

## Expression of *spoT* in *Borrelia burgdorferi* during Serum Starvation

Marc B. Concepcion and David R. Nelson\*

Department of Cell and Molecular Biology, University of Rhode Island, Kingston, Rhode Island 02881

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***Borrelia burgdorferi*, the causative agent of Lyme disease, is transmitted by the tick *Ixodes scapularis*. A 2.9-kb fragment containing a putative *spoT* gene was isolated from *B. burgdorferi* genomic DNA by PCR amplification and cloned into a pBAD24 vector. The cloned gene complemented *Escherichia coli* mutant strain CF1693, which contains deletions of both the *relA* and *spoT* genes. The *spoT* gene in *E. coli* encodes a bifunctional enzyme capable of synthesizing and degrading (p)ppGpp, which mediates the stringent response during carbon source starvation. *B. burgdorferi* has been reported to have a stress response to serum starvation. Thin-layer chromatography was used to detect (p)ppGpp extracted from  $H_3^{32}PO_4$ -labeled *B. burgdorferi* cells starved for serum in RPMI. *B. burgdorferi* *spoT* gene expression was characterized during fatty acid starvation. Northern analysis of *spoT* revealed detectable message at 2.5 min of starvation in RPMI. Expression of *spoT* during serum starvation increased ~6-fold during the 30 min that starvation conditions were maintained. Further, expression of *spoT* decreased when serum was added to serum-starved cells. Reverse transcriptase PCR (RT-PCR) was used to detect *spoT* mRNA from  $\sim 10^6$  cells starved for serum in RPMI for 2.5 to 30 min or incubated in tick saliva for 15 min. Northern blot analysis suggests that *spoT* transcript was ~900 nucleotides in length. RT-PCR amplification of the transcript using several sets of primers confirmed this finding. Additionally, a truncated clone containing only the first 950 bp of the 2,001-bp *spoT* open reading frame was able to complement *E. coli* CF1693. The data suggest that *B. burgdorferi* exhibits a stringent response to serum starvation and during incubation in tick saliva.**

The spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease (9, 50), is a tick-borne pathogen spread by members of the genus *Ixodes*. During a blood meal by the tick vector, *B. burgdorferi* cells migrate through the tick gut epithelium and pass into the hemolymph and then to various tissues, including the salivary glands. *B. burgdorferi* has been shown to enter the salivary glands of *Ixodes scapularis* during a blood meal (42, 58) and has been successfully isolated from the saliva of *I. scapularis* (17), indicating that saliva serves as the route of transmission. During its life cycle, *B. burgdorferi* must adapt to different host conditions. As a result, the pathogen survives a variety of stress conditions which include physiological and nutrient changes between hosts (28). Stress conditions encountered by *B. burgdorferi* within the tick include starvation and temperature shifts (1, 10). Heat shock occurs when cells encounter rapid increases in temperature (10, 46). Proteins synthesized in response to elevated temperatures are termed heat shock proteins. Heat shock proteins have been identified and characterized in *B. burgdorferi* (10). It is hypothesized that additional stress responses are used as mechanisms of survival by *B. burgdorferi* under adverse conditions. *B. burgdorferi* cells are probably subjected to periods of starvation within their tick hosts when the ticks molt and go months between blood meals. *B. burgdorferi*, like other bacteria, should respond to nutrient depletion by initiating the stringent response (11).

The stringent response has been characterized as a short-term stress response to amino acid and carbon starvation. This global response is brought about by the failure of tRNA ami-

noacylation to keep up with the demand of protein synthesis. It is mediated by the accumulation of guanosine 3',5'-bispyrophosphate (ppGpp) (11, 30). In *Escherichia coli* and *Vibrio angustum*, two genes, *relA* and *spoT*, govern the stringent response (4, 19, 39). The *relA* gene encodes the enzyme (p)ppGpp synthase I (PSI). The enzyme is activated when a ribosome engaged in protein synthesis binds a nonaminoacylated cognate tRNA at the acceptor site (4, 11, 41). The study of *relA* null mutants led to the observation that SpoT is a bifunctional enzyme capable of both ppGpp synthesis and degradation (21, 22, 27, 45, 57). Mutational analysis of the *spoT* gene confirmed that there are two enzymatic sites contained within the amino acid sequence. One enzyme site is responsible for synthesis of (p)ppGpp, while the other site is for degradation of (p)ppGpp (22, 57). The accumulation of (p)ppGpp due to (p)ppGpp synthase II (PSII) activity is primarily due to carbon source depletion (21, 22). The function of (p)ppGpp is to act as an intracellular alarmone for nutritional deficiency (18). As a result of (p)ppGpp accumulation, bacteria shut down processes that consume amino acids and energy reserves, including stable RNA synthesis and the initiation of new rounds of DNA replication.

Increased ppGpp concentration affects many cellular processes, including the inhibition of tRNA synthesis and rRNA synthesis and the stimulation of biosynthetic and some catabolic pathways (26). In *Myxococcus xanthus*, the accumulation of ppGpp has been shown to be required for the initiation of fruiting body formation (25). During carbon starvation, *Mycobacterium smegmatis* cells exhibit a morphological change from elongated rods to cocci in response to elevated intracellular levels of ppGpp (38). Similarly, in the bacterium *V. angustum* the carbon starvation response (ultramicrocell formation) involves the loss of motility and a change in morphology to a

\* Corresponding author. Mailing address: Department of Cell and Molecular Biology, 117 Morrill Hall, University of Rhode Island, Kingston, RI 02881. Phone: (401) 874-5902. Fax: (401) 874-2202. E-mail: dnelson@uri.edu.

TABLE 1. Primers used in this study

Primer	Primer sequence (5' → 3') <sup>a</sup>	Coordinates
SpoT F1	TTT TTT TCG ACG AGC TC*C GGT AGT GGA TGA GC	195219–195233 <sup>b</sup>
SpoT F2	TT GCA CAT TTA ATC AAG ATA AAT G	195713–195736 <sup>b</sup>
SpoT R1	CGG ATT AGC TGG CAC AT	196044–196028 <sup>b</sup>
SpoT R2	TTT TTC TGC AG*G GTT TTG TTC TAA AGA ATC ATC C	198165–198142 <sup>b</sup>
RT F –25	GAA AAG AGA GCA AGG GCG CTG ATA	195657–195680 <sup>b</sup>
RT F –50	ACA TTT AAA AAC AAT TTA CGA TCT T	195632–195656 <sup>b</sup>
RT R +800	TAA GTT TGT TTG TTC TTG TTT GCA T	196507–196483 <sup>b</sup>
RT R +850	TTT TGT TTT TTG CAA ATT ATT CTT A	196556–196532 <sup>b</sup>
RT R +950	TTG ATA TTT ATT TTC TTT TGG GCT T	196656–196632 <sup>b</sup>
OspA F1	GGG AAT AGG TCT AAT ATT AGC CTT	9411–9434 <sup>c</sup>
OspA R1	CTG CTG ACC CCT CTA ATT TGG TGC C	10171–10147 <sup>c</sup>

<sup>a</sup> Bold letters indicate recognition sites for the restriction endonucleases used. SpoT F1 includes *SacI* and *SalI* sites. SpoT R2 contains a *PstI* site. \* indicates the start of the *B. burgdorferi*-specific sequence.

