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Characterization of the Stringent Response and rel_{Bbu} Expression in *Borrelia burgdorferi*

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The stringent response is a global bacterial response to nutritional stress mediated by (p)ppGpp. We previously found that both noninfectious *Borrelia burgdorferi* strain B31 and infectious *B. burgdorferi* strain N40 produced large amounts of (p)ppGpp during growth in BSK-H medium and suggested that the stringent response was triggered in *B. burgdorferi* under these conditions. Here we report that (p)ppGpp levels in *B. burgdorferi* growing in BSK-II or BSK-H medium are not further increased by nutrient limitation or by serine hydroxamate-induced inhibition of protein synthesis and that the presence of (p)ppGpp during growth of N40 in BSK-H medium is not associated with decreased 16S rRNA synthesis. Decreased 16S rRNA synthesis was associated with the decreased growth rate of N40 seen during coculture with tick cells, which are growth conditions that were previously shown to decrease (p)ppGpp levels. One-half as much of the mRNA of the gene encoding the Rel protein of *B. burgdorferi* (rel_{Bbu}) was produced by B31 as by N40 during in vitro growth (2 ± 0.5 and 4 ± 0.8 fg of rel_{Bbu} mRNA/ng of total *Borrelia* RNA, respectively). Although the amounts of N40 rel_{Bbu} mRNA were identical during growth in vitro and in rat peritoneal chambers, they were markedly decreased during growth in nymphal ticks. In contrast to the lack of change in rel_{Bbu} mRNA levels, larger amounts of a 78-kDa protein that was cross-reactive with antibodies to *Bacillus subtilis* Rel_{Bsu} were detected in immunoblots of N40 lysates after growth in rat peritoneal chambers than after growth in vitro. Differences in the level of production of (p)ppGpp between B31 and N40 could not be explained by differences in rel_{Bbu} promoters since identical transcriptional start sites 309 nucleotides upstream from the B31 and N40 rel_{Bbu} ATG start codon and identical σ^{70}-like promoters were identified by primer extension and sequencing analysis. rel_{Bbu} complemented an *Escherichia coli* CF1693 *relA spoT* double mutant for growth on M9 minimal medium, and the transformed cells produced rel_{Bbu} mRNA. These results indicate that rel_{Bbu} is functional and that its transcription and translation and production of (p)ppGpp are affected by environmental conditions in strains N40 and B31. They also suggest that in *B. burgdorferi*, an organism with few rRNA operons that grows slowly, the role of (p)ppGpp may differ from the classic role played by this molecule in *E. coli* and that (p)ppGpp may not be responsible for growth rate control.

Alternating periods of starvation and surfeit characterize the life cycles of many bacteria. *Borrelia burgdorferi* does not escape this pattern while infecting, growing, and persisting in the microenvironments of its arthropod and mammalian hosts (9, 29). Some of these microenvironments favor multiplication of *B. burgdorferi*, while others are antagonistic to growth of this organism (18, 19). Differential gene expression and tight modulation appear to be essential for the ability of *B. burgdorferi* to survive in dissimilar host environments that may sometimes foster and at other times inhibit bacterial proliferation and persistence (2, 26, 30, 40). Variations in *B. burgdorferi* gene expression related to cycling between arthropod and mammalian hosts have been identified and characterized under both in vitro and in vivo conditions (1, 13, 22, 30, 33, 41).

For *B. burgdorferi* growing in vitro, changes in pH, cell concentration, temperature, or the presence of tick cells are accompanied by well-characterized changes in expression of different lipoproteins and other proteins (5, 24, 30, 40). Changes in expression of lipoproteins (e.g., a shift from OspA to OspC) are associated with passage of *B. burgdorferi* from ticks to the mammalian host (10, 33, 34). It has been postulated that these changes are secondary to differences in the nutritional milieus encountered by *B. burgdorferi* in these two hosts along with differences in pH, temperature, and bacterial cell concentration (40). Modulation of gene expression appears to be mediated at least in part by upregulation of σ^S by upregulated expression of σ^{54} (20, 23). The stimuli that trigger this loop and the manner in which σ^S mediates this regulation have not been characterized.

The stringent response is a global bacterial stress response to nutritional stress (7, 20, 37). It is classically characterized by a regulon-type response with extreme downregulation of stable RNA and protein synthesis and upregulation of protein degradation and amino acid synthesis. In *Escherichia coli*, the stringent response is triggered by uncharged tRNA and is

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mediated by production and degradation of the alarmon (p)ppGpp by the *relA* and *spoT* gene products (7). It was originally thought that the increased concentration of (p)ppGpp generated by RelA activated by stalled ribosomes in *E. coli* was the sole factor responsible for the metabolic changes of the stringent response and slow growth (7), but it was subsequently shown that rRNA synthesis in this organism is regulated by both stringent and growth rate-dependent controls (15).

Although a chromosomal gene encoding the Rel protein of *B. burgdorferi* (rel_{Bbu}) has been identified in the *B. burgdorferi* genome (BB0198) which is orthologous to genes that encode other bacterial (p)ppGpp synthetases and hydrolases (14, 28), there is no evidence that the activity of the enzyme encoded by this gene mediates the stringent response in *B. burgdorferi*. rel_{Bbu} has, however, been found to be upregulated in *B. burgdorferi* growing in BSK-H medium at 34°C and pH 7.6 compared with the response in *B. burgdorferi* growing in coculture with tick cells at the same temperature and pH (5). It is also upregulated in *B. burgdorferi* growing in BSK-H medium at 23°C and pH 7.5 compared with the response in organisms growing in BSK-H medium at 37°C and pH 6.8 or in rat peritoneal dialysis chambers (30).

