

Genetic Transformation of the Lyme Disease Agent *Borrelia burgdorferi* with Coumarin-Resistant *gyrB*

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No useful method to genetically manipulate *Borrelia burgdorferi*, the causative agent of Lyme disease, has been developed previously. We have used resistance to the coumarin antibiotic coumermycin A₁, an inhibitor of DNA gyrase, as a genetic marker to monitor the transformation of *B. burgdorferi* by electroporation. Introduction of site-directed mutations into the *gyrB* gene demonstrated that transformation was successful, provided evidence that homologous recombination occurs on the chromosome, and established that mutations at Arg-133 of DNA gyrase B confer coumermycin A₁ resistance in *B. burgdorferi*. The coumermycin A₁-resistant *gyrB* marker and genetic transformation can now be applied toward dissecting the physiology and pathogenesis of the Lyme disease agent on a molecular genetic level.

Lyme disease is the most common arthropod-borne infection in the United States, with almost 10,000 cases diagnosed annually (2, 40). *Borrelia burgdorferi*, a bacterium in the spirochete phylum, is the causative agent of Lyme disease in North America (5, 6, 22, 41). The *B. burgdorferi* genome is atypical for a bacterium: it is composed of both linear and circular DNA molecules (3, 4, 7, 11, 15, 18, 38). The lack of a system to genetically manipulate *B. burgdorferi* has frustrated attempts to study its biology and pathogenesis. We have shown that treatment of *B. burgdorferi* with the coumarin antibiotic coumermycin A₁ inhibits bacterial growth and relaxes circular plasmids (33). Coumermycin A₁ is an inhibitor of the bacterial type II DNA topoisomerase DNA gyrase, which catalyzes the introduction of negative supercoiling into DNA molecules (14, 16, 25, 29, 45). DNA gyrase is a tetramer composed of two A subunits and two B subunits (16, 29); the latter are the target of the coumarin drugs (1, 17, 25, 26, 39, 42).

Recently, we have isolated spontaneous mutants of *B. burgdorferi* that are resistant to coumermycin A₁ (36). These represent the only *B. burgdorferi* mutants resistant to an antimicrobial agent that are currently available. In fact, only a few *B. burgdorferi* mutants of any kind have been obtained by selection (8, 9, 31, 32). We found single point mutations in *gyrB*, the gene encoding the B subunit of DNA gyrase, in the coumermycin A₁-resistant mutants (36). These mutations change a conserved arginine residue, Arg-133, to Gly or Ile. This residue corresponds to Arg-136 in the *Escherichia coli* DNA gyrase B protein and Arg-137 in the *Haloflex* sp. protein, which are mutated to Leu, His, Cys, or Ser in coumarin-resistant strains (10, 12, 19, 25). In the present study, we have used coumermycin A₁ resistance as a genetic marker to select for DNA introduced into *B. burgdorferi*. This now provides a genetic system, previously unavailable, for studying fundamental processes of pathogenesis in Lyme disease.

MATERIALS AND METHODS

Bacteria and antibiotics. High-passage wild-type *B. burgdorferi* B31 (ATCC 35210) and the coumermycin A₁-resistant derivative CR10E (36) were grown at 32°C in Barbour-Stoenen-Kelly (BSK) II medium without gelatin. Coumermycin A₁ and ciprofloxacin were obtained from Sigma and Miles, respectively. Stock solutions were 50 mg of coumermycin A₁ per ml of dimethyl sulfoxide and 25 mg of ciprofloxacin per ml of water and were stored at -20°C for less than 6 months. Bacterial growth was assayed by A₆₀₀ and reported relative to growth in the absence of antibiotics as described previously (33, 36).