<sup>b</sup> Coordinates from *B. burgdorferi* main chromosome.

<sup>c</sup> Coordinates from *B. burgdorferi* linear plasmid 54 (lp54).

spherical form, which is attributed to the expression of a number of genes (29). Thus, the stringent response, like other stress responses, acts as a global regulator of the bacterial cell.

*B. burgdorferi* responds to serum starvation by inducing a starvation response that results in changes in protein expression and eventually in the transformation of motile helical vegetative cells into nonmotile spherical starvation-stress forms (1). It has been recently reported that ppGpp affects the synthesis of cyclopropane fatty acids through  $\sigma^s$  (15) and also has a role during fatty acid starvation in *E. coli* (49). This is of significance because the genome of *B. burgdorferi* contains no fatty acid synthetic pathways (20). *B. burgdorferi* cells depend upon an external source of fatty acids. Serum is rich in lipids and is the source of fatty acids for *B. burgdorferi* cells growing in pure culture or in a host. To date, most bacterial genomes that have been fully sequenced encode the proteins RelA and/or SpoT, which are capable of synthesizing or hydrolyzing (p)ppGpp (51). *B. burgdorferi* possesses a *spoT* ortholog (BB0198) and an *rpoS* gene (BB0771), but no *relA* gene (20). Since SpoT has been shown to be a bifunctional enzyme in other bacteria, it is likely that the *B. burgdorferi* SpoT is bifunctional and responsible for the synthesis and degradation of ppGpp. Other bacteria with putative *relA* or *spoT* homologs have been cloned and characterized (2, 19, 34–36, 39, 53–55, 57), suggesting that it is not uncommon for ppGpp synthesis and degradation to be controlled by a single gene. Therefore, if the *spoT* ortholog were functional in *B. burgdorferi*, the organism should have a stringent control mechanism. This mechanism could contribute to the ability of *B. burgdorferi* to adapt to and survive various host environments. In this study we show that the *B. burgdorferi spoT* ortholog is functional, able to complement an *E. coli relA spoT* mutant, and also is expressed in a manner similar to that in other bacteria that exhibit a stringent response.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** *B. burgdorferi* B31 (high passage) was grown in BSK H medium (Sigma, St. Louis, Mo.) supplemented with 6% rabbit serum (Life Technologies, Grand Island, N.Y.). For most experiments, cells were grown for 48 h at 33°C. Additional media used for *B. burgdorferi* was RPMI 1640 Select-Amine (RPMI) (Life Technologies) and saliva collected from *I. scapularis* (T. Mather, University of Rhode Island). RPMI is a defined medium containing glucose, vitamins, and all essential amino acids. *I.*

*scapularis* saliva was collected as described by Ewing et al. (17). BSK II without albumin fraction V and lacking rabbit serum (termed BSK) was also used (5).

*E. coli* strains XL1 MRF', CF1648 (wild-type MG1655; *relA*<sup>+</sup> *spoT*<sup>+</sup>), CF1652 ( $\Delta relA251::Kn$ ), CF1693 ( $\Delta relA251::Kn \Delta spoT207::Cm$ ) (a gift from Michael Cashel, National Institutes of Health) were routinely grown in Luria broth (LB) containing the appropriate antibiotics overnight in a shaking water bath at 37°C. *E. coli* was also grown in M-9 minimal medium to induce the stringent response (43, 45).

The plasmid pBAD24 (a gift of Jon Beckwith, Harvard University, Cambridge, Mass.) was used to clone the putative *spoT*. The plasmid contains an ampicillin resistance marker (23). The plasmid was introduced into *E. coli* CF1693 by electroporation.

**DNA isolation and PCR conditions.** Genomic DNA was isolated from *B. burgdorferi* B31 cells by using a Qiagen DNeasy DNA isolation kit (Qiagen Inc., Valencia, Calif.) following the protocol of the manufacturer. The DNA was used to PCR amplify a digoxigenin (DIG)-labeled probe for Southern and Northern analysis. PCR was performed using either an Applied Biosystems GeneAmp 9600 or 9700 thermocycler (Applied Biosystems, Foster City, Calif.). Primers used to amplify the DIG-labeled probe were SpoT F1 and SpoT R1 (Table 1). The probe was synthesized according to the protocol of the manufacturer (Roche Molecular Biochemicals, Indianapolis, Ind.), utilizing cycles of 94°C (5 min; denaturation), 35 cycles of 94°C (1 min), 51°C (2 min), 72°C (3 min), and final extension at 72°C (7 min).

A 2.9-kb fragment containing *spoT* was amplified by PCR from *B. burgdorferi* genomic DNA using primers spoT F1 and spoT R2 (Table 1). The PCR amplification followed the protocol above with the exception of an annealing temperature of 61°C. The resulting amplicon was digested with the endonucleases *PstI* and *SalI* (Promega, Milwaukee, Wis.) according to the protocol of the manufacturer. The restricted amplicon was then ligated into the vector pBAD24 (23) using *T<sub>4</sub>* ligase (Promega) according to protocols of the manufacturer. The recombinant plasmid was transformed into *E. coli* CF1693 by electroporation. Transformants were selected on LB plates containing ampicillin (200  $\mu$ g/ $\mu$ l). Colonies resistant to ampicillin were replica plated onto M-9 minimal medium plates containing ampicillin (200  $\mu$ g/ml), kanamycin (25  $\mu$ g/ml), and chloramphenicol (30  $\mu$ g/ml) to test for complementation of the *spoT* mutation (43).

Reverse transcriptase (RT) reactions were performed using either a Superscript II kit (Life Technologies) or a Qiagen RT kit (Qiagen Inc.) according to the protocols of the manufacturers. The primers used for the RT reactions were SpoT R1, SpoT RT R +800, SpoT R +850, RT R +850, and RT R +950 (Table 1). Prior to the RT reaction, total RNA was treated with RQ1 DNase (Promega) to eliminate any residual DNA contamination. Total RNA was quantified and RT reactions were performed on equal amounts of RNA (1  $\mu$ g) from each sample. PCR amplification was performed on 2  $\mu$ l of each RT reaction mix (22- $\mu$ l total volume). Following the RT reaction, amplification was performed by PCR using forward primers SpoT F2, RT F –50, and RT F –25 and reverse primers SpoT R1, RT R +800, RT R +850, and RT R +950 to yield amplification products. Sizes of the amplification products were determined using Kodak digital imaging software (Kodak 1D image analysis software, version 3.0.2; Eastman Kodak Co., Rochester, N.Y.) by comparison to a 1-kb DNA ladder (Promega).

