INFECTION AND IMMUNITY, Aug. 2005, p. 4972–4981 Vol. 73, No. 8
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.8.4972–4981.2005

Borrelia burgdorferi rel Is Responsible for Generation of Guanosine-3′-Diphosphate-5′-Triphosphate and Growth Control

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Received 11 January 2005/Returned for modification 1 March 2005/Accepted 14 March 2005

The global transcriptional regulator (p)ppGpp (guanosine-3′-diphosphate-5′-triphosphate and guanosine-3′,5′-bisphosphate, collectively) produced by the *relA* and *spoT* genes in *Escherichia coli* allows bacteria to adapt to different environmental stresses. The genome of *Borrelia burgdorferi* encodes a single chromosomal *rel* gene (BB0198) (*B. burgdorferi rel* [rel_{Bbu}]) homologous to *relA* and *spoT* of *E. coli*. Its role in (p)ppGpp synthesis, bacterial growth, and modulation of gene expression has not been studied in detail. We constructed a rel_{Bbu} deletion mutant in an infectious *B. burgdorferi* 297 strain and isolated an extrachromosomally complemented derivative of this mutant. The mutant did not synthesize rel_{Bbu} mRNA, Rel_{Bbu} protein, or (p)ppGpp. This synthesis was restored in the complemented derivative, confirming that rel_{Bbu} is necessary and sufficient for (p)ppGpp synthesis and degradation in *B. burgdorferi*. The rel_{Bbu} mutant grew well during log phase in complete BSK-H but reached lower cell concentrations in the stationary phase than the wild-type parent, suggesting that (p)ppGpp may be an important factor in the ability of *B. burgdorferi* to adapt to stationary phase. Deletion of rel_{Bbu} did not eliminate the temperature-elicited OspC shift, nor did it alter *bmp* gene expression or *B. burgdorferi* antibiotic susceptibility. Although deletion of rel_{Bbu} eliminated *B. burgdorferi* virulence for mice, which was not restored by complementation, we suggest that rel_{Bbu}-dependent accumulation of (p)ppGpp may be important for in vivo survival of this pathogen.

The stringent response is a regulatory response that allows bacteria to adapt to a lack of nutrients and other environmental stresses (6). It causes accumulation of guanosine-3′-diphosphate-5′-triphosphate (pppGpp) and guanosine-3′,5′-bisphosphate (ppGpp), collectively referred to as (p)ppGpp or "magic spots." These nucleotides are synthesized by enzymatic phosphorylation of GDP and GTP to ppGpp and pppGpp, respectively, using ATP as a phosphate donor (6). In *Escherichia coli*, two different but highly homologous proteins are involved in (p)ppGpp synthesis: RelA, bound to ribosomes and activated by the presence of uncharged tRNA at the ribosomal A site which generally synthesizes (p)ppGpp in response to amino acid limitation (47), and SpoT, a cytosolic (p)ppGpp synthetase (15) which is responsive to changes in the availability of carbon, phosphate, and fatty acids as well as to changes in temperature and osmolarity (6, 34). SpoT is also a (p)ppGpp hydrolase (21, 31). Many gram-positive bacteria have only a single *rel* ortholog which exhibits both (p)ppGpp synthetase and hydrolase activity (29).

(p)ppGpp acts as a global transcriptional regulator. The general effect of the stringent response is a decrease in rRNA, tRNA, and protein synthesis and a decrease in growth rate that results in bacterial adaptation to an environment scarce in nutrients (6). (p)ppGpp also influences many other bacterial physiological functions including competence (23), morphological and physiological differentiation and production of clavulanic acid and cephamycin C (25, 26), production of actinorhodin and undecylprodigiosin antibiotics (46), thermotolerance (48), adaptation to oxidative stress (30), and sensitivity to antibiotics. As regards the latter, *E. coli* strains able to synthesize (p)ppGpp in either a RelA- or a SpoT-dependent manner show a greater resistance to antimicrobials than strains that cannot produce (p)ppGpp (17). In *E. coli*, production of (p)ppGpp is also required for the accumulation of inorganic polyphosphate needed for degradation of proteins during starvation mediated by the Lon protease (28), and lack of inorganic polyphosphate synthesis in enteric bacteria is accompanied by a reduction in virulence (27).

The *relA* and *spoT* gene products and (p)ppGpp mediate important aspects of virulence in a number of pathogens (8, 20, 34). For example, *Vibrio cholerae relA* is involved in the ability of *V. cholerae* to display pathogenicity in in vitro and in vivo models of infection (20), *Mycobacterium tuberculosis rel*-mediated adaptation to stationary phase is critical to long-term persistence of *M. tuberculosis* in mice (8), and mutations in *Salmonella enterica* serovar Typhimurium *relA* and *spoT* result in attenuation in animals (34). Not all *rel* mutants show decreased virulence. Mutation of *Listeria monocytogenes relA*, while accompanied by loss of (p)ppGpp synthesis and a decrease in osmotolerance, does not diminish in vivo virulence (32).

Borrelia burgdorferi is the etiologic agent of Lyme disease (44). During its life cycle, this spirochete thrives in both ticks and mammals and is able to adapt to various environmental conditions, including changes in temperature, pH, osmolarity, and nutrient availability (10, 41). Preliminary work by ourselves and others has indicated that the stringent response is

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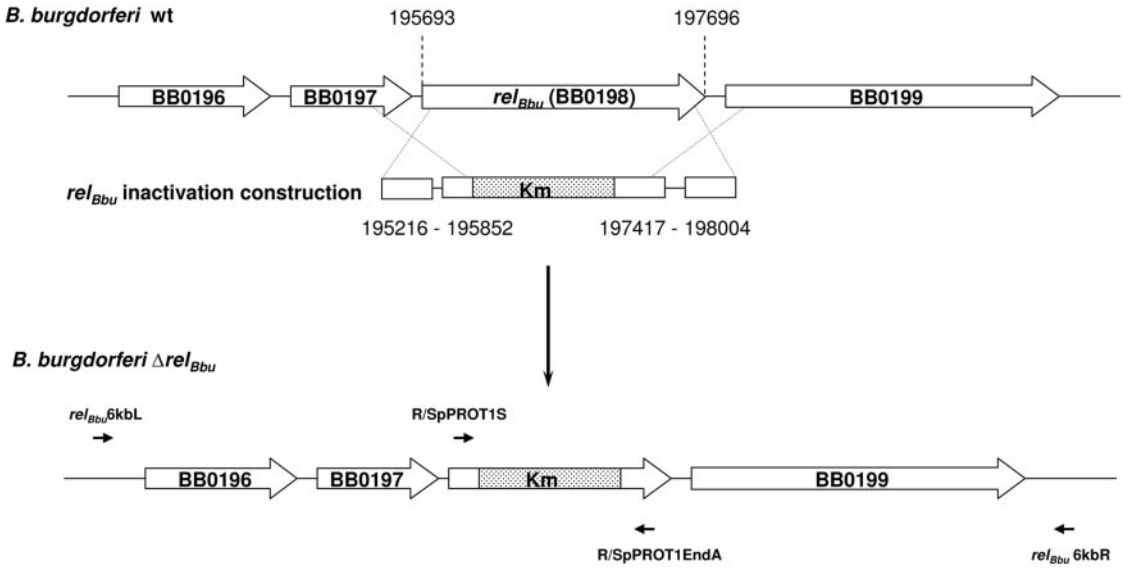

FIG. 1. Production of the *B. burgdorferi* 297 rel_{Bbu} deletion mutation by replacement of central region of rel_{Bbu} with the kanamycin resistance gene *aph*(*3′*)-*IIIa* (Km) from *Enterococcus faecalis* (43). Numbers showing the positions of nucleotides on *B. burgdorferi* chromosome indicate the start and the end of rel_{Bbu} gene and positions of flanking rel_{Bbu} fragments used in inactivation construction. Primers used for analysis of rel_{Bbu} deletion are shown by arrows. wt, wild type.