Electroporation and selection. Five hundred milliliters of a log-phase culture of *B. burgdorferi* B31 ($\sim 3 \times 10^7$ to 7×10^7 cells per ml) was pelleted in a GSA rotor (Sorvall) at 5,000 rpm ($4,000 \times g$) for 20 min at 4°C, washed twice with 60 ml of cold phosphate-buffered saline (PBS; Dulbecco's PBS without divalent cations), pelleted in an SS34 rotor (Sorvall) at 5,000 rpm ($3,000 \times g$) for 10 min at 4°C, washed three times with 20 ml of cold EPS (272 mM sucrose, 15% glycerol) pelleted in a TMA3 rotor (Tomy) at 5,000 rpm ($2,000 \times g$) for 10 min at 4°C, and resuspended with 0.6 ml of cold EPS (final volume of ~ 0.9 ml). Fifty microliters of cell suspension was mixed with 0.3 to 1 μ g of either total DNA partially digested with *Sau3A* or a PCR product (in 1 to 5 μ l of water) on ice and transferred to a 0.2-cm electroporation cuvette (BTX) at 4°C. One pulse was delivered from a Gene Pulser with Pulse Controller (Bio-Rad) set at 2.5 kV, 25 μ F, and 200 Ω , producing a time constant of 4 to 5 ms (13). One milliliter of BSK II medium was immediately added to the cuvette, and the cells were transferred to a 15-ml culture tube with an additional 9 ml of BSK II medium. Cultures were incubated at 32°C for 24 to 48 h in the absence of coumermycin A₁. Then 0.1 ml of culture was plated in solid BSK II medium with 0.1 μ g of coumermycin A₁ per ml as described previously (23, 36) except that 100-mm-diameter dishes were used with 15 ml of bottom agarose and 20 ml of top agarose. The remaining 9.9 ml of culture was pelleted in a TMA3 rotor at 10,000 rpm ($4,000 \times g$) for 10 min, resuspended in 1 ml of supernatant fraction, and plated. Plates were incubated for 14 days at 32°C in a humidified 5% CO₂ atmosphere.

DNA and site-directed mutations. An 840-bp region of the *gyrB* gene that encodes the first 280 amino acids of the

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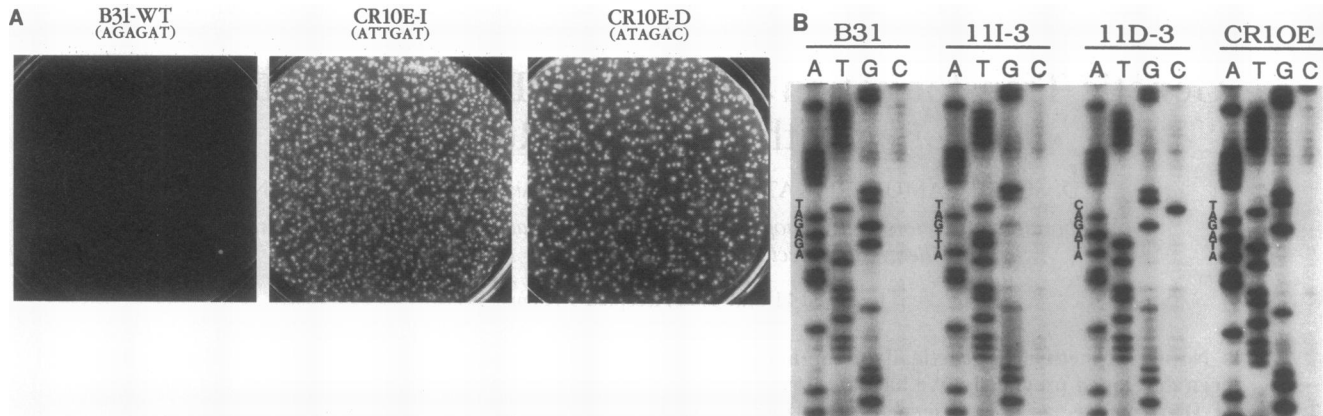