Truncated and full-length *spoT* DNA sequences were amplified by PCR, and

the amplicons were cloned into pSTBlue-1 using a Novagen Perfectly Blunt cloning kit (Novagen, Inc., Madison, Wis.). PCR was performed using pBAD24.spoT as the template. The forward primer for all reactions was SpoT F1 with the following reverse primers: SpoT R2, SpoT R1, and SpoT R + 950. PCR amplicons yielded full-length *spoT* (2.9 kb) and two truncated DNA sequences of ~0.4 and ~1.1 kbp, respectively. The clones were designated pMBC04, pMBC05, and pMBC06. The truncated clones were then transformed into *E. coli* CF1693 as previously described and grown on M-9 agar containing isopropyl- $\beta$ -D-galactopyranoside to determine whether the clones could complement *E. coli* CF1693. Plasmids were purified from the appropriate *E. coli* strains grown overnight using a Qiagen spin miniprep kit (Qiagen) according to the protocol of the manufacturer.

**<sup>32</sup>P labeling and detection of (p)ppGpp by thin-layer chromatography (TLC).** *E. coli* cells were grown in phosphate-limited medium and labeled with [<sup>32</sup>P]phosphate as described by Bochner and Ames (8, 37). Briefly, cells were grown for 16 h in LB at 37°C. Cells were harvested by centrifugation (8,000 × g, 8 min, 4°C) and then incubated in glucose-limited (0.04%) phosphate-limited medium for 16 h at 37°C. Supplementation with glucose differed in that for glucose-limited culture 0.04% glucose was used, and otherwise 0.4% was utilized. To label *E. coli* cells with H<sub>3</sub><sup>32</sup>PO<sub>4</sub>, cells were harvested by centrifugation (8,000 × g, 8 min, 4°C), washed, resuspended at 2 × 10<sup>6</sup> CFU/ml in phosphate-limited medium supplemented with H<sub>3</sub><sup>32</sup>PO<sub>4</sub> (specific activity, 500 mCi/mmol; ICN Biomedical, Irvine, Calif.) at 150 μCi/ml, and allowed to incubate for 3 h (~2 generations). For induction of the stringent response, the cells were transferred to M-9 broth and allowed to incubate for 15 min at 37°C and then extracted for (p)ppGpp as described below.

To label *B. burgdorferi* cells with H<sub>3</sub><sup>32</sup>PO<sub>4</sub>, cells were grown in BSK H as previously described, harvested by centrifugation (8,000 × g, 8 min, 4°C), washed in RPMI, and then resuspended (~1 × 10<sup>7</sup> cells/ml) and labeled in RPMI medium without phosphate (Life Technologies) supplemented with 6% rabbit serum for 4 h. Cells were labeled with H<sub>3</sub><sup>32</sup>PO<sub>4</sub> (specific activity, 500 mCi/mmol; ICN Biomedical) at 150 μCi/ml. The <sup>32</sup>P-labeled cells were then washed and resuspended in RPMI at 10<sup>6</sup> cells/ml and incubated for 15 min at 33°C. Samples of labeled cells (200 μl) taken before and after the stress were extracted with formic acid for analysis of (p)ppGpp (8). Briefly, 20 μl of cold 13 M formic acid was added to the samples, and samples were stored overnight at -75°C. Prior to separation and analysis by TLC, cell debris was removed by centrifugation (15,000 × g for 5 min). A 10-μl sample of the extracted supernatant was spotted onto a polyethyleneimine (PEI) cellulose TLC plate (Sigma) and separated in 1.5 M H<sub>3</sub>PO<sub>4</sub> (pH 3.4) buffer for 2.5 h. Digital images of the TLC plate were captured using a Molecular Dynamics Storm 840 PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.). TLC analyses were repeated three times.

**RNA isolation.** *B. burgdorferi* B31 cells grown in BSK H (60 ml) with 6% rabbit serum at 33°C (~1 × 10<sup>7</sup> cells/ml) were harvested by centrifugation (8,000 × g, 8 min, 4°C), washed in RPMI, and resuspended in the appropriate medium (10<sup>8</sup> cells/ml) for induction of the *spoT* gene (RPMI, RPMI with 6% rabbit serum, BSK, and a 1:1 dilution of tick saliva [in sterile nuclease-free H<sub>2</sub>O]). Samples (1 ml) were taken at various times (0, 2.5, 5, 10, 15, and 30 min) after incubation had begun, and total RNA was isolated using RNA Purescript (Gentra, Minneapolis, Minn.) according to the protocol of the manufacturer. Purified RNA samples were stored at -75°C until use. The RNA was quantified by absorption at 260 nm using an Ultraspec 4000 spectrophotometer (Amersham Pharmacia Biotech, Piscataway, N.J.) (43).

**DNA sequencing.** DNA sequencing was performed by the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, Conn.). Sequence analysis was carried out on Applied Biosystems 377 and 3100 DNA analysis instruments. The sequencing reactions utilized fluorescently labeled dideoxynucleotides (Big Dye terminators) and *Taq* DNA polymerase (Applied Biosystems) in a thermal cycling protocol. Premixed samples of DNA template along with primers (Table 1) were sent to the facility, which then performed the sequencing reactions.

**Southern and Northern analysis.** *B. burgdorferi* genomic DNA (2 μg) was treated with the endonucleases *EcoRI* and *EcoRV* according to the protocol of the manufacturer (Promega). The digested fragments were separated by electrophoresis in a 1% agarose gel run in Tris-acetate-EDTA (TAE) buffer (43). The gel was prepared for Southern blot analysis as described by Seldon and Ausubel (48). After the washes, the DNA was transferred from the agarose gel onto a nylon membrane (Magna Graph; Osmonics, Minnetonka, Minn.) using the Turboblotter system (Schleicher & Schuell, Inc., Keene, N.H.), employing a downward transfer with 20× SSC (1× SSC contains 0.03 M sodium citrate and 0.3 M sodium chloride) made in diethyl pyrocarbonate (DEPC) (Sigma)-treated sterile distilled water (dH<sub>2</sub>O). The DNA was bound to the membrane by UV

cross-linking using a UV cross-linker (FB-UVXL-1000; Fisher Scientific). The blot was then developed according to the specifications of the manufacturer (Life Technologies). Sizes of hybridizable bands were determined by comparison with a DNA standard and using Kodak 1D digital imaging software.

For Northern blot analysis, RNA samples (5 to 15 μg) were treated by heating at 55°C for 15 min (47). The RNA samples were separated in a formaldehyde-agarose gel, which was made with DEPC-treated dH<sub>2</sub>O, 10× MOPS buffer solution (0.4 M 3-[N-morpholino] propanesulfonic acid, 0.2 M sodium acetate, and 10 mM EDTA, pH 7.0, in DEPC-treated dH<sub>2</sub>O) and 37% formaldehyde and containing 0.634 nM ethidium bromide. Equal amounts of RNA were loaded into each gel lane for each experiment. The gel was run at 80 V for 1.5 h. Following electrophoresis, the 23S and 16S rRNA subunits were observed in the gels by UV light. Northern analysis was only performed if both subunits were observed and were present in equal amounts for each lane of the gel. The gel was prepared for transfer to the nylon membrane by washing with 10× SSC for 45 min (47). The RNA was transferred to a nylon membrane (Magna Graph; Osmonics) using a Turboblotter (Schleicher & Schuell, Inc.). The RNA was then bound to the nylon membrane by using a UV cross-linker.

