10-1996

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Rosa, Patricia; Samuels, D. Scott; Hogan, Daniel; Stevenson, Brian; Casjens, Sherwood; and Tilly, Kit, "Directed Insertion of a Selectable Marker into a Circular Plasmid of Borrelia Burgdorferi" (1996). Biological Sciences Faculty Publications. 4.
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Directed Insertion of a Selectable Marker into a Circular Plasmid of *Borrelia burgdorferi*

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Received 6 June 1996/Accepted 12 August 1996

Studies of the biology of *Borrelia burgdorferi* and the pathogenesis of Lyme disease are severely limited by the current lack of genetic tools. As an initial step toward facile genetic manipulation of this pathogenic spirochete, we have investigated gene inactivation by allelic exchange using a mutated borrelial gyrB gene that confers resistance to the antibiotic coumermycin A1 as a selectable marker. We have transformed *B. burgdorferi* by electroporation with a linear fragment of DNA in which this selectable marker was flanked by sequences from a native borrelial 26-kb circular plasmid. We have identified coumermycin A1-resistant transformants in which gyrB had interrupted the targeted site on the 26-kb plasmid via homologous recombination with the flanking sequences. Antibiotic resistance conferred by the mutated gyrB gene on the plasmid is dominant, and transformed spirochetes carrying this plasmid do not contain any unaltered copies of the plasmid. Coumermycin A1 resistance can be transferred to naive *B. burgdorferi* by transformation with borrelial plasmid DNA from the initial transformants. This work represents the first example of a directed mutation in *B. burgdorferi* whereby a large segment of heterologous DNA (gyrB) has been inserted via homologous recombination with flanking sequences, thus demonstrating the feasibility of specific gene inactivation by allelic exchange.

*Borrelia burgdorferi* is maintained in nature through an infectious cycle between wild mammals and ticks (25). Lyme disease, the most common arthropod-borne disease in the United States, is acquired when an infected tick feeds upon a human and transmits *B. burgdorferi* (8, 20, 52). There are currently very few genetic tools with which to study the biology of *B. burgdorferi* and the pathogenesis of Lyme disease. To date, there are no descriptions of any phage or plasmid vectors that can be used to efficiently introduce recombinant DNA into borreliae. Transformation of *B. burgdorferi* by electroporation was only recently achieved (47); those experiments provided definitive evidence for homologous recombination between exogenous DNA introduced by electroporation and related sequences on the chromosome. In these transformations, a segment of a chromosomal gene was replaced by donor DNA that differed by only a small (2-bp) mismatch.

*B. burgdorferi* has an unusual genome composed of a linear chromosome (6, 11, 15, 16) and multiple linear and circular plasmids (2, 5, 17, 19, 50, 54). It has been proposed that these various DNA molecules represent components of a segmented genome and that the distinction between plasmid and chromosome may not be relevant to borreliae (4, 7, 16). Genes encoding a number of different proteins have been cloned and mapped to the chromosome or plasmids (10, 11, 12, 15). Cellular localization, pattern of expression, and putative function have been determined for a number of these proteins. However, a definitive understanding of the roles that any of these gene products or genomic components play in the spirochete, their essential or dispensable nature, or their significance to the infectious cycle or disease requires the ability to genetically manipulate the spirochete.

We would like to investigate the potential role of genes located on a 26-kb circular plasmid (cp26) of *B. burgdorferi* in environmental sensing and adaptation to the tick vector and mammalian host. All *B. burgdorferi* isolates analyzed contain cp26 (26, 28, 53). Genes carried by cp26 include those encoding the differentially synthesized outer surface protein C (ospC) (28, 44, 49), the purine biosynthetic enzymes GMP synthetase (guaA) and IMP dehydrogenase (guaB) (30), and a homolog of the peptide-binding component of the oligopeptide permease system (oppA) (17a). OspC appears on the surface of spirochetes in ticks following a blood meal but preceding transmission to the mammal (49). GMP synthesis is probably essential in mammals but not in ticks (30). In other bacteria, OpaA can be a receptor for peptide pheromones that signal adaptive responses to environmental conditions (22, 27, 34, 39, 40, 55). Given this information, each of these gene products, and perhaps genes located elsewhere on the 26-kb plasmid, may play an essential role in the adaptation of *B. burgdorferi* to either the tick or the mammalian environment or may facilitate transmission between these two hosts. To study the functions of these and other genes in *B. burgdorferi*, we have undertaken to develop a method for specific gene inactivation. *B. burgdorferi* variants that are resistant to the DNA gyrase inhibitor coumermycin A1 (i.e., are CouR) have been isolated (48), and the mutations have been mapped to the gyrB gene (47). With the long-term goal of inactivating specific genes by allelic replacement, our initial aim was to insert a copy of the *B. burgdorferi* gyrB gene that confers CouR (gyrB′) into a non-coding site on cp26. We chose this approach for several reasons. First, to demonstrate the feasibility of the technique, we wanted to begin with a presumably nonessential site to ensure that there was no counterselection against the desired insertion. Second, the transformed spirochetes would have both a
N.Y. (8). The clone B31-NGR was derived from wt B31 by transformation and selection on m coumermycin A1 (Sigma) permland incubated at 35°C. The 50% inhibitory concentration for wt B31 is 0.04 μg of coumermycin A1 per ml (48).

TABLE 1. Courmerycin A1 sensitivity of gyrB gene products from strains used in this study

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Chromosomal gyrB</th>
<th>cp26 gyrB</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt B31</td>
<td>Sensitive</td>
<td>—</td>
</tr>
<tr>
<td>B31-NGR</td>
<td>Resistant</td>
<td>—</td>
</tr>
<tr>
<td>B31-67</td>
<td>Sensitive</td>
<td>Resistant</td>
</tr>
<tr>
<td>B31-9</td>
<td>Sensitive</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

* wt B31 is the uncloned high-passage-number coumermycin A1-susceptible strain from which clones B31-NGR, -3, -9, and -67 were derived by transformation. The 50% inhibitory concentration for wt B31 is 0.04 μg of coumermycin A1 per ml (48).

