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# *groEL* Expression in *gyrB* Mutants of *Borrelia burgdorferi*

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**GroEL protein and *groEL* mRNA transcript were up-regulated in *gyrB* mutants of *Borrelia burgdorferi*, a causative agent of Lyme disease. Furthermore, the protein and transcript levels in *gyrB* mutants were greater than those in experimentally heat-shocked cultures of wild-type *B. burgdorferi*. Circular DNA in the *gyrB* mutants was more relaxed than in wild-type cells, although *groEL* is on the linear chromosome of *B. burgdorferi*. To our knowledge, this is the first evidence, albeit indirect, for the effect of DNA topology on gene expression from a linear DNA molecule in a bacterium.**

Lyme disease is a multisystem disorder caused by the spirochete *Borrelia burgdorferi* (4, 24). Pathology to host tissues may be due in part to an autoimmune response to *B. burgdorferi* heat shock proteins (HSPs) (13). HSPs are synthesized when cells are exposed to elevated temperatures or to a variety of other stresses (11). Some HSPs have been shown to act as chaperones for the assembly of complex and oligomeric proteins (2, 8). The major HSP of ~72 kDa, the DnaK homolog (1, 25), is immunoreactive, and antibodies to DnaK are commonly seen in sera from Lyme disease patients (1). GroEL is a major HSP of ~60 kDa. After heat treatment, DnaK and GroEL were synthesized continuously in *gyrA* mutants of *Escherichia coli* but only transiently in wild-type cells (16). Inhibitors of DNA gyrase also induce HSPs (11, 17, 26). These responses are due to relaxation of DNA supercoiling (12). We observed that coumermycin $A_1$-resistant *gyrB* mutants of *B. burgdorferi* had increased levels of an ~68-kDa protein, which was subsequently identified as GroEL (Fig. 1 and 2A).

*B. burgdorferi* strain X32, a clone of strain B31 carrying a coumermycin $A_1$-resistant *gyrB* mutation (Arg 133 → Leu) (22) (D. S. Samuels, B. J. Kimmel, D. C. Criswell, C. F. Garon, W. M. Huang, and C. H. Eggers, unpublished data), synthesizes the up-regulated 68-kDa protein. A crude lysate of X32 was prepared from a 1.5-liter culture grown in BSK-H medium (Sigma) at 32°C as previously described (15) with the following modifications. Cells from a 1.5-liter culture (in three 500-ml bottles) were collected at 10,500 × *g* for 20 min in a Sorvall GSA rotor. The cell pellet was washed twice in 30 ml of Dulbecco's phosphate-buffered saline (DPBS; 138 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$). Cells were collected in an SS-34 rotor at 7,500 × *g* for 10 min after the first wash and at 6,000 × *g* after the second wash. Cells were resuspended in 1.5 ml of 50 mM Tris-HCl (pH 8.0; the pH of Tris solutions was measured at 25°C)–15% sucrose and stored at −80°C. Four 1.5-ml aliquots were thawed at 37°C, and dithiothreitol (DTT; final concentration, 2 mM), EDTA (final concentration, 1 mM), and phenylmethylsulfonyl fluoride (final concentration, 0.5 mM) were added to each aliquot. The cells were then lysed by sonication (eight 15-s pulses at 3.5 in a Fisher Scientific Sonic Dismembrator 550 with a microtip probe for each of the four aliquots). Nucleic acid was precipitated by slowly adding 1/5 volume of 1 M KCl and 2/5 volume of 5% streptomycin sulfate (pH 7.2 with $NH_4HCO_3$) followed by rotation at 4°C for 10 min. The lysate was clarified first by centrifugation at 7,500 × *g* for 10 min in an SS-34 rotor and then by ultracentrifugation at 435,000 × *g* for 30 min in a TLA-100.2 (Beckman).