The nylon blots for Southern and Northern analysis were hybridized at 55°C with a *spoT*-specific DIG-labeled probe, which targets both DNA and RNA, according to the protocol of the manufacturer (Roche Molecular Biochemicals). The detection of the hybridized DIG-labeled probe followed the protocol of the manufacturer. Briefly, the blots were probed for 18 h with the DIG-labeled probe; following hybridization, the membranes were washed, blocked, and visualized by chemiluminescence according to the protocol of the manufacturer of the DIG synthesis kit.

## RESULTS

**Cloning of *B. burgdorferi spoT*.** The putative *B. burgdorferi spoT* gene was PCR amplified from *B. burgdorferi* genomic DNA using the primers *spoT* F1 and *spoT* R2 (Table 1). This resulted in a 2.9-kb amplicon, which was treated with the endonucleases *PstI* and *SalI* and ligated into the plasmid vector pBAD24 to yield pBAD24.spoT. The recombinant plasmid was introduced into competent *E. coli* CF1693 cells by electroporation. Southern blot analysis was used to confirm the presence of the 2.9-kb PCR amplicon in pBAD24.spoT. *B. burgdorferi* genomic DNA treated with the endonucleases *EcoRI* and *EcoRV* and pBAD24.spoT DNA treated with *PstI* and *SalI* were separated by electrophoresis on a 1% agarose gel in TAE buffer and then examined by Southern blot analysis (47). The DNA was probed with a DIG-labeled *spoT* probe synthesized by PCR. Southern analysis (Fig. 1) revealed hybridization of the DIG-labeled probe with the PCR control (lane 1), as well as hybridization to a 5.4-kb fragment contained within the *EcoRI*- and *EcoRV*-digested *B. burgdorferi* genomic DNA (lane 2), and to a 2.9-kb *PstI*- and *SalI*-digested fragment of the cloned *spoT* gene (lane 4). No hybridization to the pBAD24 vector (lane 3) was detected. DNA fragment length was determined by comparison to a 1-kb molecular mass marker (Promega).

To verify that the cloned DNA contained the putative *B. burgdorferi spoT* gene (BB0198), the clone was sequenced. The recombinant plasmid pBAD24.spoT served as template PCR amplicons sequenced. The sequence data were compared to the *B. burgdorferi* genome and showed >99% sequence identity with the published *spoT* (BB0198) DNA from *B. burgdorferi* (20).

**Complementation of *E. coli* CF1693 with *B. burgdorferi spoT*.** The cloned *B. burgdorferi spoT* gene was tested for function and ability to complement *E. coli* CF1693, which has deletions in both the *relA* and *spoT* genes, resulting in a phenotype unable to grow on M-9 agar (45). Figure 2 illustrates the



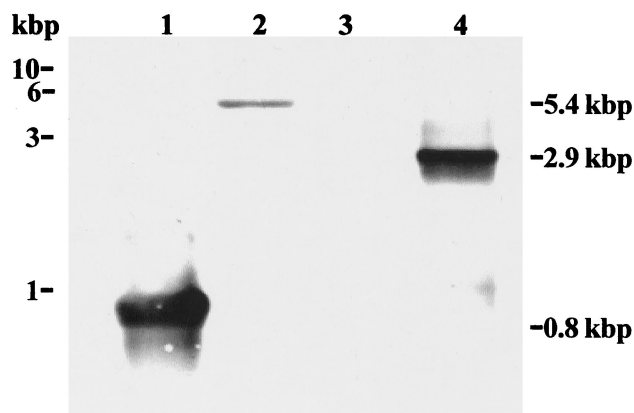


FIG. 1. Southern analysis of the cloned *B. burgdorferi spoT*. A DIG-labeled *spoT* probe (see Materials and Methods) was used to probe a Southern blot containing the PCR product of *spoT* F1 and *spoT* R1 primers, which yielded a 0.8-kbp band (lane 1); *B. burgdorferi* genomic DNA digested with the restriction endonucleases *EcoRI* and *EcoRV*, yielding a 5.4-kbp band (lane 2); pBAD24 digested with the restriction endonucleases *PstI* and *SalI* (negative control) (lane 3); and pBAD24.*spoT* digested with *PstI* and *SalI*, yielding a 2.9-kbp band (lane 4). The positions of DNA size markers are shown on the left in kilobase pairs (kbp). The sizes of the detected DNA bands are shown on the right.

growth of *E. coli* strains CF1648, CF1693, CF1693(pBAD24), and CF1693(pBAD24.*spoT*) on LB and M-9 media. As expected, the wild-type strain CF1648 grew on both LB and M-9 agar. In contrast, CF1693 and CF1693(pBAD24) grew on LB (Fig. 2A), but did not grow on M-9 agar (Fig. 2B). However, CF1693(pBAD24.*spoT*) did grow on both LB and M-9 agar plates (Fig. 2A and B), indicating that the *B. burgdorferi spoT* gene was able to complement the lack of functional *relA* and *spoT* genes in CF1693. The bacteria were also grown in LB and M-9 broth to determine the growth characteristics of the various strains. All strains grew at identical rates and to identical cell densities ( $\sim 3 \times 10^9$  CFU/ml) in LB. In contrast, while the

wild-type *E. coli* CF1648 and *E. coli* CF1693(pBAD24.*spoT*) grew at identical rates in M-9 medium, *E. coli* CF1693 and *E. coli* CF1693(pBAD24) grew more slowly than either *E. coli* CF1648 or CF1693(pBAD24.*spoT*). After 9 h of growth in M-9 broth, the final cell density of *E. coli* CF1648 and CF1693(pBAD24.*spoT*) was  $\sim 1 \times 10^8$  CFU/ml. The final cell density of *E. coli* CF1693 and CF1693(pBAD24) was  $\sim 50$ -fold less, at  $\sim 2 \times 10^6$  CFU/ml.

**Detection of the intracellular alarmone (p)ppGpp.** In order to detect the metabolic product of *spoT*, the alarmone (p)ppGpp, *B. burgdorferi* cells were grown and labeled with  $H_3^{32}PO_4$  as described in Materials and Methods. Formic acid extracts from the  $^{32}P$ -labeled cells were prepared and separated by TLC and analyzed using a phosphorimager (see Materials and Methods). Intracellular nucleotide pools from *B. burgdorferi* cells in the presence and absence of serum were compared with those of *E. coli* CF1648 cells grown in LB or M-9. The accumulation of pppGpp and ppGpp was detected in *B. burgdorferi* cells starved for 15 min in RPMI and in *E. coli* cells grown in M-9 (Fig. 3). GTP was also detected in the cell extracts. A GTP (10 mmol) control was used to confirm the TLC separation and was viewed by UV light absorption (data not shown).