Previously, infectious and noninfectious strains of *B. burgdorferi* have been shown to produce large amounts of (p)ppGpp and to transcribe rel_{Bbu} during growth in BSK-H medium at 34°C and pH 7.6 (5). Under these conditions, 97% of *B. burgdorferi* N40 *bmpD* gene expression was associated with monocistronic mRNA from the *bmpD* promoter and only 3% of *bmpD* expression was associated with polycistronic messages with *rpsL* from the upstream promoter. In contrast, *B. burgdorferi* N40 cocultured with tick cells produced no (p)ppGpp and grew more slowly than it grew during culture in BSK-H medium, while total *bmpD* transcription decreased eightfold and only polycistronic *rpsL-bmpD* was transcribed. These observations suggested that the stringent response could modulate *B. burgdorferi* gene expression. The presence of a rel_{Bbu} homologue in the genome of *B. burgdorferi*, production (p)ppGpp, and expression of *B. burgdorferi* rel_{Bbu} mRNA encouraged us to continue our studies to characterize the putative stringent response and rel_{Bbu} in *B. burgdorferi*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Unless otherwise specified, high-passage, noninfectious *B. burgdorferi* strain B31 (= ATCC 35210) was grown in BSK-II medium (32) or BSK-H medium (Sigma, St. Louis, Mo.) supplemented with 6% rabbit serum (Sigma). Low-passage infectious *B. burgdorferi* strain N40 (from Linda Bockenstadt, Yale University) and *B. burgdorferi* strain BL206, a recent clinical isolate from a human blood sample (from Ira Schwartz, New York Medical College), were grown in BSK-H medium at 34°C. *E. coli* CF1648 (wild type) and CF1693 (containing a double deletion of *relA* and *spoT*), obtained from Michael Cashel, National Institutes of Health (39), were grown with shaking at 37°C in Luria-Bertani (LB) medium (Life Technologies, Paisley, Scotland), in MOPS (morpholinepropanesulfonic acid) medium (6), or on M9 medium (31)–1.5% agar (Difco Laboratories, Detroit, Mich.) plates. Kanamycin and chloramphenicol (Sigma) were added at concentrations of 50 and 20 μg/ml, respectively, when *E. coli* CF1693 was grown (39).

(p)ppGpp detection. *B. burgdorferi* cells from a log-phase culture (2×10^7 to 5×10^7 cells/ml) grown with 10 μCi of [^{32}P]orthophosphate (Amersham Pharmacia Biotech, Piscataway, N.J.) per ml were centrifuged and were resuspended at a concentration of 7×10^6 cells/ml for B31 in complete medium or at a concentration of 0.7×10^6 cells/ml for N40 in media containing 10-fold-lower concentrations of rabbit serum, yeastolate (Difco), or neopeptone (Difco) or in complete medium containing 1 mg of DL-serine hydroxamate (Sigma) per ml. All of the resuspension media contained 10 μCi of uniformly labeled [^{32}P]orthophosphate per ml. After various times of growth in these media, labeled cells were harvested from 1- to 5-ml cultures, (p)ppGpp was extracted with 50 μl of 2 M formic acid on ice for 30 min, and 5 μl of supernatant was loaded on a cellulose polyethyleneimine thin-layer chromatography plate (Selecto Scientific, Suwanee, Ga.). Samples were fixed in methanol (5 min) and dried. The thin-layer chromatography plates were developed in 1.5 M KH_2PO_4 (pH 3.4), air dried, and exposed to Hyperfilm MP (Amersham Pharmacia Biotech) for 18 to 48 h at −20°C or were counted by using a Molecular Dynamics Storm System 860 phosphorimager. To detect (p)ppGpp in *E. coli*, overnight colonies were inoculated into various media as described below, and (p)ppGpp was extracted from 200 μl of culture and detected as described above. *B. burgdorferi* spots were identified by comigration with *E. coli* (p)ppGpp and with ATP and GTP, which were used as standards (Sigma), as described previously (5, 6).

DNA isolation, sequencing, and cloning of *B. burgdorferi* rel_{Bbu}. DNAs of *B. burgdorferi* B31, N40, and BL206 cells from mid-log-phase cultures were isolated by using a High Pure PCR template preparation kit for isolation of genomic DNA (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's directions. DNA manipulations were performed by standard methods (31). The complete *B. burgdorferi* rel_{Bbu} gene (BB0198), including nucleotides from position −546 upstream from the *B. burgdorferi* rel_{Bbu} start codon to position 109 downstream from the *B. burgdorferi* rel_{Bbu} stop codon, was cloned into pBluescript IISK+ as described previously (5). The *B. burgdorferi* N40 and BL206 rel_{Bbu} DNA regions were sequenced by the dideoxy chain termination method by using a dye terminator *Taq* cycle sequencing kit and a model 377 DNA sequencer (Perkin-Elmer, Foster City, Calif.). Comparative analysis of *B. burgdorferi* B31 (14), BL206, and N40 rel_{Bbu} regions was performed by using OMIGA 2.0 software (Oxford Molecular).

Infection of tick cell lines. IDE8 tick cells (kindly provided by Ulrike Munderloh, University of Minnesota, St. Paul) were infected with a preparation containing 10^7 *B. burgdorferi* N40 cells/ml at a multiplicity of infection of 5 in L15BS medium at 32°C for 3 days as previously described (5).

Infection of ticks. Four-week-old C3H/HeN mice were infected intradermally with *B. burgdorferi* N40 (10^4 bacteria/mouse). Two weeks after infection, ear punches were taken from the mice and checked for the presence of *B. burgdorferi* by culture in BSK-H medium containing 50 μg of rifampin (Merrel Dow Pharmaceuticals Inc., Cincinnati, Ohio) per ml and 2.5 μg of amphotericin B (Sigma) per ml. *Ixodes scapularis* larvae were fed to repletion on infected mice, collected, and allowed to molt into nymphs. Molted nymphs were engorged on mice or 2-month-old Sprague-Dawley rats (Harlan Sprague-Dawley Inc., Indianapolis, Ind.) for 48 to 72 h. Two groups of engorged nymphs were pooled for use in reverse transcription (RT)-PCR experiments. The first group contained 91 nymphs, and the second group contained 246 nymphs.

RNA isolation, RT-PCR, and competitive RT-PCR. Total RNAs from infected engorged nymphal ticks, from *B. burgdorferi*, and from *E. coli* were isolated by the guanidine thiocyanate-phenol-chloroform method (8) and were treated with RQ1 RNase-free DNase (Promega) to eliminate DNA contamination. RT-PCR and competitive RT-PCR were performed by using the Access RT-PCR system (Promega, Madison, Wis.) as described previously (5, 12). The primers used for RT-PCR are described in Table 1. Constitutively expressed *B. burgdorferi flaB* (11) was used as a control to compare different RNA isolates in competitive RT-PCR experiments.

