the stationary phase relative to the exponential growth phase, and our results indicate that the borrelial RpoS
FIG. 7. Susceptibility of *rpoS* wild-type B31-A to 1 N NaCl. (A) Live/dead stain with the LIVE/DEAD BacLight bacterial viability kit of a B31-A log-phase culture without and 40 min after addition of 1 N NaCl. (B) Susceptibility of B31-A to 1 N NaCl determined with a microdilution assay. Successive wells represent twofold dilutions. Row I, after 40 min of exposure to 1 N NaCl. Row II, control culture not exposed to NaCl. The first well of the control received an initial inoculum of $3.5 \times 10^5$ bacteria (row II, asterisk). An equivalent inoculum was treated with 1 N NaCl for 40 min prior to addition to the first well (row I). Solid arrow, growth endpoint of the untreated control culture, corresponding to a calculated number of 1.3 spirochetes in the initial inoculum; open arrow, growth endpoint of the NaCl-exposed culture. A $>90\%$ reduction in viability was calculated as the inverse of the ratio of the number of viable spirochetes as assessed by growth endpoint following salt treatment relative to the untreated control culture.

FIG. 8. Comparison of *rpoS* wild-type (wt) B31-A and *rpoS* mutant A74 in their resistance to 1 N NaCl. Spirochetes were grown for 3, 5, and 7 days in liquid culture. At days 3, 5, and 7, the number of viable spirochetes was determined without (0 min) and 10, 40, and 100 min after addition of NaCl. The percent survivors represents the number of viable spirochetes in salt-treated cultures relative to that in comparable sham controls. Error bars indicate the standard error obtained from three independent countings.
homolog participates in this stationary-phase response. This is consistent with the previous demonstration of RpoS induction in stationary-phase spirochetes (Frye et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999). However, RpoS is not solely responsible for these changes, since the osmotic resistance of the rpoS mutant also increased during stationary phase, although to a lesser extent. Growth phase-related factors, such as pH change of the BSK medium, could influence osmotic resistance independently of RpoS. RpoS in B. burgdorferi is not required for resistance to oxidative stress, because survival of rpoS mutant organisms exposed to 15 mM hydrogen peroxide in stationary phase was unaltered compared with the isogenic rpoS wild-type strain (data not shown). We note that in Legionella pneumophila, RpoS does not participate in stationary-phase-dependent resistance to environmental factors such as oxidative stress but is required for growth within the protozoan host Acanthamoeba castellanii (20).

Further characterization of the rpoS mutant will provide information about how B. burgdorferi adapts to variable environmental conditions. Identification of the 11 proteins differentially made by rpoS wild-type and mutant spirochetes will address which B. burgdorferi genes are regulated by RpoS, either directly or indirectly. In this regard, several borrelial proteins have previously been shown to be differentially regulated in log phase versus stationary phase (23, 44; Frye et al., Abstr. 99th Gen. Meet. Am. Soc. Microbiol. 1999), but their dependence upon RpoS expression has not been investigated. Osmotolerance requires de novo protein synthesis (25), and some of the proteins made in greater abundance in the rpoS wild-type than in the rpoS mutant could be responsible for the higher osmotic resistance. For example, proX, proW, and proV on the chromosome of B. burgdorferi encode an ABC transporter for osmoprotectants such as proline and betaine (ProU). In E. coli, ProU participates in osmoregulation and is regulated by RpoS (32).

RpoS is important for the survival of Salmonella serovar Typhimurium and L. pneumophila in their hosts and regulates virulence genes in Salmonella (14, 20, 29) and V. cholerae (60). Inactivation of rpoS in a low-passage, infectious B. burgdorferi strain will enable us to directly test the function of RpoS in the infectious cycle of this important pathogen.

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ADDENDUM IN PROOF

While this article was in press, Knight et al. reported the disruption of the gac gene located on the chromosome of B. burgdorferi (S. W. Knight, B. J. Kimmel, C. H. Eggers, and D. S. Samuels, J. Bacteriol. 182:2048–2051, 2000).

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