Modulation of *Borrelia burgdorferi* Stringent Response and Gene Expression during Extracellular Growth with Tick Cells

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Borrelia burgdorferi* N40 multiplied extracellularly when it was cocultured with tick cells in L15BS medium, a medium which by itself did not support *B. burgdorferi* N40 growth. Growth of *B. burgdorferi* N40 in the presence of tick cells was associated with decreased production of (p)ppGpp, the stringent response global regulator, a fourfold decrease in *relA/spoT* mRNA, an eightfold net decrease in *bmpD* mRNA, and a fourfold increase in *rpsL-bmpD* mRNA compared to growth of *B. burgdorferi* in BSK-H medium. As a result, the polycistronic *rpsL-bmpD* mRNA level increased from 3 to 100% of the total *bmpD* message. These observations demonstrate that there are reciprocal interactions between *B. burgdorferi* and tick cells in vitro and indicate that the starvation-associated stringent response mediated by (p)ppGpp present in *B. burgdorferi* growing in BSK-H medium is ameliorated in *B. burgdorferi* growing in coculture with tick cell lines. These results suggest that this system can provide a useful model for identifying genes controlling interactions of *B. burgdorferi* with tick cells in vitro when it is coupled with genetic methods to isolate and complement *B. burgdorferi* mutants.

Borrelia burgdorferi, the cause of Lyme disease, modulates its gene expression in the presence of host cells. As an example of this modulation, the relative expression of the *ospA* and *ospC* genes is altered as *B. burgdorferi* passes from a tick to a mammalian host (32); a similar down-modulation–up-modulation of OspA and OspC is observed during coculture of *B. burgdorferi* with tick cells in vitro (27). This oscillation in gene expression may be mediated by a regulatory loop involving changes in the expression of σ^{54} and σ^S, suggesting that *B. burgdorferi* growing in the presence of tick cells may undergo a series of as-yet-uncharacterized global regulatory changes (36). It also suggests that study of the reciprocal interactions between *B. burgdorferi* and tick cells could illuminate aspects of gene modulation in *B. burgdorferi* and the genes and gene products involved (17, 36).

Flexible adaptation by bacterial pathogens to rapidly changing host environments requires the interplay of regulators generated in response to signals triggered by environmental stimuli. One such adaptive response is the stringent response, a global response triggered by nutritional stress when protein synthesis is blocked by amino acid starvation (10, 13, 34). The stringent response causes up-regulation of protein degradation and amino acid synthesis, down-regulation of nucleic acid and protein synthesis, and alteration of many cellular functions, including transcription, translation, replication, transport, and metabolism, and it is accompanied by slow growth (10, 34). In *Escherichia coli*, the stringent response involves RelA, a synthase, and SpoT, a hydrolase, which synthesize and degrade, respectively, ppGpp and pppGpp [(p)ppGpp], the alarmon (master regulator, second message) that is the proximate mediator of this response (9, 10). In some bacterial pathogens,

such as *Mycobacterium tuberculosis*, in which the stringent response is related to induction of genes involved in the long-term persistence of the pathogen in the host (28), a single *relA/spoT* homologue codes for a protein with both synthase and hydrolase activities (1). Synthesis of (p)ppGpp by RelA (or RelA/SpoT) is triggered by uncharged tRNA in the ribosome and results in changes in levels of gene transcription by directly influencing transcription initiation and elongation (33, 34) and by modulating transcription levels of transcriptional factors, such as σ^S (20, 21).

The genome of *B. burgdorferi* contains a single chromosomal *relA/spoT* homologue (BB0198) (15), suggesting that the stringent response might be present in this species. Here we show that extracellular multiplication of *B. burgdorferi* in the presence of tick cell lines (25, 27) is associated with amelioration of the *B. burgdorferi* stringent response compared to the response in organisms cultured in BSK-H medium and is accompanied by changes in transcription of *relA/spoT* and *bmpD*, a member of a chromosomally located paralogous gene family encoding in vivo-expressed lipoproteins (14). These results suggest that this system can provide a useful model for identifying genes controlling interactions of *B. burgdorferi* with tick cells in vitro and, by extension, in whole ticks, when it is coupled with recently developed genetic methods to isolate and complement *B. burgdorferi* mutants (4, 7, 31).

MATERIALS AND METHODS

***B. burgdorferi* strains and culture.** Infectious, low-passage-number *B. burgdorferi* N40 was kindly provided by Linda Bockenstadt, Yale University. Noninfectious, high-passage *B. burgdorferi* B31 (ATCC 35210) was obtained from the American Type Culture Collection (Manassas, Va.). *B. burgdorferi* strains were maintained at 34°C in BSK-H medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 6% rabbit serum (Sigma).