**Northern analysis of *spoT* expression.** Expression of *spoT* was examined by Northern blot analysis. RNA extracted from *B. burgdorferi* cells incubated in either RPMI or BSK (without BSA or rabbit serum, sources of fatty acids) or RPMI plus rabbit serum was separated by agarose gel electrophoresis and examined by Northern blot analysis using a DIG-labeled probe (Fig. 4A, B, and C). A single transcript of  $\sim 900$  nucleotides (nt) in length was detected within 2.5 min of the initiation of serum starvation. Expression of *spoT* continued to increase ( $\sim 6$ -fold) during the 30 min that starvation conditions were maintained. In contrast, cells incubated in RPMI plus rabbit serum exhibited only a low basal level of *spoT* expression.

In order to determine whether *spoT* expression could be repressed by the addition of serum, cells were serum starved for 15 min to induce *spoT* expression and then rabbit serum

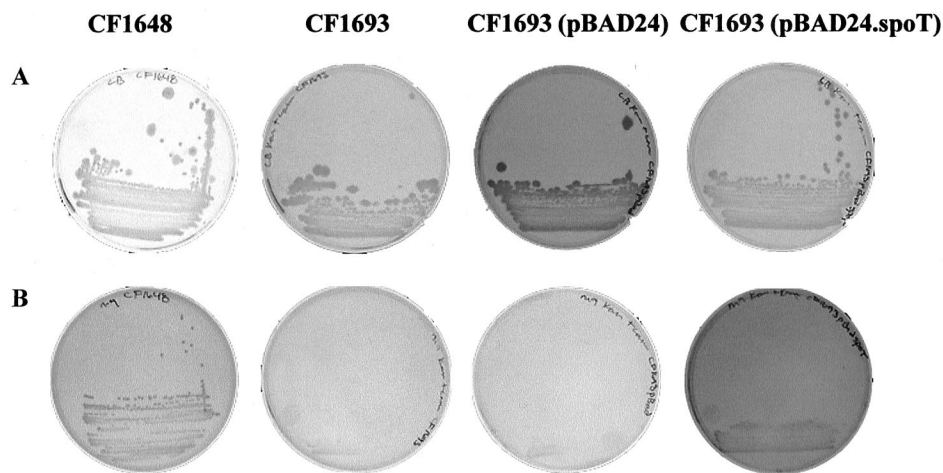


FIG. 2. Complementation of *E. coli* CF1693 with the *B. burgdorferi spoT* gene cloned into the pBAD24 vector. *E. coli* CF1648, *E. coli* CF1693, *E. coli* CF1693(pBAD24), and *E. coli* CF1693(pBAD24.*spoT*) were streaked onto LB agar (A) and M-9 agar (B), incubated for 24 h at 37°C, and examined for growth.

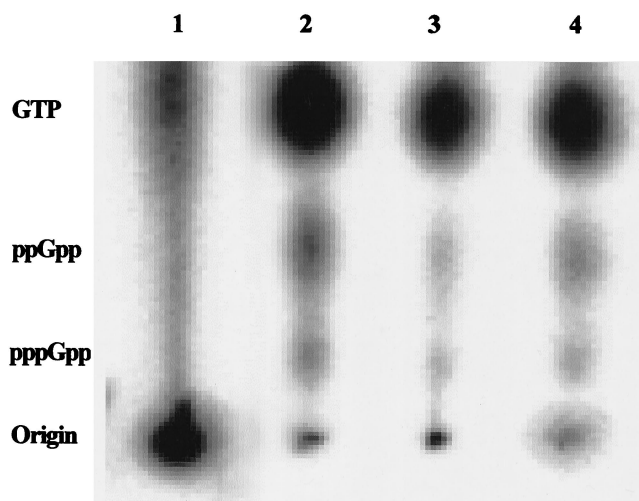


FIG. 3. Detection of (p)ppGpp in formic acid extracts of  $H_3^{32}PO_4$ -labeled *E. coli* and *B. burgdorferi* cells by PEI TLC and phosphorimaging. Cells were labeled with  $H_3^{32}PO_4$ , starved, and then extracted for (p)ppGpp (see Materials and Methods). Lane 1, *E. coli* CF1648 incubated in LB; lane 2, *E. coli* CF1648 incubated in M-9 for 15 min; lane 3, *B. burgdorferi* cells labeled for 4 h and incubated in RPMI plus rabbit serum; lane 4, *B. burgdorferi* cells labeled for 4 h and then incubated in RPMI for 15 min.

(6% final concentration) was added to the culture (Fig. 4C, row 1). Samples were taken before and after the addition of rabbit serum extracted for RNA; serum-starved cells ( $T = -15$ ) exhibited elevated levels of the *spoT* transcript. As expected, the amount of *spoT* mRNA remained high immediately after the addition of rabbit serum ( $T = 0$ ). However, within 15 min after the addition serum to the starved cells, *spoT* expression exhibited a decline. Thirty minutes after the addition of serum, *spoT* expression returned to the basal level exhibited by cells not starved for serum (compare Fig. 4B, row 1, and C, row 1). In contrast, when the blot was stripped and probed with a DIG-labeled *ospA* probe, expression of the predicted transcript ( $\sim 1,000$  nt) was observed (Fig. 4C, row 2). No decline in expression of *ospA* was observed, suggesting that the relief of serum starvation did not trigger a general mRNA degradation and that the effect was specific to the repression of *spoT* expression.

Conditions known to induce the stringent response in *E. coli* include starvation for amino acids and starvation for glucose (11). In order to determine whether these starvation conditions affect *spoT* expression in *B. burgdorferi*, the effects of starvation for amino acid (methionine) or for glucose were examined. Briefly, cells starved for methionine were incubated for up to 30 min in RPMI plus serum without methionine (Fig. 4D, row 1). Samples were collected at various times after the initiation of methionine starvation and extracted for RNA, and *spoT* expression was determined by Northern analysis (Fig. 4D, row 1). The amount of methionine contributed by the 6% serum was  $\sim 1.3 \mu M$ . In a similar experiment, cells starved for glucose were incubated for up to 30 min in RPMI plus serum without glucose (Fig. 4D, row 2). Samples were collected at various times after the initiation of glucose starvation and extracted for RNA, and *spoT* expression was determined by Northern analysis (Fig. 4D, row 2). The amount of glucose contributed by the

6% rabbit serum was  $0.26 \mu M$ , which was depleted during the experiment. Expression of *spoT* throughout the experiment remained at low basal levels and did not increase in response to the absence of methionine or glucose depletion.