FIG. 1. Introduction of site-directed mutations into *B. burgdorferi*, using coumermycin A_1 resistance as a selectable marker. (A) Coumermycin A_1 -resistant transformants were selected in solid BSK II medium containing 0.1 μg of coumermycin A_1 per ml. The following 840-bp *gyrB* PCR fragments were used for electroporation. B31-WT encodes Arg-133, and CR10E-I as well as CR10E-D encode Ile-133 and contain site-directed mutations. In CR10E-I, the Arg-133 codon (AGA) was changed to ATT (Ile-133). In CR10E-D, the Arg-133 codon (AGA) was changed to ATA (Ile-133) and the Asp-134 codon (GAT) was silently changed to GAC (Asp-134). Plates were photographed with dark-field illumination. (B) Sequence from *gyrB* of coumermycin A_1 -resistant transformants containing site-directed mutations. Ten transformants of each mutant were picked from plates and grown under selection. Partial sequences are shown from wild-type B31, one representative transformant from the electroporation with CR10E-I (11I-3) as well as one from the electroporation with CR10E-D (11D-3), and the spontaneous coumermycin A_1 -resistant mutant CR10E.

amino-terminal domain, which include Arg-133, was amplified by PCR from wild-type B31 or coumermycin A_1 -resistant CR10E, using cloned *Pfu* polymerase (Stratagene) in a PTC-100 Programmable Thermal Controller (MJ Research) with primers 1F (5'-ATGAATTATGTTGCTAGTAACATT-3') and 840R (5'-AACATGAGTTCCCCCTTCTCTTGT-3') (20, 28, 34). The PCR program was 1 cycle of 94°C for 1 min, 25 cycles of 92°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and 1 cycle of 72°C for 5 min; 1 μl (1%) of reaction product was reamplified in a second PCR, in order to increase yield and decrease total cellular DNA, and purified by using Wizard PCR Preps (Promega). Arg-133 in strain B31 (34) corresponds to Arg-138 in *B. burgdorferi* 212 (GenBank accession number L14948 [28]). Site-directed mutations were introduced by amplifying the 840-bp fragment in two pieces. The CR10E-I mutant *gyrB* was created by using 1F and 411R/398A399A (5'-AATTTTTCCATCAATATTAACATAAAC-3') as primers for the 5' portion and 385F/398T399T (5'-GTTTATGT TAATATTGATGGAAAATT-3') and 840R as primers for the 3' portion. The amplification products were purified, diluted to 30 ng per reaction, and assembled by using a two-step amplification that included thermal cycling in the absence (2 cycles) and then presence (25 cycles) of primers 1F and 840R. The CR10E-D mutation was created as described above except that 414R/398A402G (5'-AAAAATTTTTCCGT CTATATTAACATA-3') and 388F/398T402C (5'-TATGTTA ATATAGACGGAAAATTTTT-3') were used as primers instead of 411R/398A399A and 385F/398T399T, respectively. DNA was isolated, amplified by PCR, and sequenced as described previously (36).

Agarose gel electrophoresis and Southern blotting. DNA was isolated in liquid or in agarose plugs as described previously (35). DNA in liquid was digested with restriction enzymes (*Cla*I and *Pvu*II from Boehringer Mannheim Biochemical and *Eco*RI from Life Technologies) and fractionated by conventional 0.8% agarose gel electrophoresis in Tris-acetate-EDTA. Molecular weight standards were lambda DNA-*Hind*III digests (New England Biolabs). DNA isolated in agarose plugs was fractionated by electrophoresis in 1% aga-

rose gels (FMC SeaKem GTG) by clamped homogeneous electric fields (Bio-Rad CHEF Mapper) in 0.5 \times TBE (44 mM Tris, 44 mM borate, 1 mM EDTA) at 6 V/cm, 120°C, for 18:19 h (run time calibration of 2.0) with temperature set at 12°C (actual run temperature of \sim 14°C) and switch times from 0.18 s to 1:13.19 min (determined by CHEF Mapper XA interactive software for separation of DNA between 5 and 1,000 kb with a ramping constant of -1.1 in Molecular Biology Agarose). Gels were vacuum blotted (VacuGene XL; Pharmacia LKB) to Hybond-N (Amersham), with two depurination and two denaturation steps instead of extended steps (24), and UV cross-linked (Stratalinker; Stratagene). The membranes were probed in QuikHyb (Stratagene) at 68°C in a hybridization oven (Autoblot; Bellco) with the 840-bp *gyrB* PCR fragment amplified in two sequential reactions and labeled with [α - ^{32}P]ATP by random priming (Boehringer Mannheim Biochemical). The blots were washed twice for 15 min at 24°C in 2 \times SSC (0.3 M NaCl and 30 mM sodium citrate)-0.1% sodium dodecyl sulfate (SDS), washed once for 30 min at 60°C in 0.2 \times SSC-0.1% SDS, and exposed to Hyperfilm-MP (Amersham).