RT and real-time PCR. RNA from *B. burgdorferi* growing in BSK-H medium (2×10^7 to 5×10^7 cells/ml) or in a coculture with tick cells was isolated as described above. cDNA synthesis was performed with 1 μg of total *B. burgdorferi* RNA by using random primers and avian myeloblastosis virus (AMV) reverse transcriptase (Promega) according to the manufacturer's recommendations. To quantify *flaB* or 16S rRNA in *B. burgdorferi* growing in BSK-H medium or with tick cells, the resulting cDNAs were amplified and analyzed with a LightCycler real-time PCR instrument (Roche). The PCR was performed in glass capillaries by using a 10-μl (final volume) mixture containing 1× LightCycler master mixture (Roche), 3 mM $MgCl_2$, each primer at a concentration of 1 μM (primers flaBd and flaBrc for *flaB* and primers 16SrRNAd and 16SrRNArc for 16S rRNA [Table 1]), and DNA template. The amplification program consisted of denaturation at 95°C for 2 min, followed by 35 cycles of 95°C for 1 s, 55°C (for *flaB*) or 57°C (for 16S rRNA) for 5 s, and 72°C for 10 s. Fluorescent product was detected at the last step of each cycle. Unless otherwise specified, the cooling or heating rate was 20°C/s. After amplification, a melting curve was acquired by heating the product to 95°C, cooling it to 60°C, and slowly heating it at a rate of 0.2°C/s to 95°C, with fluorescence collection at 0.2°C intervals. PCRs were performed twice for each RNA isolate. Each experimental sample was analyzed in triplicate. Data were analyzed with the Lightcycler software provided by the

TABLE 1. Primers used in this study

Reaction	Gene	Primer	Sequence (5′–3′)	Size of amplified fragment (bp)	
				Wild type	Competitor
Competitive RT-PCR	*flaB*	49	CTAGTGGGTACAGAATTAATCGAGC	880	691
		50	TAACATAAAAATATCCTCCTTGC		
	bmpA	21	CCAAGGTTGCGGCTCTTC	307	219
		22	CTTCTACCAGCTTCAAGGTCAG		
	bmpB	23	TGGTGATGATGTTCAGATTCC	339	241
		24	TTTGCTGCCTCAATAACACC		
	bmpC	1	GATGAGGCAATGACTGAGGATGC	489	337
		2	GCAGCGTCATAAACTCCAAGACC		
	bmpD	19	CTGATGATGGCAAGTCGGAG	610	506
		20a	CCTATACCAGAAAGCCCTGC		
	rpsL-bmpD	55	GGAACAAAAAAGCCTAAAGC	694	564
		52	CGACTTGCCATCATCAGAGC		
	rel_{Bbu}	ST3	CAAAAAAGCGGAATTGAAGCAG	446	371
		ST4	GAATATTGACTTTTGTTGGCCG		
Real-time PCR	*flaB*	flaBd	TCATTGCCATTGCAGATTGTG	278	
		flaBrc	ACCTTCTCAAGGCGGAGTTAA		
	16S rRNA	16SrRNAd	GGCCCGAGAACGTATTCACC	288	
		16SrRNArc	CGAGCGCAACCCTTGTTATC		
Primer extension	rel_{Bbu}	SText1	CTAGCTTTTTCAAGATCATTTATCTTG	—[a]	
		SText2	CCCAAAAGAGCTTTTGTGGGTTC	—[a]	

[a] In the primer extension reaction the amplified fragment obtained with SText1 was 32 to 58 nucleotides downstream from the putative rel_{Bbu} translation start site on the *B. burgdorferi* chromosome, and the amplified fragment obtained with SText2 was 207 to 229 nucleotides upstream from the putative rel_{Bbu} translation start site.

manufacturer. Only the log-linear portion of the amplification results was used for analysis. Background fluorescence was removed by setting a noise band, and a standard curve was prepared by plotting the crossing point versus the log of copy number based on standards included in each run. Copy numbers for the experimental samples were calculated by comparing the crossing points of the samples with those of the standards. Melting curves were used to determine the specificities of the PCR products. Genomic DNA from 10^3 to 10^6 *B. burgdorferi* cells was used as a standard to estimate the copy number of *flaB* or 16S rRNA. Samples with identical amounts of *flaB* were assumed to contain the same amount of *B. burgdorferi* total RNA (11).

Detection of *B. burgdorferi* in ticks. DNA was isolated from ticks by using an Isoquick nucleic acid extraction kit (ORCA Research Inc., Bothell, Wash.) according to the manufacturer's protocol. *B. burgdorferi* DNA was detected by PCR performed with primers 49 and 50 for *flaB* (12) (Table 1).

Growth of *B. burgdorferi* in peritoneal chambers in rats. Spectra/Por dialysis membrane chambers (Spectrum Medical Industries Inc., Los Angeles, Calif.) containing 10^3 *B. burgdorferi* N40 cells/ml in 5 ml of BSK-H medium were implanted in the peritonea of 4- to 6-week-old Sprague-Dawley rats (1). The chambers were removed on the eighth day after implantation. The concentration of *B. burgdorferi* at this time was approximately 10^7 cells/ml.

Immunoblot analysis. *B. burgdorferi* N40 cell lysates were electrophoresed in 10 to 20% polyacrylamide gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and the resolved proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech) and screened with either mouse anti-*B. burgdorferi* FlaB monoclonal antibodies H9724 (4), mouse anti-*B. burgdorferi* OspA, monoclonal antibodies H5332 (3), or rabbit anti-*Bacillus subtilis* Rel_{Bsu} polyclonal antibodies (38). Blots were developed with peroxidase-conjugated anti-mouse immunoglobulin G (Fc specific) (Sigma) or peroxidase-conjugated anti-rabbit immunoglobulin G (Sigma). Proteins were detected by using enhanced chemiluminescence technology (ECL Plus; Amersham Pharmacia Biotech).

Primer extension analysis. *B. burgdorferi* B31 and N40 nucleic acids were isolated as described above. The rel_{Bbu} transcription start was identified by a primer extension reaction performed with primer SText1 or SText2 (Table 1). Extension reactions were performed with the Primer Extension System-AMV Reverse Transcriptase (Promega). Extension products were resolved in a 6% denaturing polyacrylamide gel containing 8 M urea and were visualized by autoradiography. To generate a sequence ladder for the extension product, sequencing reactions with pBlue-ST DNA were performed with a T7 Sequenase Quick-Denature plasmid sequencing kit (Amersham Pharmacia Biotech) directed by the same primer that was used for primer extension.

Nucleotide sequence accession numbers. The nucleotide sequences of the *B. burgdorferi* BL206 and N40 rel_{Bbu} genes have been deposited in the GenBank database under accession numbers AY074789 and AY074790, respectively.