likely to be present in *B. burgdorferi* (3, 4, 7). The *B. burgdorferi* genome contains a single chromosomal *rel* gene (BB0198) (*B. burgdorferi rel* [rel_{Bbu}]) that shows 35 and 39% amino acid homology, respectively, to *E. coli relA* and *spoT* (13). Microarray studies have shown increased expression of rel_{Bbu} mRNA in *B. burgdorferi* grown in BSK-H at pH 7.5 and 23°C (unfed tick conditions) compared to *B. burgdorferi* grown in BSK-H at pH 6.8 and 37°C (fed tick conditions) or in rat peritoneal chambers (37). (p)ppGpp synthesis by *B. burgdorferi* and the ability of rel_{Bbu} to complement a *relA-spoT E. coli* double mutant for growth on minimal medium (3, 4, 7) provide additional support for the existence of a functional stringent response in *B. burgdorferi* mediated by the product of rel_{Bbu}. We previously suggested that the stringent response in *B. burgdorferi* could modulate expression of *bmpD*, a lipoprotein gene jointly transcribed with the ribosomal protein genes *rpsL* and *rpsG* (3, 11). The discovery that *B. burgdorferi* RpoS (σ^S) is responsible for the expression of OspC, the adhesin crucial for the ability of *B. burgdorferi* to infect the mammalian host (18, 33) and evidence from other bacterial systems that (p)ppGpp is a positive regulator of *rpoS* expression (16), strongly suggests that (p)ppGpp might modulate expression of lipoproteins involved in the *B. burgdorferi* life cycle and virulence (22).

To confirm that rel_{Bbu} is necessary for (p)ppGpp production in *B. burgdorferi* and to clarify its role in *B. burgdorferi* physiology, rel_{Bbu} was deleted in infectious *B. burgdorferi* 297. This deletion was complemented extrachromosomally, and the *B. burgdorferi* derivatives obtained were assayed for rel_{Bbu} mRNA transcription, Rel_{Bbu} protein synthesis, and the ability to produce (p)ppGpp. Growth characteristics, OspA and OspC protein levels, *bmp* mRNA levels, sensitivity to antibiotics, and virulence in mice were also compared between wild-type, mutant, and complemented strains. The rel_{Bbu} gene was clearly responsible for (p)ppGpp synthesis. Its deletion affected *B. burgdorferi* growth in vitro and ablated in vivo virulence in mice. In contrast, deletion of rel_{Bbu} did not alter OspA protein levels, *bmp* gene expression, or *B. burgdorferi* antibiotic susceptibility and did not eliminate the temperature-elicited OspC shift, suggesting that the role of (p)ppGpp in *B. burgdorferi* physiology is different from that in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Transformable clone BbAH130 of infectious *B. burgdorferi* 297 was kindly provided by M. V. Norgard, University of Texas Southwestern Medical Center. *B. burgdorferi* was maintained at 34°C in BSK-II (38) or BSK-H (Sigma-Aldrich, St. Louis, MO). Both media were supplemented with 6% rabbit serum (Sigma). BSK-II was used for preparing electrocompetent *B. burgdorferi* cells and for the selection of transformants. BSK-H was used to compare growth and levels of mRNA, protein, and (p)ppGpp in *B. burgdorferi* and its derivatives. The *B. burgdorferi* rel_{Bbu} mutant was grown in the presence of kanamycin, 400 μg/ml (Sigma). Complemented *B. burgdorferi* was grown in the presence of kanamycin, 400 μg/ml, and streptomycin, 50 μg/ml (Sigma). *E. coli* DH5α (GIBCO/Life Technologies, Grand Island, NY) grown in the presence of spectinomycin, 100 μg/ml (Sigma), was used for propagation of the pKFSS1 and pKFSS1-rel_{Bbu} plasmids.

Generation of rel_{Bbu} deletion. Deletion of *B. burgdorferi* rel_{Bbu} was done by homologous recombination with a rel_{Bbu} inactivation construct (Fig. 1) using PCR-based fusions (43). In brief, nucleotides 195216 to 195852 (primers 1 and 2) (Table 1) and nucleotides 197417 to 198004 (primers 3 and 4) (Table 1) were amplified from *B. burgdorferi* chromosomal DNA, and the kanamycin resistance gene *aph*(*3′*)-*IIIa* from *Enterococcus faecalis* with its own promoter was amplified from plasmid pBLS500 (primers III and IV) (Table 1). All PCR assays used an initial denaturation at 94°C for 2 min and 32 cycles of 94°C for 15 s, annealing at 56°C for 20 s, and extension at 68°C for 3 min. The three amplicons were fused (43), and the final PCR product containing the rel_{Bbu} inactivation construction was precipitated with ethanol and resuspended in sterile water at 4 μg/μl. Forty micrograms was used for each transformation (38).

Construction of *B. burgdorferi* pKFSS1-rel_{Bbu}. A PCR fragment containing full-size rel_{Bbu} with 446 bp upstream from its start codon and 109 bp downstream from its stop codon was amplified from *B. burgdorferi* genomic DNA using forward primer ST1, containing a BamHI restriction site, and reverse primer ST2, containing a PstI restriction site (Table 1). After digestion with BamHI and PstI (New England Biolabs, Inc., Beverly, MA), the resulting fragment was

TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)	Reference
1	CGGGATCCGCTGCGGTAGTGGATGCATAGG	This work
2	GCTCTGATAAATATGAACATGATGAGTGATCGGGCCATAGTGGAGCTGTTCTGC	
3	CTGCATCCCTTAACTTGTTTTTCGTGTGTCTAGATTGAGTGGGAAGCAACACCAAC	This work
4	CCTAGCTAGCTTGCGTTTTTCCCTTTGATTTTAGA	
III	ATATCTAGAAAATTCTATCATAATTGTGGTTTCAA	43
IV	CTATCTAGAGGTACTAAAACAATTCATCCAGTAAA	
ST1	GTTGGATCCAGAATGTTTAGCAGAAG[a]	3
ST2	TTCTCTGCAGTTTCTATGCTTGTAAT[b]	
ST3	CAAAAAAGCGGAATTGAAGCAG	3
ST4	GAATATTGACTTTTGTTGGCCG	
49	CTAGTGGGTACAGAATTAATCGAGC	11
50	TAACATAAAAATATCCTCCTTGC	
R/SpPROT1S	AGGAGAAAGGTACCCATGATACAAGCATACGAAATTGCACA	This work
R/SpPROT1EndA	GACGTCGGATCCGGCCTGTTGAGCTAAGCAATAACA	
rel_{Bbu} 6kbL	GGCTGGAACAGGTGGAGAAGAAG	This work
rel_{Bbu} 6kbR	TGTGCAGCAATGATGGTCTTCAG	
BB0199RT_F	GTACAATCTATATCGTGATGAATCTATACCC	This work
BB0199RT_R	GGTTGATATTGCTAGGTATTTAAGTTGTCTG	

[a] The BamHI restriction site sequence is underlined.
[b] The PstI restriction site sequence is underlined.

ligated into pKFSS1 (12) digested with the same enzymes. The resulting plasmids were propagated in *E. coli* DH5α to obtain pKFSS1-rel_{Bbu} encoding rel_{Bbu} under its own promoter; 10 μg of this plasmid was electroporated into *B. burgdorferi* (38). Transformation of *B. burgdorferi* with pKFSS1 was used as a control.

Confirmation of rel_{Bbu} inactivation and complementation in *B. burgdorferi*. DNA from *B. burgdorferi* transformants was purified (High Pure PCR template preparation kit; Roche Diagnostics Corporation, Indianapolis, IN) and analyzed by PCR (primers R/SpPROT1S and R/SpPROT1EndA) (Table 1 and Fig. 1) to confirm the presence of the rel_{Bbu} deletion or complementation. The PCR fragment obtained with primers rel_{Bbu} 6kbL and rel_{Bbu} 6kbR (Table 1; Fig. 1) was sequenced using primers R/SpPROT1S and R/SpPROT1EndA (Table 1; Fig. 1) to provide further confirmation of the rel_{Bbu} deletion (Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY). DNA from complemented *B. burgdorferi* strains was transformed into *E. coli* DH5α. DNA from *E. coli* clones selected on spectinomycin plates was subjected to restriction analysis using BamHI and PstI for comparison with the plasmids originally used for electroporation of *B. burgdorferi*.