RESULTS

Genetic transformation with total DNA. Electroporation, the application of high-voltage electric pulses to effect genetic transformation, has been successfully used with a large number of bacterial species (44), including the spirochete *Serpulina hydovysenteriae* (43). Therefore, DNA from wild-type *B. burgdorferi* B31 and the isogenic coumermycin A_1 -resistant mutant CR10E, in which Arg-133 of DNA gyrase B is mutated to Ile, were isolated and partially digested with *Sau*3A. The resulting DNA fragments were electroporated into wild-type B31 in nine separate experiments. Total DNA from B31 cells, a negative control, yielded a mean of 0.1 coumermycin A_1 -resistant colony per μg of DNA (standard error [SE] 0.1), which represents the background level of spontaneous mutations ($\sim 10^{-8}$). Total DNA from CR10E cells yielded a mean of 12 colonies per μg of DNA (SE 3). The means of these two treatments differ significantly ($P = 0.0003$ as determined by a

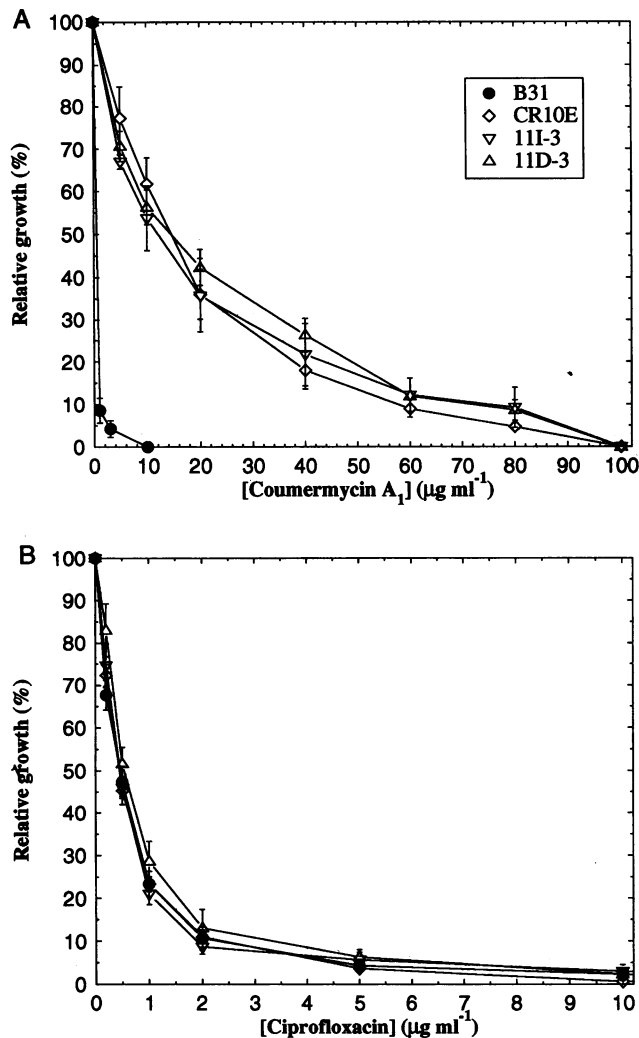


FIG. 2. Susceptibility of coumermycin A₁-resistant transformants of *B. burgdorferi* to growth inhibition by coumermycin A₁ (A) and ciprofloxacin (B). Wild-type B31, coumermycin A₁-resistant CR10E, and transformants 11I-3 and 11D-3 were grown in BSK II medium containing various concentrations of antibiotics. Growth in the absence of antibiotics was defined as 100% relative growth. Error bars represent standard errors of the means derived from at least three independent experiments.