RESULTS

Detection of (p)ppGpp in *B. burgdorferi* during growth under different conditions. (p)ppGpp is produced by the noninfectious B31 and infectious N40 *B. burgdorferi* strains growing in BSK-H medium at 34°C and pH 7.6 (5). To determine if the levels of (p)ppGpp and the ATP/GTP/(p)ppGpp ratio changed under increased-starvation conditions, B31 and N40 were grown in BSK-II and BSK-H media containing 10-fold less rabbit sera than the complete media, in BSK-II medium containing10-fold less yeastolate and neopeptone than the complete medium, and in BSK-II and BSK-H media containing a competitive inhibitor of protein synthesis, serine hydroxamate (36). Serine hydroxamate (1 mg/ml) completely inhibited the growth of *B. burgdorferi* B31 in BSK-II medium (Fig. 1) and the growth of N40 in BSK-H medium (data not shown). These changes, while decreasing the growth rate, did not alter the observed high basal levels of ppGpp (Fig. 2) and the ATP/GTP/ppGpp ratio in either strain (1:4:1 for N40 and 8:2:1 for B31) compared to the basal levels of ppGpp and the ATP/GTP/ppGpp ratio in *B. burgdorferi* grown in complete BSK-II or BSK-H medium. Synthesis of ppGpp was constant for both *B. burgdorferi* strains in all growth media from 10 min to 5 days.

Expression of *B. burgdorferi* rel_{Bbu} mRNA, Rel_{Bbu} protein, and genes of the *bmp* chromosomal cluster during in vitro and in vivo growth. During growth in complete BSK-H medium containing 6% rabbit serum, N40 contained twice as much

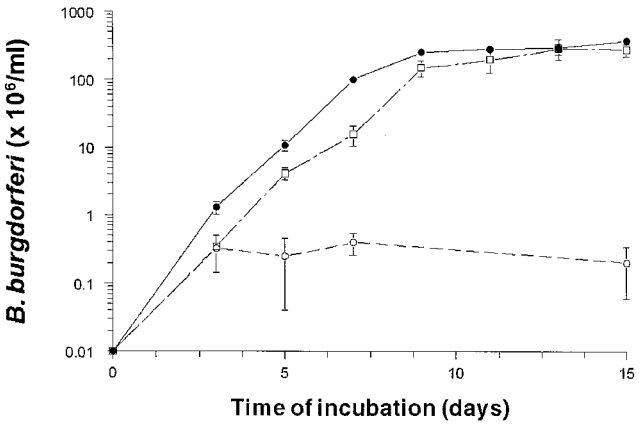

FIG. 1. Inhibition of growth of *B. burgdorferi* B31 in BSK-II medium (●) by 0.1 mg of serine hydroxamate per ml (□) and by 1 mg of serine hydroxamate per ml (○). See Materials and Methods for details.

rel_{Bbu} mRNA as B31 contained (4.0 ± 0.8 fg of rel_{Bbu} mRNA/ng of total *B. burgdorferi* RNA for N40 versus 2.0 ± 0.5 fg/ng of total RNA for B31) (Table 2, Fig. 3A). The rel_{Bbu} mRNA levels in B31 growing in BSK-II and BSK-H media were identical (data not shown). There was no difference in rel_{Bbu} mRNA levels between N40 grown at 37°C in complete BSK-H medium and N40 grown in BSK-H medium containing only 0.6% rabbit serum (data not shown), nor was there any difference in rel_{Bbu} mRNA levels between N40 grown in complete BSK-H medium at 37 and 23°C (data not shown). The N40 rel_{Bbu} mRNA levels were the same (4.0 fg/ng of total RNA) during growth in rat peritoneal chambers as during growth in BSK-H medium at 37°C (Table 2, Fig. 3A). In contrast, rel_{Bbu} mRNA was not detected during N40 growth in engorged nymphal ticks (Table 2, Fig. 3B).

TABLE 2. Expression of mRNAs of selected *B. burgdorferi* genes under various culture conditions

Gene(s)	mRNA expression (fg of mRNA/ng of total *B. burgdorferi* RNA)			
	BSK-H medium		Nymphal ticks (strain N40)	Rat peritoneal chamber and BSK-H medium (strain N40)
	Strain B31	Strain N40		
rel_{Bbu}	2	4		4
bmpA	6.5	6.5	6.5	6.5
bmpB	2	2	2	2
bmpC	0.1	0.5		0.5
bmpD	2	2	2	2
rpsL-bmpD	0.4	0.06	ND[a]	ND

[a] ND, not determined.

Previous studies indicated that there is a correlation between attenuation of the stringent response in *B. burgdorferi* cocultured with tick cells [as manifested by undetectable levels of (p)ppGpp] and modulation of expression of *rpsL-bmpD* and *bmpD* messages (5). Moreover, the fact that expression of *bmpD* was linked to expression of the ribosomal protein gene

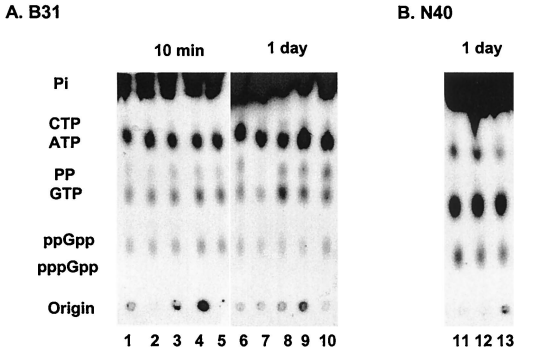

FIG. 2. (p)ppGpp accumulation in *B. burgdorferi* under various culture conditions. (A) *B. burgdorferi* B31 was grown in BSK-II medium containing [^{32}P]orthophosphate for 36 h and then centrifuged and resuspended in the same amount of BSK-II medium (lanes 1 and 6), in BSK-II medium containing 10-fold-lower levels of rabbit serum (final concentration, 0.6%) (lanes 2 and 7), yeastolate (lanes 3 and 8), or neopeptone (lanes 4 and 9), or in BSK-II medium containing 1 mg of DL-serine hydroxamate per ml (lanes 5 and 10) (all resuspension media contained [^{32}P]orthophosphate) for 10 min or 1 day. (B) *B. burgdorferi* N40 was grown in BSK-H medium containing [^{32}P]orthophosphate for 2 days, centrifuged, and resuspended in the same amount of BSK-H medium (lane 11), in BSK-H medium containing 10-fold-lower levels of rabbit serum (lane 12), or in BSK-H medium containing 1 mg of DL-serine hydroxamate per ml (lane 13) (all resuspension media contained [^{32}P]orthophosphate) for 1 day. Cells were collected by centrifugation after growth for 10 min or 1 day, and (p)ppGpp was extracted as described in Materials and Methods.