**Determination of *spoT* expression in *I. scapularis* saliva by RT-PCR.** It was of interest to determine whether incubation of *B. burgdorferi* cells in *I. scapularis* saliva would affect *spoT* expression. Increased expression of *spoT* would suggest that cells induce the stringent response during transmission to the mammalian host. Since only a very limited amount of tick (*I. scapularis*) saliva was available, RT-PCR was used to examine the expression of *spoT*. To determine whether RT-PCR amplification could detect *spoT* mRNA from  $\sim 10^6$  cells incubated under conditions known to induce *spoT*, RNA extracted from *B. burgdorferi* cells starved in RPMI (0 to 30 min) was used as a template for RT-PCR with the primers SpoT F2 and SpoT R1. Data presented in Fig. 5A demonstrate that *spoT* expression was detected within 2.5 min of the initiation of serum starvation and increased during the 30-min starvation. This result was in agreement with the results of Northern analysis of *spoT* expression (Fig. 4) and suggested that RT-PCR could be used to follow expression of *spoT* in small numbers of cells. The expected RT-PCR product was gel purified and sequenced to determine whether the RT-PCR product was due to *spoT* expression. The sequence obtained was 99% identical to the expected *spoT* product. Thus, RT-PCR could serve as a viable alternative to Northern analysis when determining *spoT* expression in low numbers of cells. When  $\sim 10^6$  *B. burgdorferi* cells were incubated in tick saliva, *spoT* expression increased (Fig. 5B), suggesting that incubation in tick vector saliva results in *spoT* expression consistent with the stringent response.

**Determination of start and end transcription sites for *spoT*.** RT-PCR was used to determine the approximate start and end transcription sites for *spoT*. Since Northern analysis revealed that the *spoT* transcript was  $\sim 900$  nt long, several RT-PCR primer sets were selected to reverse transcribe and amplify possible *spoT* transcripts of this size and smaller. The RT primers selected started at positions +350, +800, +850, and +950 and the forward PCR primers selected started at positions +25, -25, and -50 from the start codon of *spoT*. RNA extracted from *B. burgdorferi* cells starved in RPMI for 15 min was used as the template for RT-PCR amplification. Examination for the resulting amplicons by gel electrophoresis revealed that RT-PCR successfully amplified products from all forward primers and from the +350, +800, and +850 primers (Fig. 6). No amplification was detected from the +950 RT reverse primer. The RT-PCR amplicons were sequenced to confirm their origin from *spoT* transcripts (data not shown) and were confirmed to be 99% identical to the expected *spoT* sequence; this experiment was repeated twice with identical results. As the *spoT* transcript is  $\sim 900$  nt long, these data suggest that *spoT* transcription begins at approximately -50 and extends to +850 to +875.

**Cloning of a truncated *B. burgdorferi spoT* gene.** In order to determine whether the truncated *spoT* mRNA observed by Northern blot analysis was capable of encoding a functional SpoT protein, three DNA sequences were amplified from pBAD24.spoT, using SpoT F1 as the forward primer with either SpoT R2 (to yield the full-length *B. burgdorferi spoT* gene), SpoT R1 (to yield the 300-bp 5' portion of *spoT*), or