Mann-Whitney U test, a nonparametric ranked analysis of variance [37]). Intact (undigested) total DNA from CR10E yielded no transformants in our experiments.

Genetic transformation with a portion of the *gyrB* gene. We used PCR to amplify the part of the *gyrB* gene that contains the mutation site from wild-type and coumermycin A₁-resistant *B. burgdorferi* to use as substrates for electroporation. In 11 experiments, the wild-type 840-bp *gyrB* fragment produced a mean of 2.2 coumermycin A₁-resistant colonies per μg of DNA (SE 1.3), and the 840-bp *gyrB* fragment encoding Ile-133 produced a mean of 220 colonies per μg of DNA (SE 100). In spite of the large variation in transformation efficiency among experiments, which appears to be due to the physiological state of the *Borrelia* cells, the difference between the two DNA sources was statistically significant ($P \leq 0.0001$ by a Mann-Whitney U test). Under optimal transformation conditions, which are reported in Materials and Methods, about 10^3

colonies per μg of DNA (840-bp *gyrB* fragment encoding Ile-133) are routinely obtained. PCR products of various sizes, from 291 to 1,905 bp (the entire *gyrB* gene [34]), have been successfully used to transform *B. burgdorferi*. Preliminary electroporation results indicate that DNA molecules of about 1,000 bp transform most efficiently.

To confirm that transformation of *B. burgdorferi* was due to introducing exogenous DNA and not to generating spontaneous mutations, as well as to localize the recombination event to within the *gyrB* gene, we created site-directed mutations in the *gyrB* gene both at and adjacent to the codon for Arg-133. These synthetic mutations silently changed the codon for Ile-133 from ATA to ATT (CR10E-I) or the codon for Asp-134 from GAT to GAC (CR10E-D). Mutant *gyrB* gene fragments were electroporated into wild-type *B. burgdorferi* cells with a transformation efficiency of about 1,000 coumermycin A₁-resistant colonies per μg of DNA (Fig. 1A). Ten transformants from each electroporation experiment were isolated, and their *gyrB* genes were partially sequenced. All 20 of the transformants tested contained the site-directed mutations at Ile-133 or Asp-134 (Fig. 1B).

Characterization of coumermycin A₁-resistant transformants. The transformants were indistinguishable in gross morphology (by dark-field microscopy) and protein content (by SDS-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining) from either wild-type B31 or the spontaneous coumermycin A₁-resistant mutant CR10E (data not shown). The transformants had levels of resistance to coumermycin A₁, the selective agent, similar to that of the spontaneous mutant (Fig. 2A). To determine if the coumermycin A₁ resistance in the transformants was a result of a factor that conferred a general resistance to antibiotics, we assayed for the ability of ciprofloxacin to inhibit the growth of *B. burgdorferi* cells. Ciprofloxacin is a potent antimicrobial agent that targets the A subunit of DNA gyrase and whose mechanism of action is different from that of coumermycin A₁ (14, 33, 45). The transformants were no different from B31 and CR10E in ciprofloxacin susceptibility (Fig. 2B), suggesting that transformation of the Arg-133-to-Ile mutation specifically conferred resistance to coumermycin A₁.

The *gyrB* gene is located in the central region of the linear chromosome of *B. burgdorferi* (7, 27). The copy number of the *gyrB* gene in the transformants was determined by restriction enzyme digestion and Southern blotting. Digestion with *Clal*, *EcoRI*, and *PvuII* produced single major bands of about 16, 8.6, and 20 kb, respectively, that hybridized to a PCR-generated 840-bp *gyrB* probe (Fig. 3A), indicating that there is only a single *gyrB* gene per genome equivalent. A minor hybridizing band of about 4.4 kb was produced by *EcoRI* digestion; the identity of this band is unknown, but it may be the result of hybridization to the *parE* homolog (20). The *gyrB* gene was mapped to the chromosome in CR10E and the transformants by pulsed-field gel electrophoresis and Southern blotting (Fig. 3B).