Tick cell lines and coculture with *B. burgdorferi*. IDE8 and ISE6 tick cell lines isolated from embryonated eggs of northern and southern populations of *Ixodes scapularis*, respectively, were kindly provided by Ulrike Munderloh, University of Minnesota, St. Paul. Tick cell lines were maintained at 32°C in antibiotic-free L15B medium (27) supplemented with 5% fetal bovine serum (Gibco, Grand

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Island, N.Y.), 0.1% bovine lipoprotein concentrate (Irvine Scientific, Santa Ana, Calif.), and 10% Difco tryptose phosphate broth (Becton Dickinson Microbiology Systems, Sparks, Md.) in Falcon 25-cm^2 tissue culture flasks (Becton Dickinson Labware, Franklin Lakes, N.J.). Before tick cell cultures were infected, *B. burgdorferi* and tick cell monolayers were each washed once with phosphate-buffered saline (PBS) (pH 7.4). Tick cell monolayers (80 to 90% confluence, approximately 2×10^6 to 4×10^6 tick cells/ml) in L15BS medium (L15B medium supplemented with 5% fetal bovine serum, 0.1% bovine lipoprotein concentrate, 10% tryptose phosphate broth, 25 mM *N*-acetylglucosamine [Sigma], 25 mM piperazine-*N*,*N*-bis-2-ethanesulfonic acid [Sigma]) (27) in 25-cm^2 tissue culture flasks (6 ml of medium/flask) or in 9.0-cm^2 slide flask chambers (Nunc, Roskilde, Denmark) (2 ml of medium/flask) were infected with 2×10^7 to 4×10^7 *B. burgdorferi* cells/ml at a multiplicity of infection (MOI) of 10 unless otherwise specified and cultured at 32°C. Concentrations of *Borrelia* and tick cells were determined by phase-contrast microscopy using a counting chamber or by epifluorescence microscopy after cells were stained with acridine orange (Fisher Scientific Co., Fair Lawn, N.J.) (14).

Localization of *B. burgdorferi* in tick cell culture. IDE8 or ISE6 tick cell cultures in plastic slide cell chambers were infected with *B. burgdorferi* N40 (1×10^7 to 2×10^7 cells/ml; MOI, 5). After 1, 3, and 5 days of infection, tick cells adherent to the slide were washed twice with PBS (pH 7.4), fixed with 4% formaldehyde in PBS at 37°C, and allowed to cool at room temperature for 20 min. Following washing of the slides with PBS–0.1 M glycine and with PBS, the spirochetes were stained with mouse monoclonal anti-OspA antibody H5332 (2) diluted in PBS–5 mM MgCl$_2$–0.5% bovine serum albumin for 1 h at room temperature, washed with PBS, reacted with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) (Sigma) for 30 min at room temperature, and washed with PBS. To determine whether *B. burgdorferi* was located intracellularly, tick cells were then permeabilized with 0.1% Triton X-100 in PBS and stained with monoclonal anti-OspA antibody H5332 and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG (Sigma). Treatment of formaldehyde-fixed *B. burgdorferi* with Triton X-100 did not change the number of *B. burgdorferi* cells stained with the anti-OspA antibody. Stained *B. burgdorferi* cells were visualized by confocal epifluorescence microscopy by using a laser scanning confocal imaging system (MRC-1000; Bio-Rad Microscience Division, Herts, United Kingdom). Samples were optically sectioned, and the number of organisms stained with FITC- and TRITC-conjugated anti-mouse IgG in five oil power fields of the integrated image on each slide were determined. The results of two independent experiments were pooled.

Phosphatidylserine externalization analysis. After 1, 3, 5, and 7 days of infection with 1×10^7 to 2×10^7 *B. burgdorferi* N40 cells/ml (MOI, 5), tick cell cultures were stained with FITC-conjugated annexin V and propidium iodide by using an ApoAlert annexin V-FITC kit according to the manufacturer's instructions for adherent cells (Clontech Laboratories, Inc., Palo Alto, Calif.). Apoptotic and necrotic cells were visualized by epifluorescence microscopy. Cells that had bound annexin V-FITC (apoptotic cells) showed green staining of the plasma membrane; necrotic cells that had lost membrane integrity showed red staining (propidium iodide) throughout the nucleus.

Detection of DNA ladders. Total DNA was extracted from IDE8 tick cells grown in 25-cm^2 flasks for 1, 3, and 5 days in the absence or presence of *B. burgdorferi* N40. To isolate DNA, tick cells were scraped from each flask, centrifuged for 5 min at $300 \times g$, and resuspended in 3 ml of 10 mM Tris-HCl (pH 7.6)–10 mM EDTA–50 mM NaCl. Fifty microliters of 10% sodium dodecyl sulfate and 50 μl of a proteinase K solution (10 mg/ml; Sigma) were added, the cell suspension was incubated at 42°C overnight, and DNA was extracted by a standard method (3). After RNase A (Sigma) treatment, samples were separated by electrophoresis in 1.8% agarose, stained with ethidium bromide, and visualized with UV light. A similar method was used for *B. burgdorferi* DNA isolation, except that the initial centrifugation was at $8,000 \times g$ for 20 min. Whole-cell DNA was quantified spectrophotometrically at 260 nm. The amount of DNA in the ladders was estimated by electrophoresis by using a standard preparation with a known concentration of DNA fragments (Hi-Lo DNA marker; Minnesota Molecular, Minneapolis, Minn.).