Estimation of pKFSS1-rel_{Bbu} copy number. A competitive PCR assay was used to determine the pKFSS1-rel_{Bbu} copy number in complemented *B. burgdorferi* 297. Competitors for *flaB* and rel_{Bbu} genes containing internal deletions were constructed as previously described (3, 39). Serial twofold dilutions of competitors were mixed with fixed amounts of total 297 DNA to perform quantitative PCR for *flaB* and rel_{Bbu}. Primers 49 and 50 (Table 1) were used for *flaB* detection; primers ST3 and ST4 (Table 1) were used for rel_{Bbu} detection. Target DNA and competitor DNA were assumed to be present at equimolar concentrations in those reactions when the competitor and target PCR products were at similar intensities in agarose gels (11).

Determination of *B. burgdorferi* plasmid content. The plasmid content in *B. burgdorferi* rel_{Bbu} mutants and complemented strains was compared with that of the wild-type parental strain using PCR as described previously (24).

RNA isolation, RT-PCR, and competitive RT-PCR. Total RNA from late-log-phase *B. burgdorferi* was isolated with TRizol reagent (Invitrogen Life Technology, Carlsbad, CA) according to the manufacturer's recommendations and was treated with RQ1 RNase-free DNase (Promega Corporation, Madison, WI) to eliminate DNA contamination. Reverse transcription (RT)-PCR was performed using the Access RT-PCR system (Promega) according to the manufacturer's recommendations (3, 11). Primers ST3 and ST4 (Table 1) were used for rel_{Bbu} mRNA detection; primers 49 and 50 (Table 1) were used for detection of the constitutively expressed *flaB* gene as a control to show the presence of RNA in a sample (11). Primers BB0199RT_R and BB0199RT_F (Table 1) were used for detection of mRNA for the BB0199 gene. Primers and conditions for competitive RT-PCR for *bmp* genes have been described previously (3, 11).

Detection of *B. burgdorferi* proteins. Anti-Rel_{Bbu} antibodies were raised in rabbits immunized with recombinant Rel_{Bbu} protein (A. V. Bryksin, T. N. Orlova, and F. C. Cabello, unpublished data). Rabbit anti-FlaB antibodies were provided by J. D. Radolf, University of Connecticut, Farmington, CT; mouse monoclonal anti-OspA antibodies (H5332) were provided by A. G. Barbour, University of California, Irvine, CA; mouse monoclonal anti-OspC antibodies were provided by R. D. Gilmore, Centers for Disease Control and Prevention, Fort Collins, CO; and mouse monoclonal anti-RpoS antibodies (6A7-101-H11) were provided by M. V. Norgard, University of Texas Southwestern Medical Center, Dallas, TX. FlaB, OspA, OspC, Rel_{Bbu}, and RpoS proteins were detected in *B. burgdorferi* lysates by Western blot analysis and the ECF Western blotting kit (Amersham Biosciences, Piscataway, NJ) and quantified using a Storm 860 PhosphorImager and ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA).

Detection of (p)ppGpp. *B. burgdorferi* (5×10^6 cells/ml) was incubated in BSK-H at 34°C containing 10 μCi/ml of uniformly labeled [^{32}P]orthophosphate (Amersham) for 2 days. Labeled cells were harvested from 10-ml cultures, and (p)ppGpp was extracted and chromatographed on cellulose polyethyleneimine thin-layer chromatography (TLC) plates (Selecto Scientific, Suwanee, GA.) (3). Plates were air dried, exposed to a phosphor screen (Molecular Dynamics) for 12 to 24 h, and scanned using a Storm 860 PhosphorImager.

***B. burgdorferi* growth assays.** Wild-type, Δrel_{Bbu}, and complemented *B. burgdorferi* strains (10^4 cells/ml) were grown at 34°C in BSK-H for 30 days. Cell numbers were determined by dark-field microscopy every other day during the first 2 weeks of growth. To measure the effect of cell death in *B. burgdorferi* stationary-phase cultures, the number of viable organisms present in each culture was determined by limiting dilution at day 30 and compared to the number of cells counted microscopically at day 14. Limiting dilution was performed using serial fourfold dilutions of 30-day-old cultures with fresh BSK-H in 96-well plates (180 μl/well; initial dilution, 1:4; final dilution, 1:16,777,216). The highest dilution yielding positive cultures was determined after two additional weeks of growth at 34°C, and the number of cells/ml in the culture on day 30 was calculated assuming that one cell was sufficient to produce cell growth in a well. The data are presented as the cell survival index (as a percentage) = viable cells/$ml_{Day\ 30}$ (by limiting dilution)/Total cells/$ml_{day\ 14}$ (by microscopy) × 100 and are presented as means ± standard deviations. Limiting dilution analysis and microscopy were done in duplicate in each of two independent experiments. Results were analyzed statistically using a one-way analysis of variance.

***B. burgdorferi* temperature shift.** *B. burgdorferi* was grown at 34°C in BSK-H to 10^7 cells/ml, then inoculated at an initial concentration of 10^6 cells/ml, and grown at 23°C. After 1 week at 23°C, *B. burgdorferi* was transferred to fresh BSK-H at 23°C, 34°C, and 37°C at an initial concentration of 10^3 cells/ml. Cells were collected when their concentration reached 3×10^7 cells/ml for the wild-type parent and 10^7 cells/ml for Δrel_{Bbu}. In other temperature shift experiments, *B. burgdorferi* was grown at 34°C as described above, then transferred to BSK-H at 37°C, and collected at early stationary phase when cell concentrations reached 10^8 cells/ml for the wild-type parent, 2×10^7 cells/ml for the rel_{Bbu} mutant and the mutant complemented with the empty pKFSS1 vector, and 5×10^7 cells/ml for the mutant complemented with pKFSS1-rel_{Bbu}.