DISCUSSION

Taken together, the results presented here strongly suggest that genetic transformation of *B. burgdorferi* by electroporation is feasible and that the introduced DNA can recombine into a homologous site on the chromosome. This is the first demonstration of a useful genetic exchange system in any species of the genus *Borrelia*. Therefore, molecular tools are now available for defining virulence factors and understanding the pathogenic mechanisms used by *B. burgdorferi* to cause Lyme disease. In addition, transformation provides an assay for the

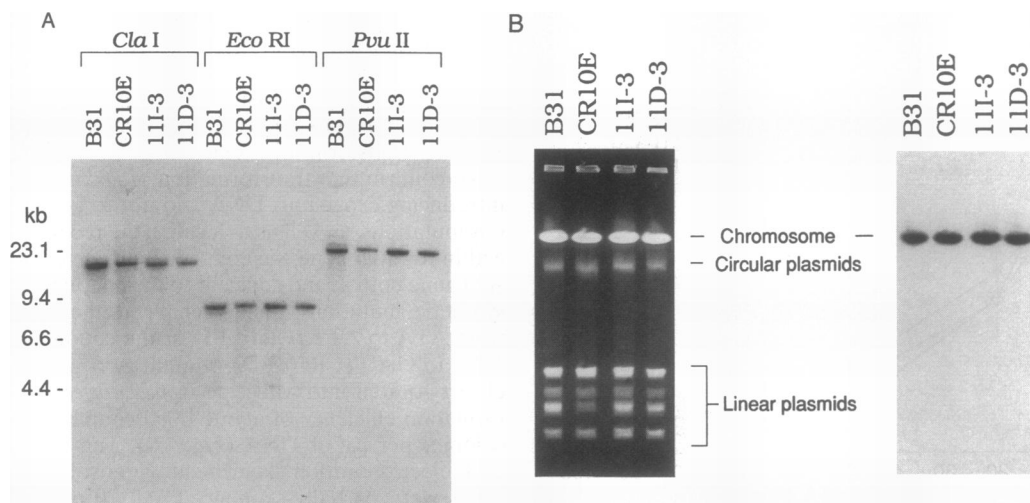


FIG. 3. Mapping of the *gyrB* gene in coumermycin A_1 -resistant transformants of *B. burgdorferi*. DNA from wild-type B31, coumermycin A_1 -resistant CR10E, and transformants 111-3 and 11D-3 was digested with *Cla*I, *Eco*RI, or *Pvu*II and fractionated by conventional gel electrophoresis (A) or fractionated intact by pulsed-field gel electrophoresis (B), Southern blotted, and hybridized with a *gyrB* probe.

ability of mutations to confer coumermycin A_1 resistance, and these results provide evidence that the Arg-133-to-Ile mutation actually confers drug resistance in *B. burgdorferi*. These experiments also indicate that *B. burgdorferi* has a homologous recombination system, consistent with previous findings (30). Twentyfold more transformants per μ g of DNA were generated by using the amplification product (the 840-bp *gyrB* fragment) than with total (partially digested) DNA. However, on a molar basis, total DNA resulted in at least 80 times more transformants than the amplification product. This effect, although modest, may be due to a restriction-modification system in *B. burgdorferi*, consistent with the report of methylated adenine residues in some strains (although not in B31) (21).

The transformation efficiency is low ($\leq 10^3$ transformants per μ g of DNA), probably because of the requirement for homologous recombination as well as DNA entry. A further explanation is that only a subpopulation of spirochetes are either susceptible to transformation (perhaps because of a difference in membrane or surface components) or able to undergo homologous recombination. This hypothesis is currently difficult to assess because of the dearth of other clearly defined genetic markers for *B. burgdorferi*. We are currently assaying other mutations in *gyrB* for the capacity to confer coumermycin A_1 resistance (34), and we are attempting to construct broad-host-range plasmids with coumermycin A_1 -resistant *gyrB* as a selectable marker for use as shuttle vectors.

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