(p)ppGpp detection. *B. burgdorferi* N40 or B31 (10^6 cells/ml) was grown in BSK-H medium containing 10 μCi of uniformly labeled [^{32}P]orthophosphate (Amersham Pharmacia Biotech, Piscataway, N.J.) per ml. *B. burgdorferi* N40 (10^7 cells/ml) was also grown in the presence of IDE8 tick cells in L15BS medium containing [^{32}P]orthophosphate. After 2 to 4 days of growth, labeled cells were harvested from 6-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 (TLC) plate (Selecto Scientific, Suwanee, Ga.). Samples were fixed in methanol for 5 min and dried. The TLC plates were developed in 1.5 M KH$_2$PO$_4$ (pH 3.4), air dried, and exposed to

Hyperfilm MP (Amersham Pharmacia Biotech) for 18 to 48 h at $-20°C$. Nucleotide identities were verified by cochromatography with standards (ATP, GTP, and ppppG [Sigma]) after two-dimensional polyethyleneimine TLC with 3.3 M ammonium formate–4.4% boric acid (pH 7.0) in the first dimension and 1.5 M KH$_2$PO$_4$ (pH 3.4) in the second dimension (8, 24).

DNA isolation and cloning of *B. burgdorferi* *relA*/*spoT*. DNA was isolated from mid-log-phase *B. burgdorferi* B31 as described above. PCR fragments containing full-size *B. burgdorferi* *relA*/*spoT* (BB0198) were amplified by using primers ST1 (5′-GTT**GGATCC**AGAATGTTTAGCAGAAG-3′; corresponding to positions -546 to -529 upstream from the *B. burgdorferi* *relA*/*spoT* start codon) and ST2 (5′-TTCT**CTGCAG**TTTCTATGCTTGTAAT-3′; corresponding to positions 92 to 109 downstream from the *B. burgdorferi* *relA*/*spoT* stop codon) (sequences recognized by *Bam*HI [for ST1] and *Pst*I [for ST2] are underlined). All restriction enzymes were obtained from New England Biolabs, Beverly, Mass. PCR was performed in capillary tubes with a RapidCycler (Idaho Technology, Salt Lake City, Utah). After restriction with *Bam*HI and *Pst*I, the resulting amplified PCR fragment was inserted into pBluescript IISK+ (5) to produce pBlue-ST encoding *B. burgdorferi* *relA*/*spoT*. DNA manipulations were performed by standard methods (30).

RNA isolation and competitive RT-PCR analysis. Total RNA from *B. burgdorferi* N40 cocultured with tick cells for 5 to 7 days and total RNA from *B. burgdorferi* B31 and N40 cultured in BSK-H medium were isolated by the guanidine thiocyanate-phenol-chloroform method (11). Competitive reverse transcription (RT)-PCR assays were performed with the Access RT-PCR system (Promega Corporation, Madison, Wis.). The reaction mixtures (10 μl) contained reaction buffer, 1.5 mM MgSO$_4$ (for *bmpA*, *bmpB*, *bmpC*, *flaB*, *rpsL*-*bmpD*, and *relA*/*spoT*) or 1.125 mM MgSO$_4$ (for *bmpD*), each deoxynucleoside triphosphate at a concentration of 0.2 mM, each primer at a concentration of 1 μM, 0.1 U of avian myeloblastosis virus reverse transcriptase per μl, and 0.1 U of *Tfl* DNA polymerase per μl. The primers used for RT-PCR for *flaB* and *bmp* detection have been described previously (14), with two exceptions. The sequence of primer 24 for *bmpB* detection was misprinted (14) and should be 5′-TTTGCT GCCTCAATAACACC-3′, and because of a difference in sequence between *B. burgdorferi* strains B31 and N40 in the region of primer 20 (G in N40 at position 848 in the *bmp* region [14] instead of C as in B31 [15]), primer 20a (5′-CCTA TACCAGAAAGCCCTGC-3′), located at *bmp* positions 828 to 847, was used to detect *bmpD* mRNA in N40. RT-PCR with primers 19 and 20a detects the total *bmpD* message consisting of *bmpD* and *rpsL*-*bmpD* mRNA, while RT-PCR with primers 52 and 55 detects only *rpsL*-*bmpD* mRNA. The primers used for determination of *B. burgdorferi* *relA*/*spoT* mRNA were ST3 (5′-CAAAAAAGCGGA ATTGAAGCAG-3′; positions 723 to 744 downstream from the *relA*/*spoT* start codon) and ST4 (5′-GAATATTGACTTTTGTTGGCCG-3′; positions 1147 to 1168 downstream from the *relA*/*spoT* start codon). *B. burgdorferi* *flaB* mRNA was used as a control to determine the total amount of *B. burgdorferi* mRNA present in the reaction mixtures. Construction of the *flaB* and *bmp* competitors has been described previously (14, 31). To construct the *B. burgdorferi* *relA*/*spoT* competitor, a 75-bp deletion was made in the *relA*/*spoT* gene by digesting pBlue-ST with *Sma*I and *Bsu*361 (New England Biolabs), followed by treatment with the Klenow fragment (New England Biolabs) in the presence of deoxynucleoside triphosphates and ligation. Competitor RNA was synthesized in vitro by using the Riboprobe in vitro transcription system (Promega) and was used as described previously (14). Both RT and PCR amplification were carried out in thin-wall PCR tubes (0.2 ml; Eppendorf Scientific, Westbury, N.Y.) with the RapidCycler under the following conditions: RT at 48°C for 45 min and denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 10 s, 52°C (for the *bmpC*, *bmpD*, *flaB*, and *rpsL*-*bmpD* genes) or 56°C (for the *bmpA*, *bmpB*, and *relA*/*spoT* genes) for 10 s, and 68°C for 50 s and final extension step at 68°C for 5 min. Target RNA and competitor RNA were assumed to be present at equimolar concentrations in the reaction mixtures in which they generated bands with similar intensities (14).