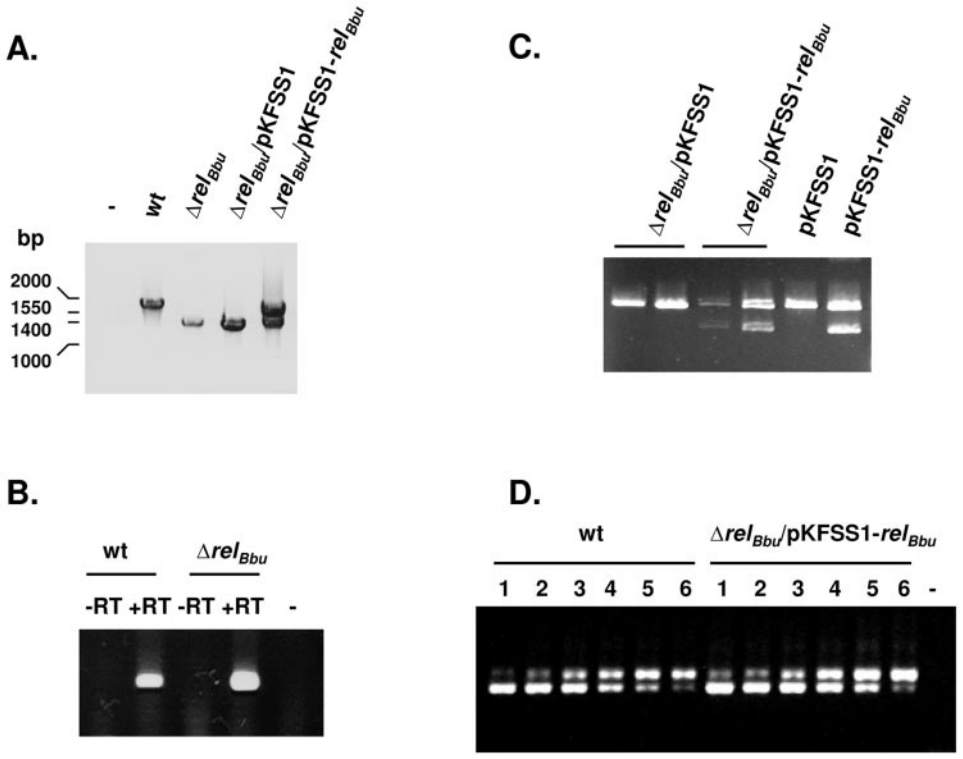

FIG. 2. Confirmation of rel_{Bbu} deletion and complementation in *B. burgdorferi*. (A) PCR analysis of the *B. burgdorferi* wild type (wt), rel_{Bbu} mutant (Δrel_{Bbu}), vector-transformed rel_{Bbu} mutant (Δrel_{Bbu}/pKFSS1), and complemented mutant (Δrel_{Bbu}/pKFSS1-rel_{Bbu}) with primers R/SpPROT1S and R/SpPROT1EndA (Fig. 1; Table 1). −, reaction with DNA omitted. (B) RT-PCR analysis of mRNA expression for BB0199 gene in the *B. burgdorferi* wild-type (wt) and rel_{Bbu} mutant (Δrel_{Bbu}). −, reaction with mRNA omitted; +RT, reaction with reverse transcriptase; −RT, reaction with reverse transcriptase omitted. (C) BamHI-PstI-digested plasmid DNA isolated from *E. coli* transformed with DNA from vector-transformed (Δrel_{Bbu}/pKFSS1) or complemented (Δrel_{Bbu}/pKFSS1-rel_{Bbu}) rel_{Bbu} mutant and BamHI-PstI-digested plasmids used for original transformation of *B. burgdorferi* (pKFSS1, pKFSS1-rel_{Bbu}). (D) Competitive PCR analysis of rel_{Bbu} gene copy number between *B. burgdorferi* wild-type (wt) and complemented rel_{Bbu} mutant (Δrel_{Bbu}/pKFSS1-rel_{Bbu}). Reaction mixtures contained the same amount of total *B. burgdorferi* DNA and six serial twofold dilutions of competitor (lanes 1 to 6). Lane 4 shows equal signal intensities from target DNA (upper band) and competitor (lower band) for the wild type and the complemented rel_{Bbu} mutant, indicating that they are present in equal concentrations in this reaction mixture. −, negative control.

Antibiotic sensitivity. MICs of ampicillin, ciprofloxacin, erythromycin, tetracycline, and vancomycin (Sigma) were determined for wild-type, Δrel_{Bbu}, and complemented *B. burgdorferi* in BSK-H. *B. burgdorferi* (10^5 cells/ml) in BSK-H was added to serial threefold dilutions of each antibiotic (initial concentration, 600 μg/ml; final concentration, 0.01 μg/ml) or no antibiotic in 96-well plates. The plates were incubated for 2 weeks at 32°C in a humidified 3% CO_2 atmosphere. The MIC was defined as the smallest antibiotic concentration preventing *B. burgdorferi* growth under these conditions.

Infection of mice with *B. burgdorferi*. Four groups of 4-week-old C3H/HeN mice (Charles River Laboratory, Wilmington, MA) were infected intradermally with *B. burgdorferi* 297 wild type (five mice), rel_{Bbu} mutant (four mice), rel_{Bbu} mutant transformed with pKFSS1 (four mice), and complemented rel_{Bbu} mutant (four mice) using 10^4 cells/mouse. Two weeks after infection, the mice were sacrificed, and ear punches and blood samples (100 μl) were taken and cultured for 2 weeks at 34°C in 1.5 ml of BSK-H containing 50 μg/ml of rifampin (Merrell Dow Pharmaceuticals, Inc., Cincinnati, OH) and 2.5 μg/ml of amphotericin B (Sigma) before being microscopically examined for growth of *B. burgdorferi*.

RESULTS

Isolation of a rel_{Bbu} mutant in *B. burgdorferi*. Construction of the rel_{Bbu} deletion is shown in Fig. 1. A PCR fragment containing the rel_{Bbu} flanking regions surrounding a kanamycin resistance gene was electroporated into infectious *B. burgdorferi* 297. This electroporation yielded 20 kanamycin-resistant clones with a deleted rel_{Bbu} gene. Of the 2,004 nucleotides of the wild-type rel_{Bbu} gene, the mutants had 160 nucleotides at the 5′ terminus and 280 nucleotides at the 3′ terminus of rel_{Bbu}, with the central 1,564 nucleotides deleted and replaced by the kanamycin resistance gene. The deletion in the rel_{Bbu} gene was confirmed by PCR analysis with primers R/SpPROT1S and R/SpPROT1EndA (Fig. 1) which showed that Δrel_{Bbu} produced a shorter PCR fragment than the fragment generated by the same primers in the wild-type *B. burgdorferi* (Fig. 2A). Only 2 of the 20 mutants had all the plasmids of parental *B. burgdorferi* 297. PCR revealed that the parental and these two rel_{Bbu} *B. burgdorferi* mutants lacked the lp56, lp38, and cp32-8 plasmids but otherwise contained the other expected *B. burgdorferi* plasmids (data not shown) (24). Chromosomal DNA fragments in these two rel_{Bbu} mutants were PCR amplified with primers rel_{Bbu}6kbL and rel_{Bbu}6kbR (Fig. 1), and the deletion of rel_{Bbu} was confirmed by DNA sequencing. Both mutants were fully characterized genetically and functionally. Identical results were obtained with both; only the data generated from one of them is shown below. Deletion of the rel_{Bbu} gene did not prevent mRNA expression of the downstream BB0199 gene (Fig. 1). Its RNA could be detected by RT-PCR in wild-type

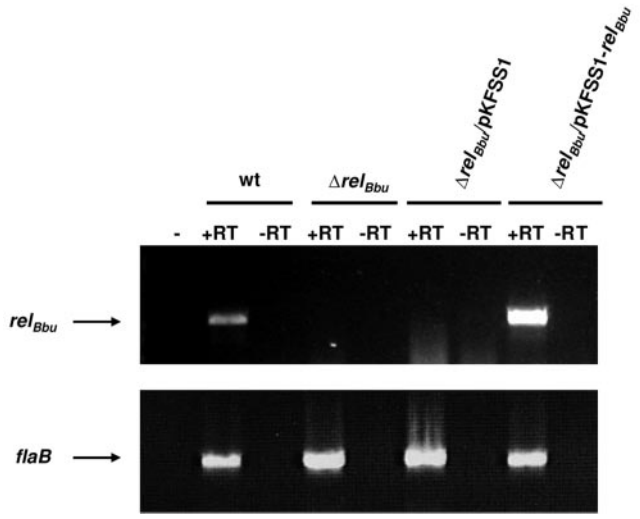

FIG. 3. rel_{Bbu} mRNA synthesis in the *B. burgdorferi* wild type (wt), rel_{Bbu} mutant (Δrel_{Bbu}), vector-transformed rel_{Bbu} mutant (Δrel_{Bbu}/pKFSS1), and complemented mutant (Δrel_{Bbu}/pKFSS1-rel_{Bbu}). RT-PCR for constitutively expressed *flaB* gene was used as a control to show the presence of RNA in a sample. −, RT-PCR in the absence of mRNA; +RT, reaction containing reverse transcriptase; −RT, reaction in the absence of reverse transcriptase. Arrows indicate PCR fragments corresponding to rel_{Bbu} or *flaB* mRNA.

and Δrel_{Bbu} *B. burgdorferi* (Fig. 2B), showing the absence of a polar effect of the rel_{Bbu} mutation.

Complementation of rel_{Bbu} mutant. To complement the rel_{Bbu} deletion, the Δrel_{Bbu} strain was electroporated with pKFSS1-rel_{Bbu} carrying the rel_{Bbu} gene under its own promoter or with the empty pKFSS1 cloning vector as a control. The presence of the wild-type and deleted rel_{Bbu} alleles was determined by PCR analysis of the complemented *B. burgdorferi* derivatives using primers R/SpPROT1S and R/SpPROT1EndA (Fig. 1). The mutant complemented with pKFSS1-rel_{Bbu} produced both mutant- and wild-type-sized amplicons, while the strain transformed with the pKFSS1 cloning vector produced only the mutant-sized amplicon (Fig. 2A). To demonstrate that the plasmids used for complementation were not modified in *B. burgdorferi*, DNA from complemented *B. burgdorferi* derivatives was transformed into *E. coli*. Restriction analysis confirmed that plasmid DNA from spectinomycin-resistant *E. coli* clones and plasmid DNA originally used for complementation were identical (Fig. 2C).