The 68-kDa protein was purified and identified (Fig. 1A) as previously described (10) with the following modifications. The clarified lysate was dialyzed overnight at 4°C against 50 mM Tris-HCl (pH 7.5)–10% glycerol–1 mM EDTA–5 mM DTT (A buffer) and loaded onto a 5-ml Econo-Pac heparin cartridge (Bio-Rad) at 2 ml $min^{-1}$. The 68-kDa protein was in the flowthrough from the column, which was combined with an equal volume of 3.4 M $(NH_4)_2SO_4$ in P buffer (50 mM Tris [pH 7.5], 1 mM EDTA, 5 mM DTT) and loaded onto a 1-ml phenyl Superose column (Pharmacia). The column was eluted with a 20-ml linear gradient from 1.7 to 0 M $(NH_4)_2SO_4$ in P buffer at 0.3 ml $min^{-1}$. Fractions containing the 68-kDa protein [which eluted at ~0.85 M $(NH_4)_2SO_4$] were dialyzed against A buffer overnight at 4°C and loaded onto a 1-ml Mono-Q column (Pharmacia). The column was eluted with a 20-ml linear gradient from 0 to 1 M NaCl in A buffer. The fractions containing the 68-kDa protein (which eluted at ~0.5 M NaCl) were concentrated with a Centricon 10 concentrator (Amicon) in an SS-34 rotor for 60 min. An equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 1.4 M 2-mercaptoethanol, 0.2% bromphenol blue) was added to the concentrated fractions and boiled for 5 min. The sample was resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore), and stained with Coomassie brilliant blue. The 68-kDa bands were excised, stored in 1 ml of distilled $H_2O$–2 mM DTT at 4°C, and subjected to Edman degradation. N-terminal sequencing and BLAST searching identified the up-regulated 68-kDa protein as GroEL (Fig. 1B).

Since GroEL is a HSP and its synthesis is induced by heat, we compared GroEL levels in cultures of experimentally heat-

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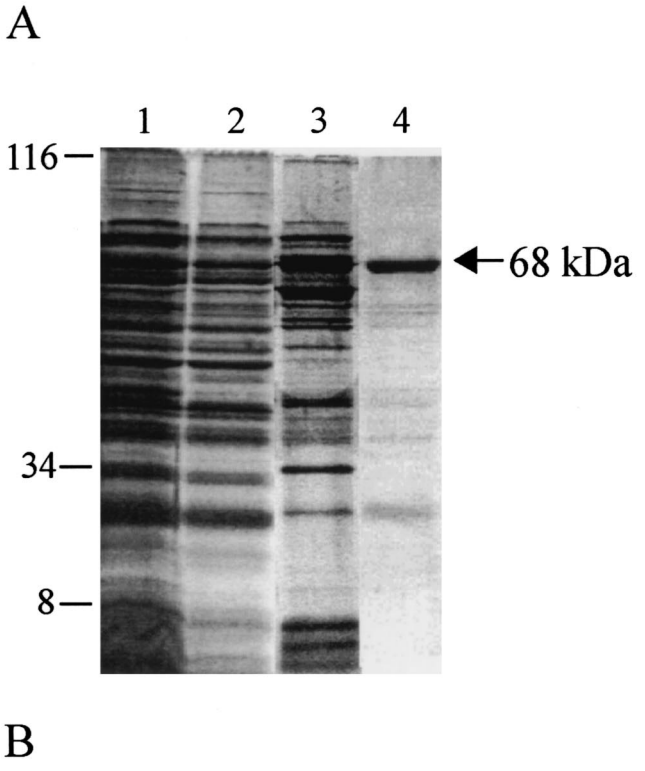


FIG. 1. Purification of GroEL from a *gyrB* mutant of *B. burgdorferi*. (A) The 68-kDa protein (identified as GroEL) was purified by column chromatography. Lane 1, cell lysate; lane 2, flowthrough from a heparin column; lane 3, pooled fractions after elution from a phenyl Superose column; lane 4, pooled fractions after elution from a Mono-Q column. Molecular masses are in kilodaltons. (B) N-terminal sequence of the 68-kDa protein and alignment with GroEL (BB0649; GenBank accession no. AE001166) (6).