Statistical analysis. The significance of differences between means was analyzed by using a two-tailed Student's t test (for two means) or a one-way analysis of variance and a Tukey-Kramer multiple-comparison post test (for three or more means).

RESULTS

Growth and localization of *B. burgdorferi* N40 cocultured with tick cells. Cultures of IDE8 and ISE6 tick cell monolayers were infected with *B. burgdorferi* N40 at an MOI of 10. After infection, *B. burgdorferi* adhered tightly to tick cells; only 5% of the infecting dose could be recovered by washing at 24 h. *B.*
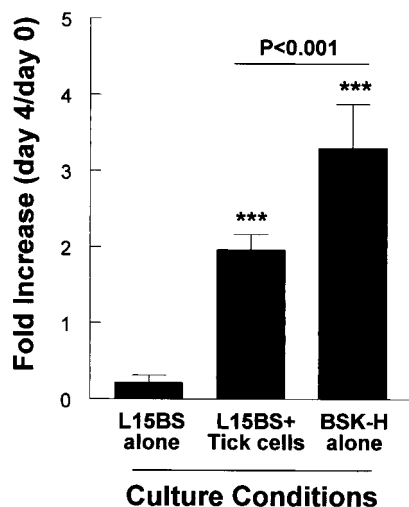
FIG. 1. *B. burgdorferi* N40 growth during coculture with tick cells: mean fold increases (± standard errors of the means) in *B. burgdorferi* N40 levels 4 days after inoculation into L15BS medium alone, into L15BS medium in the presence of tick cells (MOI, 10), and into BSK-H medium. The concentration of inoculated *Borrelia* cells was 2 × 10⁷ to 4 × 10⁷ cells/ml in each case. The results obtained with the ISE6 and IDE8 tick cell lines were similar and were pooled. Each experiment was performed at least five times. The differences in cell growth between culture conditions were highly significant. The asterisks indicate that values were significantly different from mean growth of *B. burgdorferi* in L15BS medium ($P < 0.001$, as determined by one-way analysis of variance with the Tukey-Kramer multiple-comparison post test). The difference between mean *B. burgdorferi* growth in coculture with tick cells and mean *B. burgdorferi* growth in BSK-H medium was also significant ($P < 0.001$).

burgdorferi cocultured with tick cells multiplied readily. By 4 days after infection, the *B. burgdorferi* level had increased twofold in infected cocultures of either tick cell line, but *B. burgdorferi* did not grow in L15BS medium in the absence of tick cells ($P < 0.001$) (Fig. 1). The borrelial growth during coculture with tick cells was significantly less than the growth in BSK-H medium ($P < 0.001$).

Confocal microscopy of double-stained *B. burgdorferi* in infected tick cell cultures (MOI, 5) was used to localize organisms. *B. burgdorferi* cells in these cultures were first stained with monoclonal anti-OspA and FITC-conjugated secondary antibodies, and then after tick cell permeabilization they were stained with the same anti-OspA and TRITC-conjugated secondary antibodies. Spirochetes within tick cells were stained only with TRITC, while externally located spirochetes were stained with both FITC and TRITC. Almost all *B. burgdorferi* cells in infected cell cultures (97% ± 3% [mean ± standard error of the mean] of the cells in ISE6 cultures and 99% ± 3% of the cells in IDE8 cultures) were stained with both fluorescent labels at all times examined (Fig. 2). Moreover, intracellular spirochetes were not detected within tick cells in confocal microscopy sections of infected tick cell cultures (data not shown). These data indicate that *B. burgdorferi* grows extracellularly in infected tick cell cultures.

Tick cell response to coculture with *B. burgdorferi*. The spontaneous death rate of tick cells in control cultures was less than 26%. After 4 to 5 days of coculture with *B. burgdorferi* at an

MOI of 10, cells of both tick cell lines began to detach from the flask bottoms and lyse, and the tick cell monolayers were totally destroyed by 7 to 8 days. The pH of the L15BS medium (pH 6.9 to 7.0) was unchanged throughout the course of the experiment. At an MOI of 5, the death rate of tick cells in infected cultures at 7 days was considerably less than the death rate at an MOI of 10, so that cultures infected at the lower MOI contained 7% ± 1% apoptotic cells (mean ± standard error of the mean) (surface phosphatidylserine) and 29% ± 3% necrotic cells at this time (Fig. 3). Even at the lower MOI, the levels of necrotic cells were always significantly higher in infected cultures (Fig. 3A) than in uninfected cultures, while the levels of apoptotic cells were essentially the same in both cultures (Fig. 3B). DNA fragmentation (laddering) was not demonstrable by agarose gel electrophoresis of DNA extracted from uninfected tick cell cultures but was observed with DNA extracted from tick cells incubated with *B. burgdorferi* for 1, 3, or 5 days (data not shown). The laddered DNA represented approximately 1% of the total cell DNA (up to 1 to 2 μg of fragmented DNA/100 μg of total DNA) (data not shown). The small amount of fragmented DNA in tick cells cultured with *B. burgdorferi* was consistent with the results of phosphatidylserine externalization assays and indicated that most of the tick cell death observed was due to necrosis.