Detection of rel_{Bbu} mRNA and Rel_{Bbu} protein. RT-PCR analysis showed that rel_{Bbu} mRNA was produced in wild-type *B. burgdorferi* grown in BSK-H at 34°C, while the rel_{Bbu} mutant had no rel_{Bbu} mRNA synthesis (Fig. 3). Transformation of Δrel_{Bbu} with the pKFSS1-rel_{Bbu} plasmid but not with the pKFSS1 cloning vector restored rel_{Bbu} mRNA synthesis in the mutant (Fig. 3).

Western blot analysis with rabbit anti-rRel_{Bbu} protein detected a 78-kDa band corresponding to Rel_{Bbu} protein in lysates of the wild-type *B. burgdorferi* grown in BSK-H at 34°C (Fig. 4B). This band was absent in lysates of Δrel_{Bbu} (Fig. 4B) and reappeared in lysates of Δrel_{Bbu} transformed with pKFSS1-rel_{Bbu}. Transformation with the pKFSS1 vector alone did not restore Rel_{Bbu} synthesis in Δrel_{Bbu} (Fig. 4B). The intensity of the Rel_{Bbu} band detected in the complemented derivative with pKFSS1-rel_{Bbu} was stronger than that detected in wild-type *B. burgdorferi* (Fig. 4B). Image analysis of six independent Western blots indicated that the intensity of this band was threefold greater in the complemented rel_{Bbu} mutant than in wild-type *B.*

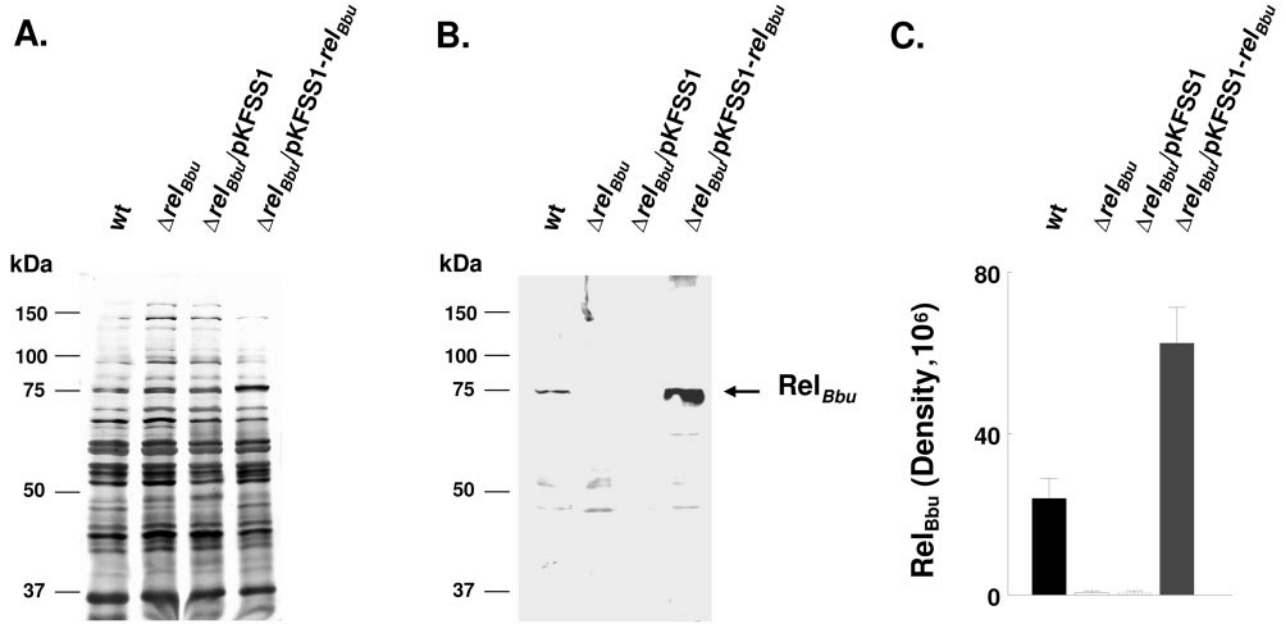

FIG. 4. Rel_{Bbu} protein synthesis in the *B. burgdorferi* wild type (wt), rel_{Bbu} mutant (Δrel_{Bbu}), vector-transformed rel_{Bbu} mutant (Δrel_{Bbu}/pKFSS1), and complemented mutant (Δrel_{Bbu}/pKFSS1-rel_{Bbu}). (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (silver stain) of total *B. burgdorferi* lysates shows that the load of total protein is similar for each lane. (B) Western blot analysis with anti-Rel_{Bbu} antibodies. (C) Relative Rel_{Bbu} band intensities estimated from six independent Western blots and presented as means ± standard deviations.

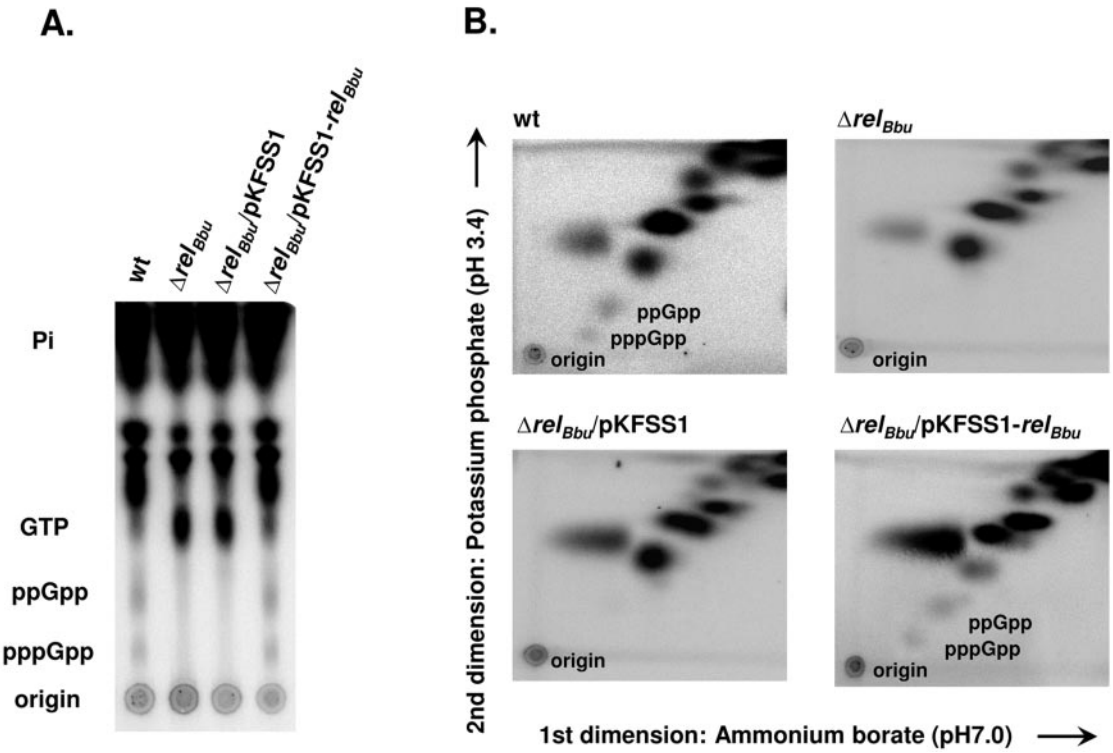

FIG. 5. (p)ppGpp synthesis in the *B. burgdorferi* wild type (wt), rel_{Bbu} mutant (Δrel_{Bbu}), vector-transformed rel_{Bbu} mutant (Δrel_{Bbu}/pKFSS1), and complemented mutant (Δrel_{Bbu}/pKFSS1-rel_{Bbu}). (A) One-dimensional TLC; (B) two-dimensional TLC. The identities of *B. burgdorferi* pppGpp and ppGpp were determined by comigration on chromatograms with (p)ppGpp of *E. coli* (3). Pi, inorganic phosphate.

burgdorferi, suggesting increased synthesis of Rel_{Bbu} protein in complemented *B. burgdorferi* (Fig. 4C).