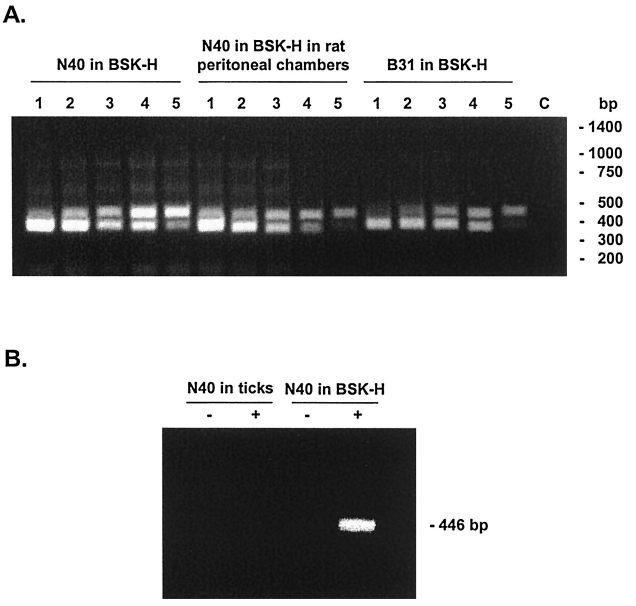

FIG. 3. RT-PCR analysis of *B. burgdorferi* rel_{Bbu} mRNA. (A) Relative concentrations of rel_{Bbu} mRNA in *B. burgdorferi* N40 grown in BSK-H medium or in BSK-H medium in rat peritoneal chambers or in *B. burgdorferi* B31 grown in BSK-H medium. rel_{Bbu} mRNA was detected by competitive RT-PCR. All reaction mixtures contained 1 ng of *B. burgdorferi* total RNA. The amounts of competitor were 16, 8, 4, 2, and 1 fg in lanes 1 to 5, respectively. Lane 3 for N40 and lane 4 for B31 showed equal signal intensities for rel_{Bbu} mRNA (upper band) and the competitor (lower band), indicating that in these reaction mixtures they were present at equal concentrations. In lane C RNA was omitted. (B) rel_{Bbu} mRNA in *B. burgdorferi* N40 in BSK-H medium and in ticks. −, reverse transcriptase omitted; +, complete reaction mixture for specific RNA detection.

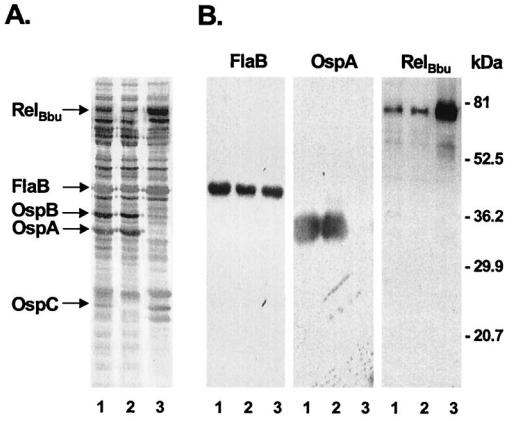

FIG. 4. Rel_{Bbu} protein in *B. burgdorferi* N40 grown in BSK-H medium at 37°C (lanes 1) or at 23°C (lanes 2) or in rat peritoneal chambers containing BSK-H medium (lanes 3). (A) Silver staining of *B. burgdorferi* proteins. Downmodulation of OspA and OspB and upregulation of OspC (lane 3) indicated that there was host adaptation of *B. burgdorferi* in rat peritoneal chambers. (B) Immunoblot analysis of *B. burgdorferi* proteins with anti-*B. burgdorferi* FlaB monoclonal antibodies (loading control), anti-*B. burgdorferi* OspA monoclonal antibodies (host adaptation control), and anti-*B. subtilis* Rel_{Bsu} polyclonal antibodies. The positions of protein molecular mass standards are indicated on the right. In the FlaB panel the similar intensities of FlaB in lanes 1, 2, and 3 indicate that the amounts of total *B. burgdorferi* protein loaded in lanes 1, 2, and 3 were the same. In the OspA panel the absence of OspA in lane 3 is characteristic of host-adapted *B. burgdorferi* growing in rat peritoneal chambers (1; Caimano et al., unpublished).

rpsL suggested that *bmpD* expression might be indirectly regulated by the stringent response by the levels of rRNA synthesis (7). Therefore, expression of the entire *bmp* gene family under several in vivo growth conditions was examined (Table 2). *B. burgdorferi*-specific RNA and DNA were not detected in uninfected tick nymphs. Growth of *B. burgdorferi* N40 in engorged nymphs was accompanied by clear downmodulation of rel_{Bbu} expression but not by changes in expression of *bmpA*, *bmpB*, or *bmpD* compared to the expression seen during in vitro growth in BSK-H medium (Table 2). The inability to detect *bmpC* mRNA in engorged ticks (Table 2) could have been a result of the relatively small amount of *B. burgdorferi* mRNA obtained under these conditions, as well as the generally limited expression of this gene (5, 12). The levels of transcription of the rel_{Bbu} and *bmp* genes were identical during growth of N40 in rat peritoneal chambers and in BSK-H medium in vitro (Table 2).

B. burgdorferi Rel_{Bbu} (14) is 43% identical to *B. subtilis* Rel_{Bsu} (38) over a 450-amino-acid span, suggesting that polyclonal anti-Rel_{Bsu} antibodies could be used for immunoblot analysis of Rel_{Bbu} in cell lysates. Immunoblot analysis with such antibodies of lysates of N40 grown in BSK-H medium detected a protein with an M_r of 78,000, which is similar to the M_r predicted for Rel_{Bbu} (Fig. 4B, Rel_{Bbu} panel, lanes 1 and 2). Despite identical rel_{Bbu} transcription levels during N40 growth in rat peritoneal chambers and BSK-H medium in vitro (Table 2, Fig. 3A), the levels of Rel_{Bbu} protein appeared to be much higher in host-adapted *B. burgdorferi* growing in rat peritoneal chambers than in an in vitro culture (Fig. 4B, Rel_{Bbu} panel, compare lane 3 with lanes 1 and 2).