***B. burgdorferi* response to coculture with tick cells: down-modulation of (p)ppGpp production and *relA/spoT* gene expression.** The growth of *B. burgdorferi* N40 in L15BS medium only in the presence of tick cells and the existence of a *relA/spoT* homologue in the *B. burgdorferi* genome (15) prompted us to determine if the *B. burgdorferi* stringent response was triggered under these conditions. As mentioned above, the stringent response is a global response characterized by synthesis of the transcriptional regulator (p)ppGpp, whose concentration is in turn regulated by the enzymatic activities of RelA/SpoT and is accompanied by modulation of the transcription of many genes (10).

B. burgdorferi N40 growing in the presence of tick cells in L15BS medium did not generate (p)ppGpp (Fig. 4A, lane 2), while *B. burgdorferi* N40 growing in BSK-H medium did (Fig. 4A, lane 1). Both *B. burgdorferi* B31 and N40 produced pppGpp and ppGpp during growth in BSK-H medium (Fig. 4B). Labeled (p)ppGpp could be detected after about 12 h of *B. burgdorferi* growth with [³²P]orthophosphate in BSK-H medium; its concentration remained unchanged for as long as 1 week after initial addition of [³²P]orthophosphate (data not shown). In addition to producing (p)ppGpp, *B. burgdorferi* B31 and N40 transcribed *relA/spoT* during growth in BSK-H medium (Fig. 5). *relA/spoT* transcription was down-modulated fourfold in *B. burgdorferi* cocultured with IDE8 tick cells (Table 1). These data indicate that growth of *B. burgdorferi* in BSK-H medium, a presumably rich medium, is accompanied by the production of (p)ppGpp and transcription of *relA/spoT*, while synthesis of this starvation-induced compound and transcription of *relA/spoT* are down-modulated during growth of *B. burgdorferi* in the presence of tick cells.

***B. burgdorferi* response to coculture with tick cells: modulation of *bmp* gene expression.** (p)ppGpp modulates expression of many bacterial genes, including those encoding ribosomal proteins (9, 10, 13). *bmpD*, a gene encoding a *B. burgdorferi* membrane lipoprotein, is transcribed in a polycistronic mRNA
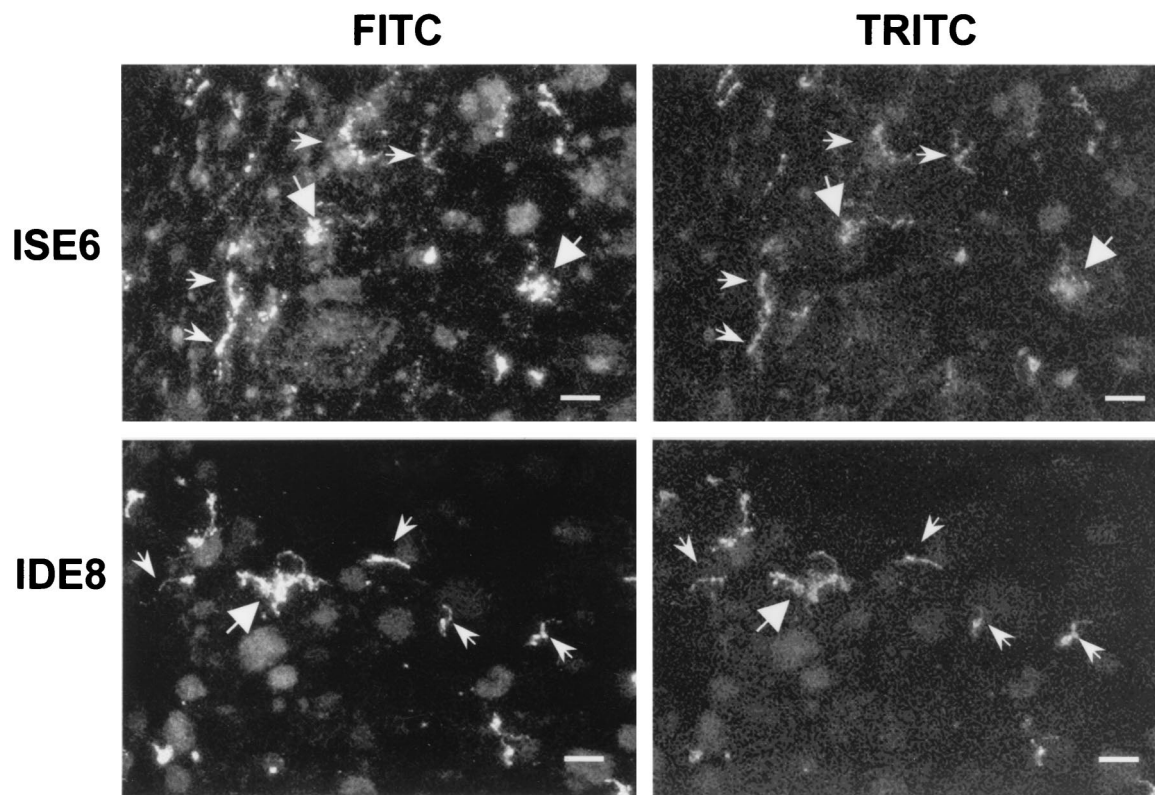
FITC