To determine whether the increased intensity of the Rel_{Bbu} band was merely a consequence of the pKFSS1-rel_{Bbu} copy number, the numbers of rel_{Bbu} genes in wild-type and complemented *B. burgdorferi* were compared by competitive PCR (39). Competitive PCR for *flaB* was used as an internal control for copy number, since both strains have only a single *flaB* gene. Wild-type and complemented *B. burgdorferi* had similar numbers of rel_{Bbu} copies (Fig. 2D), and since wild-type *B. burgdorferi* has only one rel_{Bbu} gene, the copy number of the pKFSS1-rel_{Bbu} plasmid was also one per *B. burgdorferi* cell.

(p)ppGpp synthesis. Both ppGpp and pppGpp could be easily detected in thin-layer chromatograms in wild-type *B. burgdorferi* 297 grown at 34°C in BSK-H. Spots corresponding to ppGpp or pppGpp were not detected in the rel_{Bbu} mutant (Fig. 5A and B), while (p)ppGpp synthesis was restored in Δrel_{Bbu} complemented with pKFSS1-rel_{Bbu}. There was no ppGpp nor pppGpp in the mutant transformed with the empty pKFSS1 vector (Fig. 5A and B). (p)ppGpp levels, as indicated by the intensity of the spots, appeared to be the same in wild-type and complemented *B. burgdorferi* despite the different amounts of Rel_{Bbu} protein in these two strains. These results confirm that rel_{Bbu} is involved in (p)ppGpp synthesis in *B. burgdorferi*.

Growth in culture. In a variety of bacteria, including *E. coli*, (p)ppGpp levels affect cell growth (6, 26, 32). We therefore compared the growth of the wild type, the Δrel_{Bbu} mutant, and the complemented mutant at 34°C in BSK-H (Fig. 6). Figure 6A shows that there was little difference in cell numbers between the wild type and the rel_{Bbu} mutant during the first 8 days of culture. The average doubling time was 5.2 h for wild-type *B. burgdorferi* 297 and 7.8 h for the rel_{Bbu} mutant during this time, but this difference was not statistically significant (analysis of variance, $P > 0.05$). However, at 10 to 14 days of culture, when the cultured cells were approaching stationary phase, cell densities in the wild-type cultures were approximately 10-fold greater than those in Δrel_{Bbu}, and this difference was significant (analysis of variance, $P < 0.01$). Complementation of the rel_{Bbu} mutant with pKFSS1-rel_{Bbu} increased stationary-phase cell densities significantly, about fourfold, at 10 to 14 days, but a significant twofold difference in cell densities between complemented and wild-type cell remained (analysis of variance, $P < 0.01$). There was no difference in cell growth between Δrel_{Bbu} and Δrel_{Bbu} transformed with pKFSS1 vector alone.

To determine whether cell death was responsible for the lower cell concentrations of the rel_{Bbu} mutant cultures in the stationary phase, wild-type, mutant, complemented mutant, and vector-transformed cultures were grown for 30 days and the numbers of viable organisms remaining in these cultures at that time were determined by limiting dilution. After a further 2 weeks of growth, the highest dilution that gave positive cultures for each *B. burgdorferi* strain was used to calculate a survival index which took into account the differences in cell concentrations reached by each strain on day 14 (Fig. 6B). The numbers of surviving cells at 30 days of culture in the wild-type, mutant, and complemented strains were not significantly different (analysis of variance, $P = 0.7$) and constituted approx-

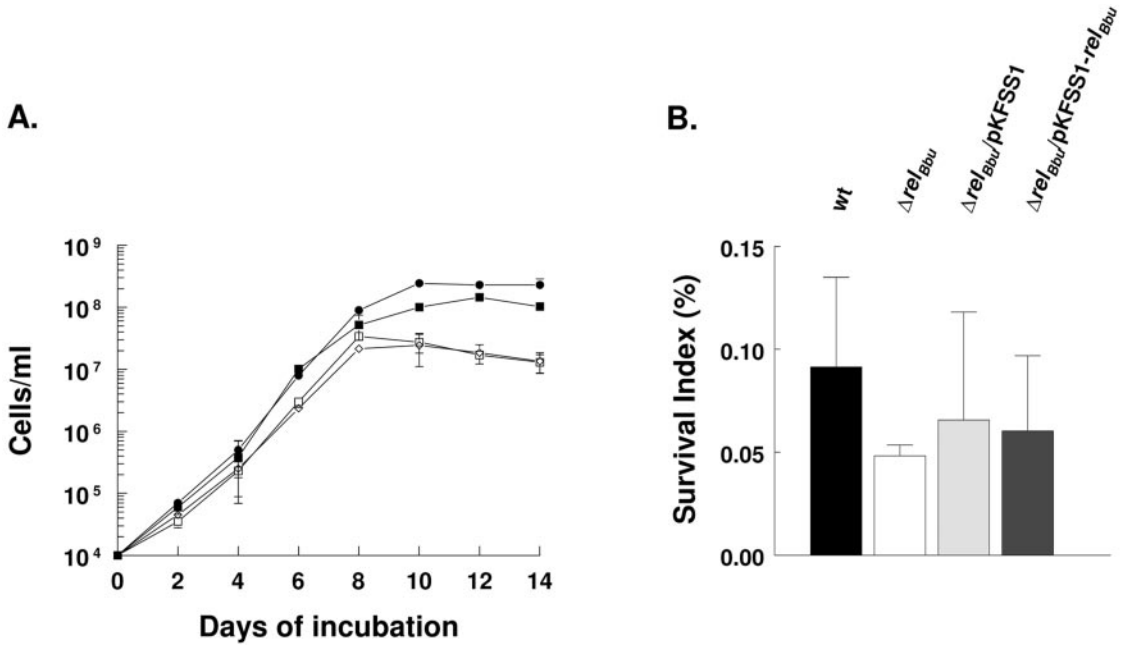

FIG. 6. Growth of the *B. burgdorferi* wild type (wt), rel_{Bbu} mutant (Δrel_{Bbu}), vector-transformed rel_{Bbu} mutant (Δrel_{Bbu}/pKFSS1), and complemented mutant (Δrel_{Bbu}/pKFSS1-rel_{Bbu}) in BSK-H at 34°C. (A) Growth curves of the *B. burgdorferi* wild type (closed circles), rel_{Bbu} mutant (open squares), vector-transformed rel_{Bbu} mutant (open diamonds), and complemented mutant (closed squares). The numbers of cells/ml of culture were calculated from the results from two independent experiments in duplicate and presented as means ± standard deviations for the corresponding day of incubation. (B) Survival index (as a percentage) = viable cells/$ml_{Day\ 30}$ (by limiting dilution)/total cells/$ml_{day\ 14}$ (by microscopy) × 100. The survival index is presented as the mean ± standard deviation calculated from the results from two independent experiments in duplicate.

imately 5 to 9 cells per 10,000 cells observed microscopically at 14 days of culture. These experiments showed that the rel_{Bbu} mutation did not lead to increased cell death in stationary-phase cultures but rather decreased growth abilities prior to this point during the late exponential growth phase.

Expression of bmp genes. *bmpD* is transcribed together with the ribosomal protein genes *rpsL* and *rpsG*, and its expression might be modulated by the stringent response (3, 11). Measurement of *bmp* mRNA by competitive RT-PCR showed no differences in transcription of *bmpA*, *bmpB*, *bmpC*, and *bmpD* genes between the wild type and the rel_{Bbu} mutant of *B. burgdorferi* (data not shown), indicating that *bmp* gene transcription is not modulated by (p)ppGpp.