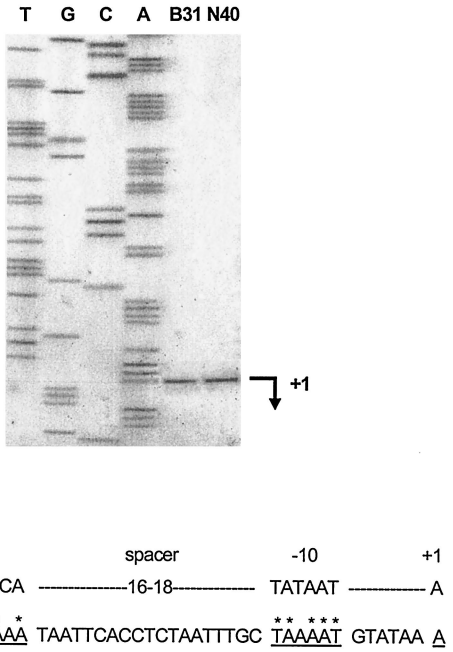

FIG. 5. Primer extension analysis of *B. burgdorferi* B31 (lane B31) and N40 (lane N40) mRNAs and sequencing ladder of the homologous region of the B31 rel_{Bbu} gene (lanes T, G, C, and A). Products having similar sizes were generated with AMV reverse transcriptase from B31 and N40. The arrow indicates the position of the primer extension products in relation to the sequencing ladder. +1, rel_{Bbu} transcription site. The putative −10 promoter sequence and the putative −35 promoter sequence are underlined in the nucleotide sequence of the region upstream of rel_{Bbu}. Homology of the deduced rel_{Bbu} promoter with the *E. coli* σ^{70} promoter (21) is indicated by asterisks.

Transcription of rel_{Bbu} from a σ^{70}-like promoter. Primer extension analysis was used to determine whether differences in the levels of production of (p)ppGpp between noninfectious and infectious *B. burgdorferi* strains could be explained by differences in rel_{Bbu} promoters. The rel_{Bbu} promoter is located within the open reading frame (ORF) that is 5′ to rel_{Bbu} (BB0197); this ORF encodes a putative protoporphyrinogen oxidase. Transcription of the rel_{Bbu} gene began from a σ^{70}-like promoter at a transcriptional start site located 308 nucleotides upstream from the rel_{Bbu} ATG translational start in both *B. burgdorferi* B31 and N40 growing in BSK-H medium (Fig. 5). The putative rel_{Bbu} promoter, like the putative *bmpD* promoter, has a 19-nucleotide space between the −10 and −35 elements (12). It is also possible that the putative rel_{Bbu} promoter has an extended −10 element.

DNA sequence analysis of rel_{Bbu} in *B. burgdorferi* B31, N40, and BL206. Since the rel_{Bbu} transcription start was the same in N40 and B31, an effort was made to determine if there might be differences in promoter sequences and other regulatory signals in regions upstream and downstream from the rel_{Bbu} ORF. DNAs from infectious strains N40 and BL206 were amplified and sequenced in a region comprising 455 nucleotides upstream of the rel_{Bbu} putative start codon and 55 nucleotides downstream of the putative termination codon, and the sequences were compared to the B31 sequence in this region (14). The DNA sequences of the entire rel_{Bbu} region of BL206 and B31 were identical. There were no sequence differences

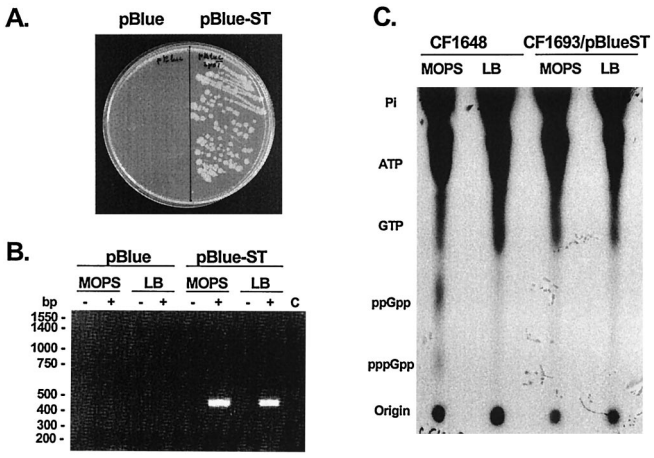

FIG. 6. Complementation of *E. coli* CF1693 (*relA*$^-$ *spoT*$^-$) with *B. burgdorferi* rel_{Bbu} homologue. (A) *E. coli* CF1693 was transformed with pBluescript IISK+ (left side) or pBlue-ST plasmid DNA (right side) and grown on a 1.5% agar–M9 medium plate. (B) *B. burgdorferi* rel_{Bbu} mRNA synthesis by CF1693 containing pBlue-ST following growth in MOPS starvation medium or LB medium. −, reverse transcriptase omitted; +, complete reaction mixture for specific RNA detection. In lane C RNA was omitted. (C) (p)ppGpp accumulation in *E. coli*, as determined with wild-type strain CF1648 and the CF1693 *relA*$^-$ *spoT*$^-$ double mutant containing pBlue-ST in MOPS starvation medium or LB medium.

among B31, BL206, and N40 in the 55 nucleotides downstream from the rel_{Bbu} coding region. Compared to the B31 and BL206 DNA sequences, the N40 DNA sequence had 5 nucleotide substitutions in the 455 nucleotides upstream from the N40 rel_{Bbu} start codon, all of which were outside the identified promoter, and 14 nucleotide substitutions in the 2,001 nucleotides of the coding sequence. Most substitutions in the coding region were silent, and only two led to amino acid changes. Substitution of an A (in N40) at position 1594 beginning from the translation start for a G (in B31) resulted in a change from Ala (B31) to Thr (N40) at amino acid 532 in the Rel_{Bbu} protein, while substitution of an A at position 1708 (N40) for a C (B31) resulted in a change from Pro (B31) to Thr (N40) at amino acid 570.