TRITC

FIG. 2. Extracellular location of *B. burgdorferi* N40 in infected tick cell cultures. *B. burgdorferi* (MOI, 5) was cultured for 3 days with IDE8 or ISE6 tick cells. Confocal integrated micrographs of *B. burgdorferi* in unpermeabilized and permeabilized tick cell cultures show similar spirochetal staining with each reagent. The small arrows indicate single *B. burgdorferi* cells, and the large arrows indicate large clumps of *B. burgdorferi* cells. Bars, 100 μm.

with ribosomal protein genes *rpsL* and *rpsG* and, as in a monocistronic mRNA, from its own promoter (14). During coculture of *B. burgdorferi* with either tick cell line, the *bmpD* mRNA levels decreased eightfold and the *rpsL-bmpD* mRNA levels increased fourfold compared to the levels found in *B. burgdorferi* growing in BSK-H medium (Table 1). As a result, *rpsL-bmpD* polycistronic mRNA, which accounted for only 3% of the total *bmpD* message in *B. burgdorferi* grown in BSK-H medium, accounted for 100% of the *bmpD* message in *B. burgdorferi* grown in the presence of tick cells. In contrast, the mRNA levels for *bmpA*, *bmpB*, and *bmpC* were similar in *B. burgdorferi* cocultured with either tick cell line and *B. burgdorferi* cultured in BSK-H medium (Table 1). *B. burgdorferi* thus responds to coculture with tick cells by down-modulation of (p)ppGpp and *relA/spoT* and by modulating *bmpD* and *rpsL* gene expression compared to its metabolic state during growth in BSK-H medium.

DISCUSSION

We have shown that *B. burgdorferi* multiplies extracellularly when it is cocultured with either IDE8 or ISE6 tick cell lines in L15BS medium but is unable to grow in this medium in the absence of tick cells. We have also demonstrated that *B. burgdorferi* has a functional stringent response, consistent with the *relA/spoT* ortholog in its genome (15) and with its encountering situations characterized by starvation in its life cycle (e.g., in

the intestines of unfed ticks) (6, 26). Surprisingly, though, the stringent response in *B. burgdorferi* is triggered during growth in the presumably rich BSK-H medium and is ameliorated during *B. burgdorferi* growth in the presence of tick cells. In the latter case, (p)ppGpp levels and *relA/spoT* transcription are down-modulated relative to the (p)ppGpp levels and *relA/spoT* transcription seen during growth in BSK-H medium. Triggering and amelioration of the *B. burgdorferi* stringent response are also associated with changes in transcription of a chromosomally located surface lipoprotein gene, *bmpD*, but not with changes in transcription of other members of this paralogous gene family.

The present study confirms that *B. burgdorferi* cells multiply when they are cocultured with tick cell lines in L15BS medium but are unable to grow in this medium in the absence of tick cells (25). It has been claimed previously that *B. burgdorferi* is found both outside and within tick and mammalian host cells (6, 12, 18, 19, 22, 26). Even though *B. burgdorferi* has been shown to occur within tick cells by electron microscopy (19), the frequency of this phenomenon and its biological relevance have been unclear. The present study provides definitive evidence for the extracellular location of *B. burgdorferi* in tick cell cultures, and the results are consistent with the extracellular location of *B. burgdorferi* in the tick gut and its migration from the gut to the hematocele and salivary glands without ever becoming intracellular (6, 26).
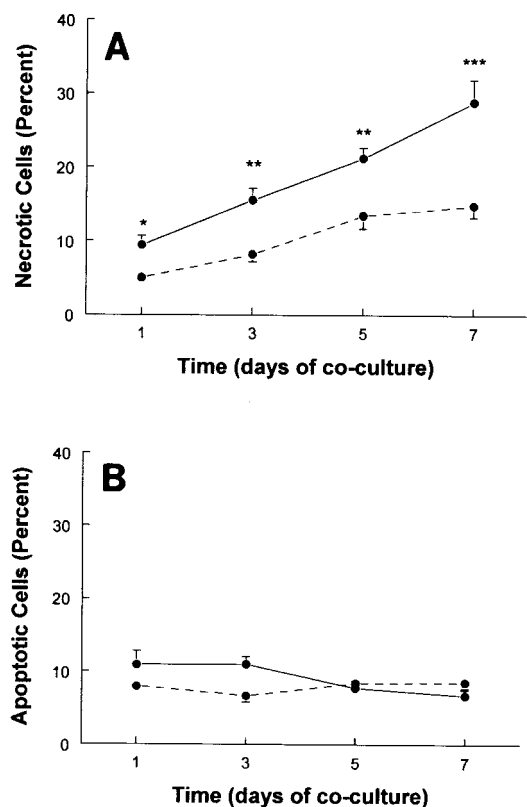
FIG. 3. Tick cell death in IDE8 tick cell cultures infected with *B. burgdorferi* N40 (1×10^7 to 2×10^7 cells/ml; MOI = 5): mean ($\pm$ standard error of the mean) percentages of necrotic (A) and apoptotic (B) tick cells in infected (solid lines) and uninfected (dashed lines) tick cell cultures. See Materials and Methods for details. Each point represents the results from 6 to 10 measurements pooled from two independent complete experiments. The differences in the percentages of necrotic cells between infected and uninfected cultures are significant; the differences in the percentages of apoptotic cells between infected and uninfected cultures are not significant (as determined by one-way analysis of variance with the Tukey-Kramer multiple-comparison post test). $*$, $P < 0.05$; $**$, $P < 0.01$; $***$, $P < 0.001$.
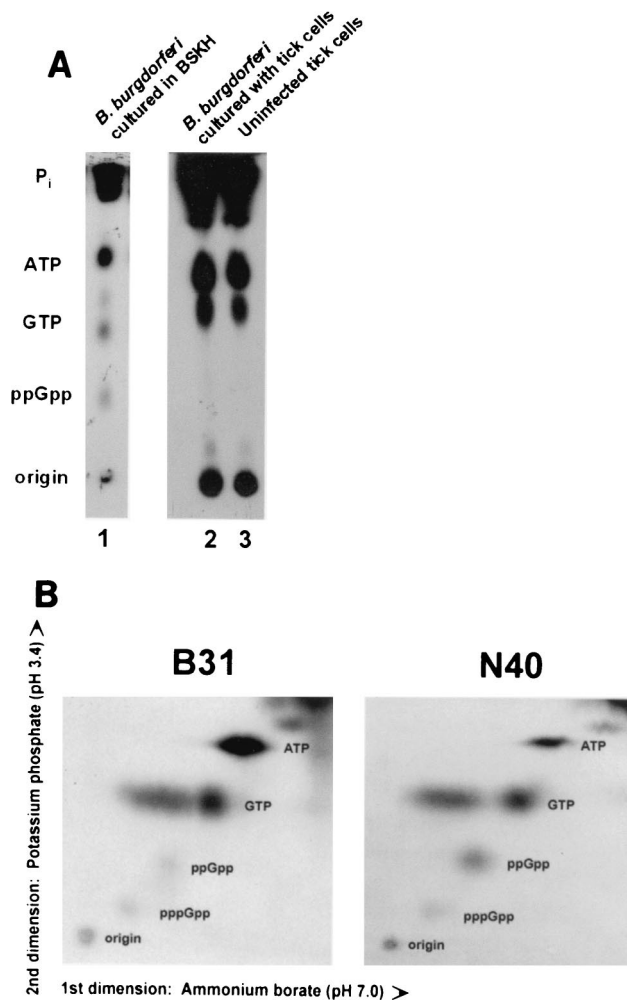