Effects of temperature shift on OspC production. *B. burgdorferi* increases OspC levels when *B. burgdorferi* is transferred from 23°C to 34 to 37°C conditions of growth (42, 45). RpoS has been shown to be responsible both for this increase in OspC levels with a shift in temperature and for activation of transcription from the *ospC* promoter at elevated temperatures (22). Because (p)ppGpp is a positive regulator of RpoS in *E. coli* (16), ablation of (p)ppGpp synthesis in the rel_{Bbu} mutant might alter the expression pattern of OspC. OspC protein was produced at 34°C and 37°C and was essentially absent at 23°C in mid-log-phase wild-type and Δrel_{Bbu} cultures grown in vitro in BSK-H (Fig. 7A). However, OspC levels were at least two-fold lower in the rel_{Bbu} mutant than in wild-type *B. burgdorferi* grown at elevated temperatures (Fig. 7A). To determine whether (p)ppGpp was needed for high-level OspC expression, OspC levels in wild-type, Δrel_{Bbu}, and complemented *B. burgdorferi* were determined in organisms grown at 37°C to early stationary phase, where the difference in growth between the wild type and the rel_{Bbu} mutant might further increase any possible effect of (p)ppGpp on OspC expression. Stationary-phase OspC levels were higher in wild-type *B. burgdorferi* grown at 37°C than in the rel_{Bbu} mutant (Fig. 7B), confirming the results obtained with mid-log-phase cultures, but complementation of the rel_{Bbu} mutant did not raise OspC levels, suggesting that (p)ppGpp is not involved in regulation of OspC synthesis. RpoS was detected in the immunoblots of the wild-type and rel_{Bbu} mutant *B. burgdorferi* growing at elevated temperature but not at 23°C (data not shown), indicating that RpoS expression does not require (p)ppGpp.

Antibiotic susceptibility of wild-type, rel_{Bbu} mutant, and complemented *B. burgdorferi*. In *E. coli*, (p)ppGpp synthesis is required for resistance to several antibiotics (17). We therefore examined whether the absence of (p)ppGpp in the rel_{Bbu} mutant modified *B. burgdorferi* antibiotic susceptibility. The MICs of ampicillin, erythromycin, tetracycline, and vancomycin were similar for wild-type *B. burgdorferi* and the rel_{Bbu} mutant (Table 2). The MIC of ciprofloxacin was three times higher for the wild-type strain than for the rel_{Bbu} mutant, but the MIC of ciprofloxacin for the complemented derivative was identical to that of the rel_{Bbu} mutant. Since complementation of the rel_{Bbu} mutant did not restore the ciprofloxacin MIC to the wild-type level, we concluded that (p)ppGpp was not involved in the change in the ciprofloxacin susceptibility of the mutant.

Infection of mice with wild-type, Δrel_{Bbu}, and complemented *B. burgdorferi*. C3H/HeN mice were infected intradermally with the *B. burgdorferi* 297 wild type, rel_{Bbu} mutant, rel_{Bbu} mutant transformed with pKFSS1, or rel_{Bbu} mutant complemented with pKFSS1-rel_{Bbu}. All the strains used for infection had been shown to carry all plasmids of the parental *B. burg-*

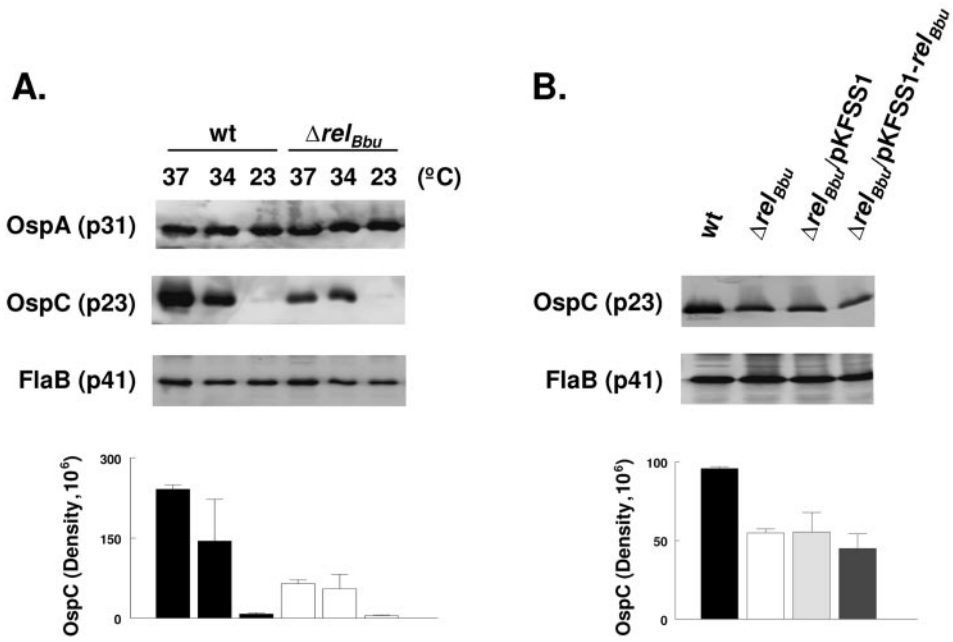

FIG. 7. Temperature shift in the *B. burgdorferi* wild type (wt), rel_{Bbu} mutant (Δrel_{Bbu}), vector-transformed rel_{Bbu} mutant (Δrel_{Bbu}/pKFSS1), and complemented mutant (Δrel_{Bbu}/pKFSS1-rel_{Bbu}). (A) Western blots for FlaB, OspA, and OspC in *B. burgdorferi* wild-type and rel_{Bbu} mutant mid-log-phase cultures in BSK-H at 23°C, 34°C, and 37°C. OspC levels in the *B. burgdorferi* wild type and rel_{Bbu} mutant in mid-log-phase cultures in BSK-H at 23°C, 34°C, and 37°C were estimated from the results from four independent Western blots and presented as means ± standard deviations. (B) Western blots for FlaB and OspC in wild-type, rel_{Bbu} mutant, and complemented *B. burgdorferi* from early-stationary-phase cultures grown in BSK-H at 37°C. OspC levels in wild-type, rel_{Bbu} mutant, and complemented *B. burgdorferi* from early-stationary-phase cultures grown in BSK-H at 37°C were compared from four different Western blots and presented as means ± standard deviations. Similar intensities of FlaB bands in panels A and B show similar protein loads for each lane.

dorferi 297 (data not shown). Two weeks after infection, *B. burgdorferi* could be cultured only from ear punches and blood samples of mice injected with wild-type *B. burgdorferi* (four of five positive ear punch cultures and five of five positive blood cultures). *B. burgdorferi* could not be cultured from ear punches and blood of any of the mice receiving Δrel_{Bbu} or its derivatives transformed with pKFSS1 or pKFSS1-rel_{Bbu}.

DISCUSSION

relA and *spoT* homologues are responsible for synthesis of (p)ppGpp, the alarmone involved in the bacterial stringent response (6). We have shown that rel_{Bbu} is the only gene involved in generation of (p)ppGpp in *B. burgdorferi*. Deletion of rel_{Bbu} did not eliminate mRNA synthesis from the BB0199 downstream gene of unknown function but completely abolished the production of rel_{Bbu} mRNA, Rel_{Bbu} protein, and (p)ppGpp in infectious *B. burgdorferi* 297. Synthesis of these molecules during growth of *B. burgdorferi* in BSK-H was restored after introduction of an extrachromosomal copy of the wild-type allele of rel_{Bbu} into the mutant. Our findings are not inconsistent with the possible existence of different rel_{Bbu} regulators that may affect (p)ppGpp production under certain conditions. The apparently increased amounts of Rel_{Bbu} protein in the complemented *B. burgdorferi* strain (Fig. 4) could not be explained by the different strengths of the rel_{Bbu} gene promoter in the chromosome and the plasmid, since rel_{Bbu} was cloned into pKFSS1 under its own promoter (located about 300 nucleotides upstream from the rel_{Bbu} ATG translational start) (4). These increased amounts of Rel_{Bbu} could also not be a result of multiple copies of the introduced plasmid, since we found only a single copy of the vector per complemented *B. burgdorferi* genome. The possibility exists that differences in DNA supercoiling for the rel_{Bbu} gene located on the pKFSS1-rel_{Bbu} plasmid and on the *B. burgdorferi* chromosome might ultimately be responsible for the different amounts of Rel_{Bbu} protein observed (1, 9). The increased amounts of Rel_{Bbu} did not increase (p)ppGpp levels in complemented *B. burgdorferi* (Fig. 5), most probably because Rel_{Bbu} has two activities, (p)ppGpp synthetic and hydrolytic (6), and both increase simultaneously with increased amounts of protein. Alternatively, the increase in the production of Rel_{Bbu} might not be accompanied by increased levels of (p)ppGpp because the excess protein might be inactively bound to the ribosomes (47).