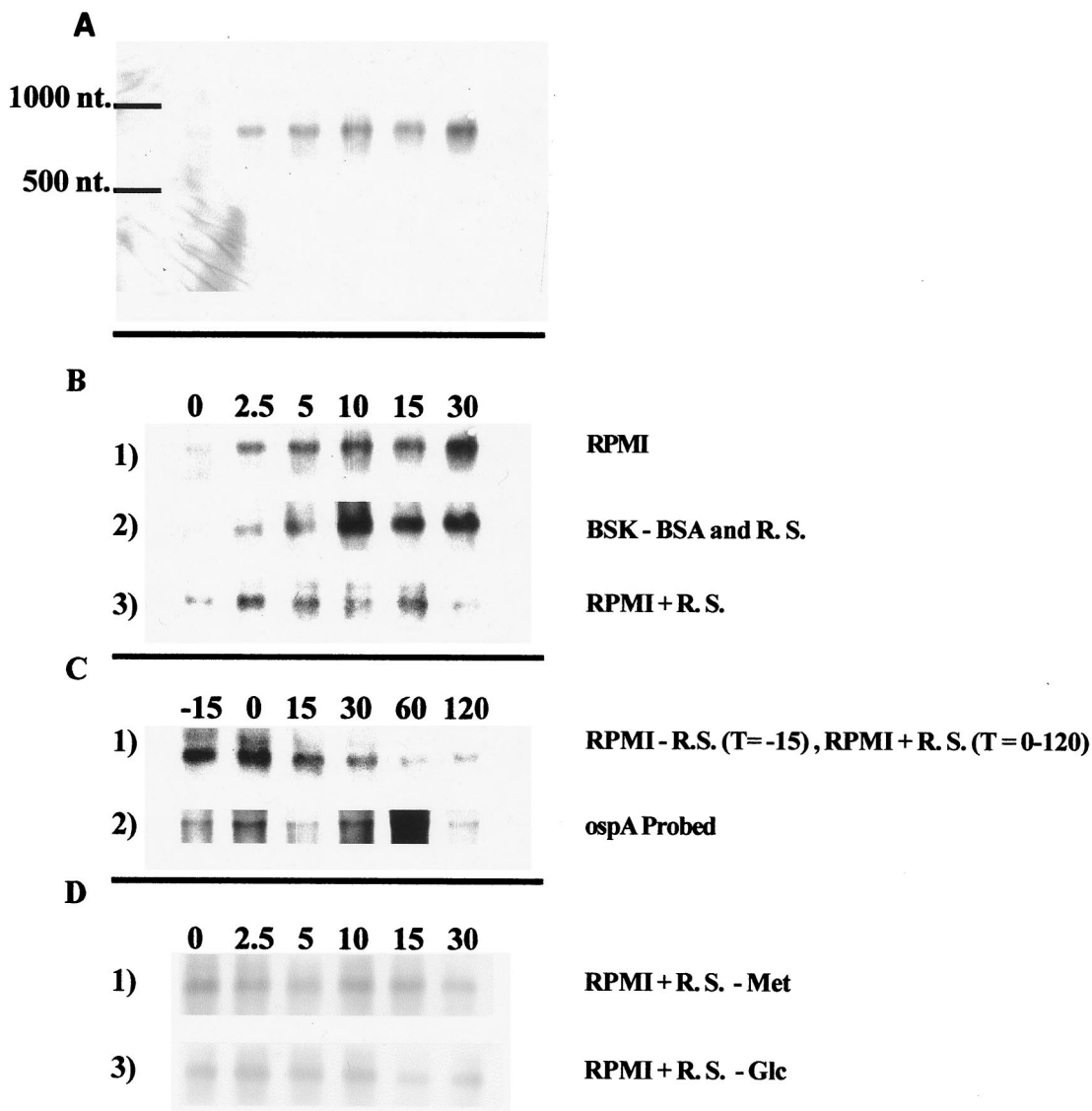


FIG. 4. Northern blot analysis of *spoT* expression by *B. burgdorferi* cells in the absence and presence of rabbit serum. Cells samples were prepared and extracted for RNA as described in Materials and Methods. (A) Entire Northern blot of RNA from cells incubated in RPMI ( $T = 0, 2.5, 5, 10, 15,$  and  $30$  min), showing specificity of the DIG-labeled *spoT* probe, and approximate nucleotide marker. (B) Northern blot showing *spoT* expression in cells incubated in RPMI (row 1), BSK (row 2), and RPMI plus 6% rabbit serum (row 3) for 0, 2.5, 5, 10, 15, and 30 min. (C) Northern blot showing effect of addition of 6% rabbit serum on expression of *spoT* (row 1) and *ospA* (row 2). Cells were starved in RPMI for 15 min ( $T = -15$ ), and then rabbit serum was added to the culture ( $T = 0$ ) and samples were taken at 15, 30, 60, and 120 min. (D) Northern blot showing *spoT* expression in cells incubated in RPMI plus 6% rabbit serum minus methionine (row 1) and RPMI plus 6% rabbit serum minus glucose (row 2). Samples were taken and prepared from cells incubated for 0, 2.5, 5, 10, 15, and 30 min.

SpoT +950 (to amplify the 950-bp 5' portion of the *spoT* gene). The three amplicons were cloned into a Novagen Perfectly Blunt cloning vector. The clones pMBC04, pMBC05, and pMBC06 were transformed into *E. coli* CF1693 and the resulting transformants were tested for growth on M-9 minimal agar as previously described. As expected, the full-length *spoT*, *E. coli* CF1693(pMBC04), complemented the *spoT* mutation and restored growth on M-9 agar. The 300-bp truncated clone, *E. coli* CF1693(pMBC05), was unable to grow on the minimal medium. However, the 950-bp truncated clone, *E. coli* CF1693(pMBC06), was able to grow on M-9 agar, indicating

that it encoded a protein capable of complementing the *spoT* mutation.

**DISCUSSION**

The *spoT* gene encodes a bifunctional enzyme capable of degrading and synthesizing (p)ppGpp (PSII). In *E. coli*, SpoT is constitutively expressed, resulting in low concentrations of (p)ppGpp during normal growth and resulting in changes to RNA polymerase promoter activity (27, 44). The SpoT/PSII system is thought to regulate growth rate by adjusting the rate



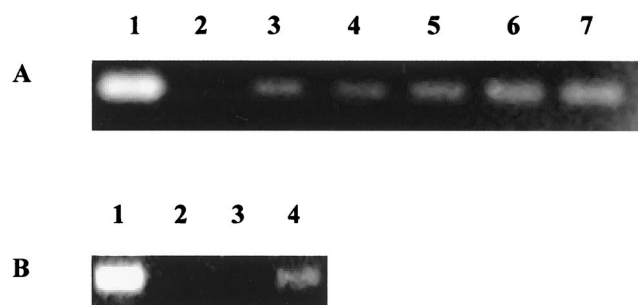


FIG. 5. RT-PCR analysis of *spoT* expression in RNA isolated from *B. burgdorferi* cells incubated in RPMI (A) or *I. scapularis* saliva (B). The 371-bp amplicon was separated by gel electrophoresis on a 1% agarose gel using TAE buffer and stained with ethidium bromide. Equal volumes (10  $\mu$ l) of the RT-PCR-amplified material were loaded onto each gel lane. (A) RT-PCR performed on RNA isolated from cells incubated in RPMI. Lane 1, PCR amplification from *B. burgdorferi* genomic DNA; lane 2, amplification using total RNA extracted at 0 min; lane 3, RNA extracted at 2.5 min; lane 4, RNA extracted at 5 min; lane 5, RNA extracted at 10 min; lane 6, RNA extracted at 15 min; lane 7, RNA extracted at 30 min. (B) RT-PCR performed on RNA extracted from *B. burgdorferi* cells incubated in the saliva of the tick vector *I. scapularis* (1:1 dilution in nuclease-free H<sub>2</sub>O). Lane 1, PCR amplification using *B. burgdorferi* genomic DNA as template; lane 2, negative control of nuclease-free H<sub>2</sub>O without DNA; lane 3, amplification using total RNA extracted from cells incubated for 0 min in tick saliva; lane 4, RNA extracted from cells incubated for 15 min in tick saliva.

of stable RNA synthesis (6, 7, 12). It is hypothesized that constitutive SpoT activity helps the cell monitor nutritional states with regard to carbon sources (11). The findings of Kvint et al. (31, 32) show that RpoS-dependent promoters require ppGpp for induction. It is proposed that RpoS-dependent promoters are positively affected by ppGpp due to the dissociation of  $\sigma^{70}$ -programmed RNA polymerase from stringent promoters, increasing the availability of free RNA polymerase. Additionally, ppGpp reduces the stability of open complexes with RNA polymerase (6). The increased levels of ppGpp also can positively affect RpoS by increasing the half-lives of the RNA polymerase complexes. It is also thought that ppGpp may directly or indirectly aid in the ability of  $\sigma^s$ -dependent promoters to bind RNA polymerase holoenzyme.

Several *relA* or *spoT* homologs have been cloned and char-

acterized (2, 19, 34, 35, 39, 53, 55, 57). These single genes appear to function like either the *relA* or *spoT* of *E. coli* or a combination of the two genes. For example, *relA* and *spoT* homologues of *Bacillus stearothermophilus*, *Bacillus subtilis*, *Streptococcus pyogenes*, and *Streptococcus rattus* are induced only under conditions of amino acid depletion. In contrast, the *relA* or *spoT* homologues of *M. smegmatis*, *Mycobacterium tuberculosis*, and *Streptococcus equisimilis* only respond to carbon starvation.

The stringent response is not only important in the regulation of short-term responses to nutritional depletion, it is also important in the regulation of long-term starvation-stress responses. For example, *M. xanthus* cells change from motile rods to nonmotile, spherical myxospores within fruiting bodies in response to starvation (25). The intracellular accumulation of (p)ppGpp is required for the initiation of fruiting body formation (25). In *M. smegmatis*, carbon starvation results in the activation RelA, increased ppGpp concentrations, and a morphological change from elongated rods to cocci (38). Thus, increased ppGpp concentration regulates FtsZ activity and, therefore, cell division. Increases in (p)ppGpp concentration have also been shown to control acid-stress tolerance in *Lactococcus lactis* (41).

*B. burgdorferi* undergoes several environmental transitions during transmission between the tick vector and a vertebrate host. These changes include temperature, nutrient availability, cell density, and pH (13, 16, 28). Some of these environmental transitions have been shown to affect gene expression in *B. burgdorferi*. Carreiro et al. (10) have demonstrated that rapid increases in temperature induce the heat shock response. More recently, Alban et al. (1) demonstrated that at least 20 proteins are induced in *B. burgdorferi* cells starved for serum. Additionally, motile helical cells convert over  $\sim$ 48 h of starvation to round nonmotile starvation forms (or "cysts"). Since there are no fatty acid biosynthesis pathways in *B. burgdorferi*, these bacteria obtain fatty acids from their environment (20). Recently, Cox and Radolf (14) demonstrated that *B. burgdorferi* obtains fatty acids by diffusion through its outer membrane, which could result in the slow growth rate of the organism.