FIG. 4. (p)ppGpp accumulation in *B. burgdorferi* under different growth conditions. (A) ppGpp accumulation in *B. burgdorferi* N40 cultured in BSK-H medium or cocultured with IDE8 tick cells. (B) Identification of ppGpp and pppGpp among ^{32}P-labeled nucleotides in *B. burgdorferi* B31 and N40 cultured in BSK-H medium by two-dimensional polyethyleneimine TLC. See Materials and Methods for details.

B. burgdorferi cocultured with tick cell monolayers adhered tightly to the tick cells (18) and led to their detachment and lysis. Tick cell death was due to necrosis rather than to apoptosis. The mechanism of this cell death is unknown, but cell death clearly was not due to intracellular growth of *B. burgdorferi*. Tick cell necrosis seen after coculture with *B. burgdorferi* may simply be the result of exhaustion of nutrients in the medium due to the growth of *B. burgdorferi*. Its relevance to *B. burgdorferi* growth in ticks is uncertain since tissue necrosis has not been described during *B. burgdorferi* growth in ticks (16, 38).

Despite evidence for triggering of the stringent response in *B. burgdorferi* growing in BSK-H medium and amelioration of this response in *B. burgdorferi* growing in the presence of tick cells, *B. burgdorferi* grew more rapidly in BSK-H medium than when it was cocultured with tick cells. Although production of (p)ppGpp in *E. coli* has been related to slow growth due to down-modulation of the synthesis of rRNA (10, 13), this does not appear to be the case in *B. burgdorferi*, and the findings

suggest that tick cells and/or their metabolic products may only partially complement the nutritional shortcomings of L15BS medium. They also suggest that this nutritional complementation may be qualitatively different from other complementations, since it eliminated the stringent response but did not completely normalize the growth rate. An alternative explanation could be that generation of (p)ppGpp in *B. burgdorferi* is regulated by factors present in BSK-H medium and absent from L15BS medium. *B. burgdorferi* has only two tandem copies of the 23S and 5S rRNA (compared to the seven copies of *E. coli*) and one copy of 16S RNA genes (15), and it is quite possible that it may have novel regulatory mechanisms that uncouple starvation, slow growth, and down-modulation of the synthesis of rRNA.

In *E. coli*, the levels of (p)ppGpp are directly linked to starvation and are independent of the levels of *relA* mRNA and RelA protein (9). The direct relationship which we observed
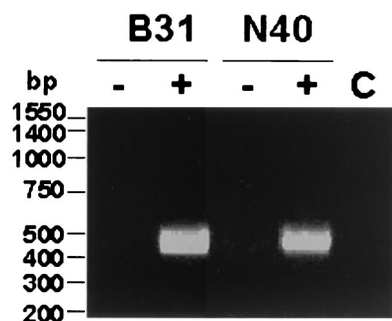
FIG. 5. Detection of *relA*/*spoT* mRNA in *B. burgdorferi* B31 and N40 during growth in BSK-H medium. −, reverse transcriptase omitted; +, complete reaction for specific RNA detection; C, RNA omitted.