The rel_{Bbu} gene and (p)ppGpp are not essential for in vitro

TABLE 2. MICs of antibiotics for wild-type, rel_{Bbu} mutant, and complemented *B. burgdorferi*

Strain	MIC (μg/ml) of:				
	Ampicillin	Ciprofloxacin	Erythromycin	Tetracycline	Vancomycin
Wild type	0.09	2.46	0.01	0.09	0.27
Δrel_{Bbu}	0.09	0.82	0.01	0.09	0.27
Δrel_{Bbu}/pKFSS1	ND[a]	0.82	ND	ND	ND
Δrel_{Bbu}/pKFSS1-rel_{Bbu}	ND	0.82	ND	ND	ND

[a] ND, not determined.

growth of *B. burgdorferi*, since the rel_{Bbu} mutant was able to multiply in BSK-H (Fig. 6). *B. burgdorferi* thus differs from *Staphylococcus aureus*, since *rel* mutants cannot be isolated in *S. aureus* because the rel_{Sau} gene and (p)ppGpp are essential in this species (14). Nevertheless, the rel_{Bbu} mutant did show an important growth defect because it only achieved 1/10 the cell concentration of wild-type *B. burgdorferi* in the stationary phase in vitro. It is not clear whether this growth defect resulted from a decrease in growth during the exponential phase or is a result of the inability of this mutant to thrive during the stationary phase, since the mutant does not display increased cell death during the stationary phase (Fig. 6). The growth deficit of *B. burgdorferi* lacking (p)ppGpp is quite different from that of *E. coli* lacking (p)ppGpp. In *E. coli*, experimental evidence indicates that (p)ppGpp can suppress expression of the *ftsZ* gene, an essential gene for cell division and septation (35), and the level of (p)ppGpp is inversely correlated with the rate of initiation of new rounds of DNA replication (40).

We previously reported that lack of (p)ppGpp synthesis in *B. burgdorferi* grown in the presence of tick cells was associated with changes in the transcription of *bmpD*, a gene transcribed with the ribosomal protein genes *rpsL* and *rpsG*, compared to that seen in *B. burgdorferi* grown in BSK-H (3, 11). Unexpectedly, we found no alteration of *bmp* gene expression in the rel_{Bbu} mutant compared to wild-type *B. burgdorferi*, indicating that perhaps the changes seen in the expression of these genes during *B. burgdorferi* growth in the presence of tick cells were more the result of their interaction with the tick cells than of the disappearance of (p)ppGpp (3). More extensive experiments to examine *bmp* gene modulation in the rel_{Bbu} mutant and its complemented derivatives growing in the presence of tick cells will be needed to clarify these differences.

It is well established that (p)ppGpp can regulate gene expression in *E. coli* through a positive control cascade mediated by the alternative bacterial σ factor RpoS (6). RpoS is involved in the OspC temperature-mediated shift in *B. burgdorferi* (22), which has been shown to be important for transmission of *B. burgdorferi* from ticks to mammals (18, 33). Although OspC expression decreased in *B. burgdorferi* Δrel_{Bbu}, the OspC temperature shift was not eliminated by the lack of (p)ppGpp. This suggests that (p)ppGpp does not modulate RpoS functions in *B. burgdorferi*. Detection of RpoS in immunoblots in the wild-type 297 and Δrel_{Bbu} strains (data not shown) also confirmed that RpoS expression does not require (p)ppGpp. The absence of modulation of RpoS-dependent genes by (p)ppGpp in *B. burgdorferi* is surprising, since there is a homologue of *dksA* in the *B. burgdorferi* genome (BB0168) (13) whose gene product mediates RpoS induction by (p)ppGpp in *E. coli* (2). An absence of modulation of RpoS levels by (p)ppGpp has also been found in *S. enterica* serovar Typhimurium (34), where (p)ppGpp can modulate expression of virulence-associated genes by an RpoS-independent pathway (34). A recent report has proposed that there are RpoS-independent virulence regulons in *B. burgdorferi* (5) and that (p)ppGpp may have a role in their regulation, but this requires further study.

(p)ppGpp mediates resistance of *E. coli* to various antibiotics by lowering bacterial metabolism and growth rate as well as by RpoS activation (17, 19, 36). Our experiments indicate that the MICs of several classes of antibiotics are similar in *B. burgdorferi* in the presence and absence of (p)ppGpp (Table 2). The absence of this phenotype could be explained by the fact that, in contrast to what occurs in *E. coli*, the lack of (p)ppGpp did not change the *B. burgdorferi* growth rate substantially and did not affect the synthesis of RpoS, two elements involved in (p)ppGpp-mediated antibiotic resistance in *E. coli*. The decreased MIC of ciprofloxacin observed in the rel_{Bbu} mutant and its complemented derivative could be the result of a spontaneous mutation. Alternatively, the difference in cell densities between complemented and wild-type *B. burgdorferi* strains might be responsible for the increased susceptibility to ciprofloxacin in the complemented strain.

Since mutation of *rel* has been shown to affect virulence in several bacterial pathogens (8, 20, 34), we compared the ability of wild-type, Δrel_{Bbu}, and complemented *B. burgdorferi* to infect mice after needle inoculation. The loss of infectivity for Δrel_{Bbu} suggested that rel_{Bbu} is necessary for *B. burgdorferi* to produce infection in mice. Since complementation, even in the presence of all parental *B. burgdorferi* plasmids, did not restore infectivity in mice, there is a possibility of a defect that cannot be determined by the present techniques. This suggests that other complementation approaches (for example, intrachromosomal) might be needed to confirm that (p)ppGpp is involved in *B. burgdorferi* virulence. The decreased levels of OspC, a major lipoprotein necessary for infection of mammalian hosts (18, 33), might partially explain the loss of infectivity in the rel_{Bbu} mutant, although complementation restored neither OspC levels nor infectivity. Increased Rel_{Bbu} levels in complemented *B. burgdorferi*, even though they did not lead to increased (p)ppGpp amounts, might be responsible for the failure of the complementation to restore infectivity in mice. Another explanation for the failure of complementation to restore the infectiousness of the Δrel_{Bbu} strain could be loss of the complementing plasmid harboring the rel_{Bbu} wild-type gene in mice (12).

In summary, we have shown that rel_{Bbu} is responsible for (p)ppGpp synthesis and affects the growth and probably the infectivity of *B. burgdorferi*. Our work also provides evidence that the absence of (p)ppGpp in *B. burgdorferi* was not accompanied by the same phenotypes as in *E. coli*. This suggests that the role of this mediator in *B. burgdorferi* physiology is different from its role in *E. coli*. Our results indicate that (p)ppGpp does not regulate RpoS-dependent promoters and does not influence susceptibility to antibiotics in *B. burgdorferi*. Further studies will be needed to ascertain the role of (p)ppGpp in *B. burgdorferi* and to define the stringent response in this pathogen.

ACKNOWLEDGMENTS

This work was supported by a grant to F.C.C. (R01 AI41-056-7).

We thank M. Cashel, National Institutes of Health, Bethesda, MD, for advice and discussions; M. V. Norgard, University of Texas Southwestern Medical Center, Dallas, TX, for *B. burgdorferi* 297, clone BbAH130, and anti-RpoS antibodies; D. S. Samuels, University of Montana, Missoula, MT, for pKFSS1; J. D. Radolf, C. H. Eggers, and M. J. Caimano, University of Connecticut, Farmington, CT, for anti-FlaB antibodies and for extensive discussions; A. G. Barbour, University of California, Irvine, CA, for anti-OspA antibodies (H5332); and R. D. Gilmore, CDC, Fort Collins, CO, for anti-OspC antibodies. We also thank Radha Iyer and Darya Terekhova, New York Medical College, Valhalla, NY, for help with analysis of *B. burgdorferi* plasmid composition.

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Editor: D. L. Burns