Expression of *B. burgdorferi* rel_{Bbu} in *E. coli*. The functionality of the *B. burgdorferi* rel_{Bbu} gene was confirmed by transformation of an *E. coli* CF1693 *relA spoT* double mutant with pBlue-ST containing the *B. burgdorferi* B31 rel_{Bbu} gene, including 446 bp upstream from its start codon and 109 bp downstream from its stop codon. Untransformed *E. coli* CF1693 grew on LB agar but not on M9 minimal agar, while *E. coli* CF1693 transformed with the recombinant plasmid containing the *B. burgdorferi* rel_{Bbu} gene grew well on both media (Fig. 6A). *B. burgdorferi* rel_{Bbu} mRNA was detected in transformed *E. coli* CF1693 cells whether they were grown in MOPS starvation medium or in LB medium (Fig. 6B). The cross-reactivity of the polyclonal anti-Rel_{Bsu} antibodies with a protein that has an M_r similar to that of Rel_{Bbu} in *E. coli* lysates (CF1648 and CF1693) made it impossible to identify Rel_{Bbu} expression in CF1693 cells transformed with pBlue-ST (data not shown). (p)ppGpp could not be detected in the double mutant or in cells transformed with *B. burgdorferi* rel_{Bbu} (Fig. 6C), while both pppGpp and ppGpp were readily demonstrable in wild-type *E. coli* grown in MOPS starvation medium but not in LB medium.

Influence of the stringent response on 16S rRNA synthesis. Because rRNA gene number and organization in *B. burgdorferi* differ so markedly from rRNA gene number and organization in *E. coli* (14, 16, 25), because the mechanisms controlling rRNA transcription in *B. burgdorferi* are uncharacterized, and because slow growth of *B. burgdorferi* in the presence of tick cells was accompanied by an abrogation of the stringent response (5), the influence of the stringent response and slow growth on transcription of the single *B. burgdorferi* 16S rRNA gene was examined. This single gene and $tRNA^{Ala}$ appear to be a single transcriptional unit (16). Real-time PCR indicated that *B. burgdorferi* N40 growing in BSK-H medium contained 294 ± 15 copies of 16S rRNA/pg of total *B. burgdorferi* RNA, while N40 cocultured with tick cells contained 102 ± 15 copies of 16S rRNA/pg of total *B. burgdorferi* RNA (means ± standard errors of the means, based on three independent experiments). The decreased synthesis of 16S rRNA in N40 cocultured with tick cells compared to the synthesis in BSK-H medium was highly significant ($P < 0.01$, as determined by a *t* test) and was correlated with slow growth of *B. burgdorferi* in the presence of tick cells rather than with the decrease in (p)ppGpp production seen under these conditions (5). Analysis of the regulatory DNA sequences of the 16S rRNA in *B. burgdorferi* failed to identify a GCGC discriminator motif immediately downstream from the −10 region for both putative 16S rRNA promoters in *B. burgdorferi* identified by sequence analysis (14, 16). In *E. coli*, this discriminator motif is associated with regulation of rRNA synthesis by the stringent response (42).

DISCUSSION

Limitation of intracellular amino acid availability and energy sources classically triggers the bacterial stringent response mediated by the alarmon (p)ppGpp (7). We previously detected production of (p)ppGpp in rapidly growing *B. burgdorferi* in BSK-H medium and the ablation of (p)ppGpp synthesis during slower growth of *B. burgdorferi* in the presence of tick cells (5). The changes in (p)ppGpp concentration were accompanied by changes in the transcription of chromosomally encoded genes, indicating the potential ability of (p)ppGpp to modulate transcription, as it does in the stringent response in other bacteria (7, 20, 37). The results described here extended our observations regarding the presence of (p)ppGpp and the functionality of *B. burgdorferi* rel_{Bbu} by identification of the promoter, determination of the amounts of rel_{Bbu} mRNA in *B. burgdorferi* strains grown under different conditions, detection the Rel_{Bbu} protein, and demonstration of the ability of the Rel_{Bbu} protein to complement an *E. coli relA spoT* double mutant.

Synthesis of larger amounts of ppGpp, expression of higher levels of rel_{Bbu} mRNA, and slower growth of the infectious N40 strain compared to the results obtained with the noninfectious B31 strain during growth in BSK-II and BSK-H media could suggest that these strains respond differently to similar environmental stimuli. The identity of the rel_{Bbu} regulatory regions in the noninfectious and infectious strains rules out the possibility that the observed quantitative differences in transcription

between the noninfectious and infectious strains result from mutations in this region.

These results raise important questions regarding the role of (p)ppGpp in *B. burgdorferi* and its role in other bacteria as an indicator of nutritional and other stresses. The presence of (p)ppGpp, rel_{Bbu} mRNA, and the Rel_{Bbu} protein in *B. burgdorferi* growing in BSK-II medium and in BSK-H medium, which putatively are rich media, and our inability to upmodulate this response in these media by nutritional manipulations were unexpected, since in other bacteria the levels of (p)ppGpp produced during growth in culture media that do not fulfill nutritional requirements can be modulated by manipulation of medium nutrients (7, 28, 37). Equally unexpected was our observation that the presence of (p)ppGpp during N40 growth in BSK-H medium was associated with rapid growth but not with a decrease in 16S rRNA synthesis, while the decreased N40 growth rate seen during coculture with tick cells, growth conditions that decreased the levels of (p)ppGpp to undetectable levels (5), was associated with decreased 16S rRNA synthesis. However, these observations may not be as surprising as first supposed since even in the paradigmatic organism *E. coli*, rRNA synthesis can be regulated by a growth rate-dependent control system, as well as by the stringent response (15).

It is clearly possible that the *B. burgdorferi* stringent response involves as-yet-uncharacterized stimuli, regulatory mechanisms, and pathways that are different from those in other bacteria, so a model based on *E. coli* fails to predict the relevant changes in *B. burgdorferi* (20). The levels of (p)ppGpp and rel_{Bbu} mRNA decreased compared to the levels seen during growth in BSK-H medium when N40 was grown with tick cells (5) or in engorged ticks (this study), suggesting that the latter conditions may modulate the production of (p)ppGpp in a similar manner. The coexistence of production of (p)ppGpp and increased transcription of rel_{Bbu} in *B. burgdorferi* provides some support for the hypothesis that the environmental stimuli that trigger and regulate the stringent response in *B. burgdorferi* are different from the environmental stimuli that trigger and regulate the stringent response in other bacteria (6, 20, 37). This is not entirely unexpected as there appear to be differences among bacteria regarding the role of the stringent response in metabolism (28). The apparently different amounts of Rel_{Bbu} protein encountered in *B. burgdorferi* growing in BSK-H medium and in rat peritoneal chambers (Fig. 4) in spite of the identical levels of rel_{Bbu} mRNA (Table 2, Fig. 3A) suggests that the concentration of this protein may be regulated posttranscriptionally. The higher level of Rel_{Bbu} expression in rat peritoneal chambers is consistent with data showing that *B. burgdorferi* strains are stressed under these conditions (30; M. J. Caimano, C. H. Eggers, J. E. Purser, S. J. Norris, and J. D. Radolf, unpublished data).