TABLE 1. Expression of *B. burgdorferi* N40 *bmp* and *relA*/*spoT* mRNA during coculture with tick cell lines in L15BS medium and in BSK-H medium

mRNA	Concn (fg/ng of total *B. burgdorferi* RNA)		
	Coculture with:		BSK-H medium culture
	Cell line IDE8	Cell line ISE6	
relA/*spoT*	1.0	ND[a]	4.0
bmpD	0.25	0.25	2.0
rpsL-*bmpD*	0.25	0.25	0.06
bmpA	6.5	6.5	6.5
bmpB	2.0	2.0	2.0
bmpC	0.5	0.5	0.5

[a] ND, not determined.

between (p)ppGpp levels and transcription of *relA*/*spoT* in *B. burgdorferi* was therefore unexpected. This could suggest that starvation in *B. burgdorferi* simultaneously stimulates enzymatic activation of RelA/SpoT and its synthesis, as seems to occur in *Bacillus subtilis* (35). Alternatively, starvation could trigger other signal systems that independently stimulate these two processes (9). These results and a recently published analysis of global gene expression in *B. burgdorferi* in which DNA microarray analysis was used (29) strongly indicate that the stringent response and its ability to modulate gene expression are important components of the *B. burgdorferi* response to nutritional stress.

Levels of expression of *bmpA*, *bmpB*, and *bmpC* mRNA were similar in *B. burgdorferi* cocultured with tick cells and *B. burgdorferi* grown in BSK-H medium, but the *bmpD* mRNA level decreased eightfold and the *rpsL*-*bmpD* mRNA level increased fourfold during coculture with tick cells. Since the stringent response mediated by (p)ppGpp is known to modulate transcription of many bacterial genes, it is reasonable to suggest that the observed changes in the levels of *rpsL*-*bmpD* and *bmpD* mRNA resulted from amelioration of the stringent response and the subsequent lowering of (p)ppGpp concentrations (10, 14, 34). Although the observed down-modulation of *bmpD* expression could be a result of the presence of tick cells or stimuli generated by the different medium compositions or both, resolution of this question appears to be currently beyond experimental reach since infectious *B. burgdorferi* N40 does not grow in L15BS medium in the absence of tick cells. We have preliminary evidence that the *bmp* genes are transcribed in ticks (Bugrysheva and Cabello, unpublished data), but further experimental work will be needed to translate our findings with tick cells in vitro to ticks in vivo regarding the modulation of these genes and the stringent response.

Decreases in (p)ppGpp concentration could affect *rpsL*-*bmpD* and *bmpD* transcription by directly interacting with the promoters of these genes and the transcriptional apparatus and its components (14, 34). Alternatively, (p)ppGpp could indirectly modulate the levels of transcription of *rpsL*-*bmpD* and *bmpD* by modulating expression of transcriptional factors, such as σ^S and σ^{54}. The latter hypothesis is consistent with the recent demonstration that up-modulation of σ^S expression by σ^{54} may be responsible for the increase in OspC expression exhibited by *B. burgdorferi* after passage from a tick to a mam-

mal (17, 36) and with extensive evidence from other bacterial systems indicating that (p)ppGpp can modulate σ^S expression itself and promoters activated by σ^S and σ^{54} (10, 20, 34). That the stringent response appears to modulate divergent expression of *rpsL*-*bmpD* and *bmpD* is not unexpected as the promoters of these genes may be transcribed by different transcriptional machinery or by the same machinery with different susceptibilities to modulation by (p)ppGpp (14). The fact that *bmpC*, *bmpA*, and *bmpB* belong to a different transcriptional unit than *bmpD* may explain the lack of modulation of the former genes in the presence of tick cells (14).

The apparent nutritional insufficiency of BSK-H medium suggested by its stimulation of the stringent response even in a laboratory strain that is adapted to it, such as B31, might explain the failure of many clinical isolates of *B. burgdorferi* to grow in this medium (23). Measurement of (p)ppGpp induction might therefore be a useful adjunct for developing improved *B. burgdorferi* culture media. Because BSK-H medium does not seem to completely fulfill the nutritional requirements of *B. burgdorferi*, caution must be used in interpreting the results of gene expression studies of *B. burgdorferi* growing in this medium (37).

Modulation of the *B. burgdorferi* stringent response and gene expression appear to be the result of direct interactions between tick cells and *B. burgdorferi*. The results for the chromosomally encoded *bmp* genes, as well as previous reports demonstrating that the divergent modulation of plasmid-encoded OspA and OspC by cultured tick cells paralleled expression in ticks (27, 32), strongly suggest that *B. burgdorferi* growth in the presence of tick cells can be used to identify factors responsible for global modulation of *B. burgdorferi* gene expression. They also illustrate the complex and reciprocal interactions resulting from contacts between *B. burgdorferi* and tick cells, delineate the potential difficulties of dissecting the symbiotic aspects, and suggest that isogenic borrelial strains generated with recently developed borrelial genetic tools could be used to dissect the relationships between *B. burgdorferi* and tick cells in the future.

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