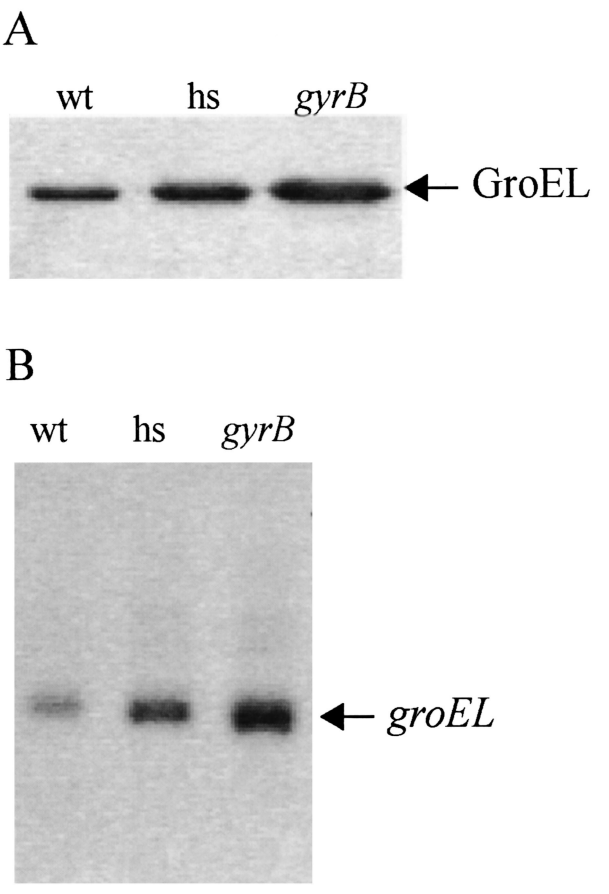


FIG. 2. Increased GroEL synthesis and *groEL* expression in a *gyrB* mutant of *B. burgdorferi*. Immunoblot (A) and Northern blot (B) analyses of wild-type B31 (wt), experimentally heat-shocked wild-type B31 (hs), and the *gyrB* mutant X32 were carried out.

shocked wild-type B31 and the *gyrB* mutant. Experimentally heat-shocked cultures were treated by incubating cells at 42°C for 1 h before harvest. *B. burgdorferi* protein extracts were prepared by pelleting 10-ml cultures of wild-type B31, experimentally heat-shocked wild-type B31, and the *gyrB* mutant, washing twice with DPBS, obtaining the optical density at 600 nm in 1 ml of DPBS, resuspending in 200 μl of $H_2O$ per unit of optical density at 600 nm, adding an equal volume of 2× Laemmli buffer, and boiling for 5 min. Protein extracts from $3.5 \times 10^7$ cells were separated by electrophoresis through SDS–12.5% polyacrylamide gels and visualized by staining with Coomassie brilliant blue. Resolved proteins were also transferred to a polyvinylidene difluoride membrane by using a Trans-Blot cell (Bio-Rad) according to the instructions of the manufacturer. The antigenic proteins were detected with an Immun-Star chemiluminescent detection system according to the instructions of the manufacturer (Bio-Rad). Membranes were blocked for 30 min at room temperature with 0.2% nonfat dry milk–20 mM Tris (pH 7.5)–500 mM NaCl (TBS) and then washed for 10 min with TBS–0.1% Tween-20 (TTBS). The membranes were then incubated overnight at 4°C with monoclonal mouse anti-GroEL antibody 149 (13) (kindly provided by Barbara Johnson and Christian Rittner, Center for Disease Control, Fort Collins, Colo.), diluted 1:2,500 in 0.2% nonfat dry milk–TTBS. The membrane was rinsed twice for 10 min each time with TTBS. Alkaline phosphatase-linked goat anti-mouse immunoglobulin G (Bio-Rad) was diluted 1:3,000 in 0.2% nonfat dry milk–TTBS and incubated for 1.5 h at room temperature. The membrane was washed three times for 10 min each time with TTBS, developed in 5 ml of the substrate solution, and exposed to radiographic film at room temperature for 3 min.

