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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 *Haloferax* 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-Stoenner-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 absorbance and reported relative to growth in the absence of antibiotics as described previously (33, 36).

**Electroporation and selection.** Five hundred microliters of a log-phase culture of *B. burgdorferi* B31 (~3 × 10⁷ to 7 × 10⁷ cells per ml) was pelleted in a GSA rotor (Sorvall) at 5,000 rpm (4,000 × 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 × 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 × 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 μg of either total DNA partially digested with Sau3A or a PCR product (in 1 to 5 μl 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 μF, 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 μg 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 × 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
amino-terminal domain, which include Arg-133, was amplified by PCR from wild-type B31 or coumermycin A₃-resistant CR10E, using cloned Pfu polymerase (Stratagene) in a PTC-100 Programmable Thermal Controller (MJ Research) with primers 1F (5'-ATGAATTATCTTGTCAATACATT-3') and 840R (5'-AACATGAGTTCCCCCCTCCTTGT-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'-AATTTTCTCATATTAACATAAAC-3') as primers for the 5' portion and 385F/398T399T (5'-GTTTGATGTAATATGATGGAAATT-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'-AAAAAAAAATCTTCCGCTAATATACACAT-3') and 388F/398T402C (5'-TATGTTAATAGACGGGAAAAATT-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).

Aagarose 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 (ClaI and PvuII from Boehringer Mannheim Biochemicals and EcoRI from Life Technologies) and fractionated by conventional 0.8% agarose gel electrophoresis in Tris-acetate-EDTA. Molecular weight standards were lambda DNA-HindIII digests (New England Biolabs). DNA isolated in agarose plugs was fractionated by electrophoresis in 1% agarose gels (FMC SeaKem GTG) by clamped homogeneous electric fields (Bio-Rad CHEF Mapper) in 0.5X 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 ~14°C) and switch times from 0.18 s to 1:13 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 depuration 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; Belco) with the 840-bp gyrB PCR fragment amplified in two sequential reactions and labeled with [α-32P]ATP by random priming (Boehringer Mannheim Biochemicals). The blots were washed twice for 15 min at 24°C in 2X 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 0.2X 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 hydysenteriae (43). Therefore, DNA from wild-type B. burgdorferi B31 and the isogenic coumermycin A₃-resistant mutant CR10E, in which Arg-133 of DNA gyrase B is mutated to Ile, were isolated and partially digested with Sau3A. 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 A₃-resistant colony per µg of DNA (standard error [SE] 0.1), which represents the background level of spontaneous mutations (~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
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* = 0.0001 by a Mann-Whitney U test). Under optimal transformation conditions, which are reported in Materials and Methods, about 10⁶ 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 *Clai*, *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
ability of mutations to confer coumermycin A₁ 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 μg 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 (<10³ transformants per μg 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 well-defined genetic markers for B. burgdorferi. We are currently assaying other mutations in gyrB for the capacity to confer coumermycin A₁ resistance (34), and we are attempting to construct broad-host-range plasmids with coumermycin A₁-resistant gyrB as a selectable marker for use as shuttle vectors.

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