Further evidence that there are differences between the stringent response in *B. burgdorferi* and the stringent response in other bacteria came from our unexpected observation that synthesis of (p)ppGpp in *B. burgdorferi* was not associated with a decreased growth rate and decreased synthesis of the single 16S rRNA gene in the *B. burgdorferi* genome, a situation at variance with what occurs in *E. coli* or in other bacteria (7, 37). This suggests that there is dissociation between the effects of the stringent response mediated by (p)ppGpp and slow growth on synthesis of 16S rRNA in *B. burgdorferi*, as the latter condition, and not the increased levels of (p)ppGpp, was associated with downregulation of rRNA synthesis. A major limitation in the analysis of rRNA synthesis in these experiments was the lack of knowledge regarding the structure of the promoters of rRNA genes in *B. burgdorferi* and the organization of the transcription and processing (16). For example, we do not know whether in *B. burgdorferi* rRNA is expressed alternatively from two promoters, as it is in *E. coli*, and whether expression is influenced by upstream DNA sequences or DNA supercoiling; we also do not know the σ and other transcriptional factors, such as FIS and H-NS homologues, that regulate initiation of transcription of rRNA genes in other bacteria (25, 37). Analysis of the regulatory DNA sequences of the rRNA genes in *B. burgdorferi* did not identify a discriminator motif associated in *E. coli* with the regulation of rRNA synthesis by the stringent response (37, 42), and this finding potentially could explain the dissociation which we found between the presence of the stringent response and the lack of downregulation of 16S rRNA synthesis.

Production of (p)ppGpp could potentially have a central role in regulating growth, gene expression, and host adaptation in *B. burgdorferi*, an extracellular bacterium whose reduced genome contains few σ factors and few identified regulatory molecules (14). Under in vitro conditions that may correspond to those in flat ticks (BSK-H medium, pH 7.5, 23°C) and engorged ticks (BSK-H medium, pH 6.8, 37°C), DNA microarray analysis of *B. burgdorferi* gene expression revealed a twofold increase in rel_{Bbu} transcription and few other changes in regulatory gene expression, while *bmpD* expression decreased sixfold at the lower temperature (30). In the present study, *bmpD* expression was the same in N40 growing in BSK-H medium (pH 7.6, 34°C) and in engorged ticks, while rel_{Bbu} expression decreased under these conditions (Table 2). These findings in turn contrast with our previous findings which showed that downregulation of rel_{Bbu} expression during coculture with tick cells was accompanied by decreases in the levels of (p)ppGpp and *bmpD* mRNA (5). However, our results are consistent with the results obtained with DNA microarrays regarding the changes in expression of rel_{Bbu} and *bmpD* when *B. burgdorferi* is shifted between media simulating different environments (30). Unfortunately, absolute comparisons between our competitive RT-PCR data and the data from microarray studies are impossible due to differences in culture conditions, differences in the genetic makeup of the strains, such as plasmid composition, and differences in the methods of analysis of gene expression.

The deduced sequence of the *B. burgdorferi* Rel_{Bbu} protein is similar to the sequences of *E. coli* SpoT and RelA, *Mycobacterium tuberculosis* Rel_{Mtu}, *Streptomyces coelicolor* Rel_{Sco}, and orthologues in other bacteria (28). The fact that the catalytic domains of these proteins are located within the N-terminal regions of the proteins (17, 27, 35) and the fact that we did not find any differences in the deduced amino acid sequence in this region in noninfectious and infectious *B. burgdorferi* strains suggest that any differences in the stringent response in these strains are not due to different enzymatic activities of their Rel_{Bbu} proteins. The finding that the levels of rel_{Bbu} mRNA paralleled the levels of (p)ppGpp suggests that expression of rel_{Bbu} might be regulated by stress-related σ^S and σ^{54} promot-

ers. Primer extension experiments ruled out transcription from a σ^{54} promoter but not transcription from a σ^S promoter, since σ^S recognizes promoters that are similar to those recognized by σ^{70} (21, 37). The location of the *rel*$_{Bbu}$ σ^{70} promoter within the ORF encoding protoporphyrinogen oxidase and the observation that many *B. burgdorferi* genes have their promoters within the structural DNA sequence of the preceding gene may simply be reflections of the compact and reduced nature of the *B. burgdorferi* genome (12, 14). The lack of detection of (p)ppGpp in the genetically complemented *E. coli relA spoT* double mutant could have been the result of low levels of expression of this gene in *E. coli*, degradation of foreign Rel$_{Bbu}$ in the *E. coli* environment, inadequate interaction with *E. coli* ribosomes, or, as is the case with *Streptococcus equisimilis* Rel$_{Seq}$, increased hydrolase activity of the enzyme (7, 27, 35). Rel$_{Bbu}$ may in fact not need to interact with ribosomes to be enzymatically active. For example, Rel$_{Seq}$ is enzymatically active in the absence of ribosomes (27).

Despite the fact that increasing or decreasing the production of (p)ppGpp had no effect on 16S rRNA synthesis, changes in (p)ppGpp levels could modulate *B. burgdorferi* lipoprotein gene expression. It has been shown that growth of *B. burgdorferi* with cultured tick cells decreases the (p)ppGpp concentration (5) and is accompanied by upmodulation of polycistronic *rpsL-bmpD* transcription (as would be expected for a gene encoding a ribosomal protein released from stringent control [37]), downmodulation of monocistronic *bmpD* transcription, and a net decrease in *bmpD* expression. (p)ppGpp is likely to be a central regulator of the *B. burgdorferi* response to nutritional and other stresses encountered during growth in hosts and in culture. Our results suggest that the potential modulation of gene expression by (p)ppGpp and the resultant changes in composition of *B. burgdorferi* could increase the ability of this organism to survive in inimical environments and influence its virulence.

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