As expected, the culture of experimentally heat-shocked wild-type B31 possessed higher levels of GroEL protein than the wild-type B31 (Fig. 2A). Interestingly, GroEL levels were even higher in the *gyrB* mutant (Fig. 2A).

We next examined *groEL* mRNA levels to determine the mechanism of increased protein levels. Total RNA was isolated from 100-ml cultures of wild-type B31, experimentally heat-shocked wild-type B31, and the *gyrB* mutant by using TRIzol reagent as described by the manufacturer (Gibco BRL). Fifteen micrograms of total RNA was fractionated on

1.2% formaldehyde–agarose gels and transferred to nylon membranes. Hybridization was performed as previously described (3, 14). Band intensity was determined by using an image acquisition and analysis system (Ambis); the mean of three independent experiments and the standard error of the mean (SEM) are reported. The hybridization probe for the *groEL* locus was generated by two rounds of PCR amplification from genomic DNA with oligonucleotides groEL 750F (5′-TG AGGATATTGAGGGGGATGC-3′) and groEL 1122R (5′-A ACTCCGCCAACAAGTTTTGC-3′) (GenBank accession no. AE001166) (6) as described previously (21).

A 2.3-fold (SEM, 0.4-fold) increase in steady-state *groEL* mRNA levels occurred in the *gyrB* mutant strain relative to the wild-type B31 parental strain (Fig. 2B). This compares to a 1.6-fold (SEM, 0.2-fold) increase in mRNA in the *gyrB* mutant relative to the heat-shocked culture of B31 (Fig. 2B). The concomitant increase in *groEL* mRNA levels suggests that the higher level of GroEL protein represents increased transcription.

We wanted to determine why *groEL* gene expression was increased in the *gyrB* mutant. We speculated that the increase was due to DNA relaxation, since *gyrB* mutants of *E. coli* have more relaxed DNA than wild-type cells (5, 16) and *groEL* is regulated by DNA supercoiling (12, 16). A small (4-kb) reporter plasmid, pGOΔ15 (23), which is transiently maintained in *B. burgdorferi*, was transformed into wild-type B31 cells and *gyrB* mutant cells to compare DNA supercoiling. A small reporter plasmid was used because the circular plasmids of *B. burgdorferi* strain X32 are too large (≥26 kb) to resolve into individual topoisomers by gel electrophoresis (J. Alverson and D. S. Samuels, submitted for publication), and the remainder of the *B. burgdorferi* genome is linear. One-dimensional agarose gels with and without chloroquine readily differentiate plasmid DNA topology (7). Plasmids were transformed into *B. burgdorferi* cells by electroporation (20). Plasmid DNA was purified using an alkaline lysis plasmid miniprep kit according to the directions of the manufacturer (Wizard; Promega). The DNA was resuspended in 30 μl of $H_2O$ and stored at −20°C. Plasmid DNA was fractionated on a 0.8% agarose gel in TAE (40 mM Tris, 20 mM acetate, 1 mM EDTA) for 4 h at 50 V with 0 or 15 μM chloroquine, which intercalates into DNA and decreases negative DNA supercoiling, added to the gel and the running buffer. The gel was rinsed in three changes of water (1 h each) to remove the chloroquine before being stained with ethidium bromide for 45 min.