Experimental data presented in this communication demonstrate that the *spoT* gene of *B. burgdorferi* is functional and able to complement *E. coli* CF1693 ( $\Delta$ *relA*  $\Delta$ *spoT*) to restore wild-

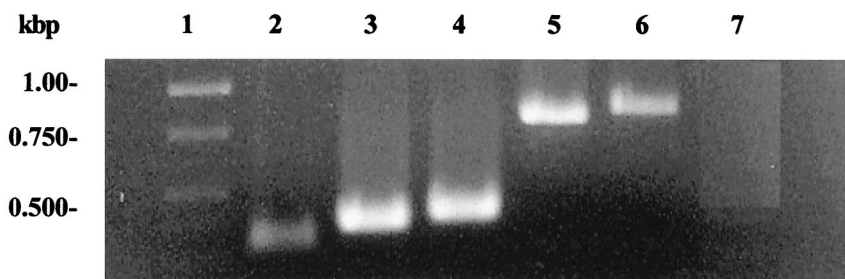


FIG. 6. Determination of the approximate start and end transcriptional sites of *spoT* by RT-PCR. RNA was extracted and prepared for RT-PCR from *B. burgdorferi* cells starved in RPMI for 15 min. RT-PCR amplification was performed using several primer sets, and the reaction products were separated by gel electrophoresis and visualized using ethidium bromide and UV transillumination. Gel lanes: 1, DNA size markers; 2, amplification using primers SpoT F2 and SpoT R1; 3, amplification using primers SpoT F -25 and SpoT R1; 4, amplification using primers SpoT F -50 and SpoT R1; 5, amplification using primers SpoT F2 and SpoT R +800; 6, amplification using primers SpoT F2 and SpoT R +850; 7, amplification using primers SpoT F2 and SpoT R +950.

type phenotypic growth in M-9 minimal medium (Fig. 2). Further, (p)ppGpp was detected in  $^{32}\text{P}$ -labeled cellular extracts of *B. burgdorferi* cells starved for serum (Fig. 3), suggesting that the *spoT* gene encodes PSII activity. Characterization of *spoT* expression was carried out by Northern blot analysis and RT-PCR.

Northern blot analysis of *spoT* expression in *B. burgdorferi* revealed that a 900-nt transcript was expressed within 2.5 min after the initiation of serum starvation (Fig. 4). Expression of *spoT* increased ~6-fold over the first 30 min of starvation and returned to basal levels after 120 min of serum starvation (data not shown). Further, when rabbit serum was added to the serum-starved *B. burgdorferi* cells, the expression of *spoT* rapidly decreased to basal levels. The decrease in *spoT* expression was specific, since *ospA* expression remained nearly constant during the 120 min after the addition of rabbit serum. Thus, serum starvation induces *spoT* expression and serum feeding represses *spoT* expression. Additionally, neither glucose nor amino acid starvation (methionine) resulted in induction of *spoT*, indicating that in *B. burgdorferi* *spoT* expression and, therefore, the stringent response is induced only by serum starvation. In comparison, the *E. coli* stringent response is activated by starvation for amino acids, glucose, and fatty acids (11). While there are no data describing the transcriptional expression of either *relA* or *spoT* in *E. coli* or organisms other than *B. burgdorferi*, both TLC data for (p)ppGpp accumulation and Western blot analyses of RelA and SpoT expression have been used to characterize PSI and PSII activities and protein expression, respectively (2, 19, 34–36, 53, 55, 56). For example, Wendrich et al. (54) demonstrated by immunoblot analysis that the RelA/SpoT homologue of *B. steartothermophilus* is induced during amino acid starvation. Additionally, (p)ppGpp accumulation was detected within 3 min of the initiation of amino acid starvation. Our observations of *spoT* expression in *B. burgdorferi* are consistent with these previous reports.

A single ~900-nt hybridizable transcript was always observed when mRNA preparations were examined using a *spoT*-specific DIG-labeled probe during Northern blot analysis. No other transcripts were observed when using the DIG-labeled *spoT* probe. The *spoT* transcript length was unexpected, because examination of *B. burgdorferi* genome data revealed a 2,001-bp open reading frame (ORF) (BB0198) identified as *spoT* (20). Further RT-PCR experiments suggest that the *spoT* transcript starts approximately 50 to 75 bp upstream of the start codon and ends approximately 850 to 875 bases downstream. The data strongly suggest that the *spoT* transcript is ~900 nt long. Additionally, when a fragment of *spoT* containing the first 950 bp of the structural gene was cloned (pMBC06) it was able to complement *E. coli* CF1693 to allow growth on M-9 agar, indicating the restoration of the stringent response. We cannot distinguish between the possibility that the transcript is rapidly processed from a longer mRNA encoded from the entire ORF (40) or that the *spoT* ORF contains an internal transcriptional terminator. However, mutational analysis of the *E. coli* SpoT demonstrates that the first 203 amino acids of the 702-amino acid SpoT are necessary for (p)ppGpp phosphohydrolyase activity and that amino acids 67 to 374 are necessary for PSII activity (22). The bacterium *Streptomyces coelicolor* has a functional *relA/spoT* homologue that has been characterized (34). Only 453 of the 874 amino

acids of the protein are necessary for both (p)ppGpp phosphohydrolyase (residues 93 to 397) and synthase (residues 267 to 453) activities (22, 35). These data support the idea that the entire *spoT* ORF is not needed to yield a functional SpoT protein.

When *B. burgdorferi* cells were incubated in the saliva of the tick vector *I. scapularis*, a 371-bp *spoT*-specific DNA fragment was amplified by RT-PCR. The RT-PCR product was sequenced and shown to be 99% identical to the expected *spoT* transcript. This result demonstrates that *B. burgdorferi* *spoT* is induced when the cells are incubated in the saliva of *I. scapularis*. This result is consistent with the hypothesis that the *B. burgdorferi* cells exhibit the stringent response during transmission from the tick vector to the mammalian host by tick saliva. Hammer and Swanson (24) demonstrated that when *Legionella pneumophila* is subjected to amino acid depletion, ppGpp accumulates and the cells convert from a replicative to a virulent state. More recently, Bachman and Swanson (3) showed that the accumulation of ppGpp activated an RpoS-dependent virulence pathway in *L. pneumophila*.

Finally, increases in intracellular concentration of (p)ppGpp have been shown to up regulate *rpoS* ( $\sigma^s$ ), the main sigma factor involved in the expression of stationary-phase genes as well as the expression of genes involved in long-term starvation conditions (15, 33, 52). Recently, Elias et al. (16) showed that the *B. burgdorferi* genome contains a functional *rpoS*. It will be interesting to determine whether *rpoS* expression responds to serum starvation.

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