The plasmid DNA was negatively supercoiled in wild-type B31 and relaxed in the *gyrB* mutant (Fig. 3). The relaxation of DNA supercoiling has previously been shown to be associated with the induction of HSPs (16). Furthermore, DnaK plays a role in protecting negative DNA supercoiling in *E. coli* during heat shock (18). We observed increased synthesis of GroEL in a *gyrB* mutant of *B. burgdorferi*. DNA supercoiling is more relaxed in cells with coumermycin $A_1$-resistant DNA gyrase (5; J. Alverson and D. S. Samuels, submitted for publication). We wondered whether the relaxation of DNA directly regulates *groEL* expression or whether the presence of a defective protein (DNA gyrase) indirectly induces GroEL synthesis. The GroEL in the latter case could stabilize DNA gyrase, resulting in the return of DNA supercoiling levels to those seen in the wild-type cells. The GroEL protein in this scenario would be

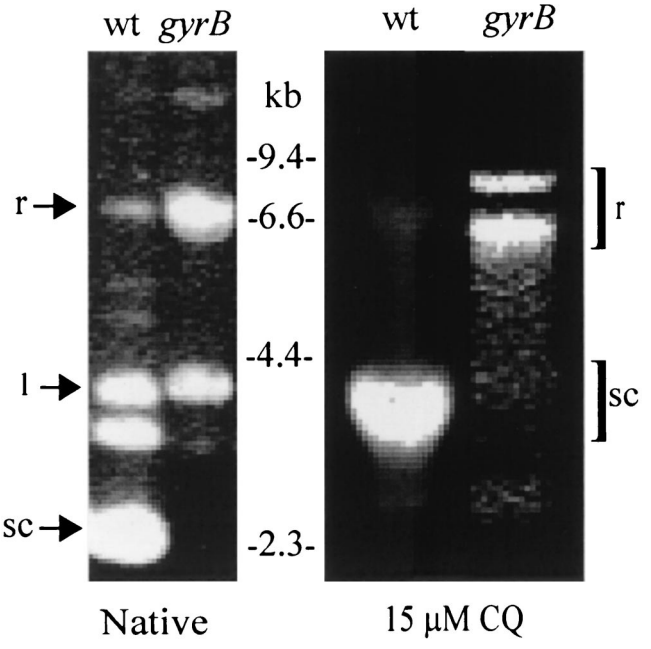


FIG. 3. Effect of a *gyrB* mutation on DNA topology. Plasmid pGOΔ15 was transiently introduced into wild-type B31 and the *gyrB* mutant X32. The topology of the plasmid was determined by one-dimensional 0.8% agarose gel electrophoresis in the absence (native) or presence of 15 μM chloroquine (CQ). The more negatively supercoiled molecules migrate faster through the gel; chloroquine intercalates into the plasmid DNA, introducing positive supercoiling that retards migration of negatively supercoiled DNA (sc) and expedites migration of relaxed DNA (r). The migration of the linearized (l) form of the plasmid is not affected in the mutant. Molecular size standards are in kilobase pairs.

acting similarly to DnaK in maintaining the negative supercoiling in the cell in response to a stress (18). This stress would be the mutation in the *gyrB* gene. However, our results suggest that DNA supercoiling is relaxed in the *gyrB* mutant. Therefore, the increase in GroEL is likely a response to DNA relaxation, which indicates that the *groEL* gene may be directly or indirectly regulated by DNA topology. DNA supercoiling is related to changes in topoisomerase levels (19). Reduced topoisomerase levels result in decreased DnaK and GroEL levels as well as attenuated thermotolerance in *E. coli* (19). DNA gyrase inhibitors relax DNA supercoiling and increase synthesis of DnaK, another HSP (9). In this study we observed decreased levels of DNA supercoiling in a *gyrB* mutant, as described previously (5). Notably, *groEL* maps to the chromosome (6), which is a linear DNA molecule in *B. burgdorferi*. The correlation between *groEL* regulation and DNA supercoiling suggests that the linear chromosome of *B. burgdorferi* may be subject to superhelical torsion. Therefore, the ends could be topologically constrained, perhaps by binding to the cell membrane or to a higher-order nucleoprotein complex. An alternative but more complicated explanation is that DNA supercoiling affects expression of a gene on a circular plasmid whose product directly regulates *groEL*.

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