**, absence of gyrB* on cp26. Where present, the gyrB* gene is located between the 3′ ends of the oppA and guaB genes.

wild-type (wt) (chromosomal) and mutated (plasmid) copy of the gyrB gene, which would render them heterozygous for the B subunit of DNA gyrase and could affect DNA supercoiling (31). Insertion of gyrB into a noncoding site would serve as a control for possible pleiotropic effects of altered supercoiling in subsequent gene inactivation experiments. Finally, we chose gyrB*, despite this and other potential shortcomings, because it was the only antibiotic resistance marker for which there were data demonstrating a selectable phenotype in B. burgdorferi (47, 48). Previous attempts to transform B. burgdorferi with other antibiotic resistance markers have been unsuccessful (56).

In this report, we describe targeted insertion of gyrB* into cp26. This represents the first demonstration of directed mutation in B. burgdorferi whereby a large segment of heterologous DNA encoding a selectable marker has been inserted via homologous recombination with flanking sequences, thus demonstrating the feasibility of specific gene inactivation by allelic exchange.

MATERIALS AND METHODS

Bacterial strains and DNA. B. burgdorferi strains used in this study are listed in Table 1. Spirochetes were grown in liquid BSK-H medium (Sigma, St. Louis, Mo.) at 34°C (1). The protocol for plating B. burgdorferi in solid medium (24) was modified as follows: spirochetes were plated within a layer of top agarose rather than on the surface, and plates were incubated at 35°C in a humidified 1% CO2 tissue culture incubator rather than in a candle jar (35, 36). wt B31 (ATCC 35210) is a high-passage-number, noninfectious, uncloned B. burgdorferi sensu stricto prototype strain, originally isolated from a tick collected on Shelter Island, N.Y. (8). Cour clone B31-NGR was derived from wt B31 by transformation and contains the following mutations in gyrB that together confer high-level Cour*: asparagine 102 to aspartate (N102D), glycine 104 to aspartate (G104D), and arginine 153 to isoleucine (R153I) (46a). The donor DNA containing these mutations was created by PCR with overlapping primers that encoded the N102D and G104D mutations, using DNA from an R153I Cour transformant (47) as the template. Cour clones B31-3, -67, and -9 were derived from wt B31 by transformation with recombinant plasmid pKK58 (described below), which contains a copy of the gyrB* gene from B31-NGR.

Cloning. The gyrB* gene of B31-NGR was amplified by PCR (32, 45) with primers U178F + BglII and 1905R + BclI (Table 2). This fragment extends from 178 nucleotides upstream of the gyrB start codon to the stop codon at the end of the gene; the 5′ end of this fragment is 11 nucleotides from the first codon of the divergently transcribed dnaA gene as described by Saint Girons et al. (45a) (corresponding to nucleotide 1320 of GenBank accession number U64527) and presumably includes the gyrB promoter. This fragment was digested with restriction enzymes BglII and BclI and ligated into BglII-digested clone pDH63, a genomic clone isolated from a B. burgdorferi B31 DNA library constructed in Lambda ZapII (Stratagene, La Jolla, Calif.) (30), whose 3.5-kb insert is derived from cp26 and spans the oppA and guaB genes (17a). The resulting plasmid, pKK58, which contains a complete copy of gyrB* inserted between the 3′ ends of oppA and guaB, is diagrammed in Fig. 1A. There are approximately 1.75 and 1.4 kb of cp26 sequence flanking the 5′ and 3′ ends, respectively, of gyrB*. pKK58 was digested with NdeI to inactivate the amplicon and plating gene, thus withdrawal from plating of the recombinant bacteria was prepared from a denser culture, which resulted in more bacteria per aliquot. This is also consistent with obtaining approximately 10-fold more transformants per microgram of DNA than in previous experiments. We did not note a decrease in the ability to transform B. burgdorferi with increasing density of the initial culture from which the competent bacteria were prepared.

PCR. Oligonucleotide primers used for PCR are described in Table 2 and specified in individual experiments. PCR-amplified fragments used in cloning and transformation as probes were produced in a T ClI DNA Thermal Cycler (Perkin Elmer/Applied Biosystems, Foster City, Calif.); PCR conditions for fragment production were 25 cycles of 94°C for 1 min; 55°C for 0.5 min, and 72°C for 3 min, with 100 ng of total genomic DNA as the template. Fragments were purified by ammonium acetate-isopropanol precipitation. PCR screening of Cour B. burg-

TABLE 2. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer No.</th>
<th>Designation</th>
<th>Sequence (5′ → 3′)</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>gb.18</td>
<td>GCAATAAATATGGAAGAGATT</td>
<td>oppA</td>
</tr>
<tr>
<td>2</td>
<td>gb.33</td>
<td>CAAAATTTATAACATACAC</td>
<td>gnuA</td>
</tr>
<tr>
<td>3</td>
<td>gb.45</td>
<td>TAGACTGCTTCAACATAC</td>
<td>gnuA</td>
</tr>
<tr>
<td>4</td>
<td>pc.42</td>
<td>GGACTGAACTTGTTCGCTGAA</td>
<td>5′-oppA</td>
</tr>
<tr>
<td>5</td>
<td>1969F</td>
<td>CTTTCAAGATGAAAAGGCCTGGG</td>
<td>gyrB</td>
</tr>
<tr>
<td>6</td>
<td>246R</td>
<td>CCTTCTATGATAGTTCGTTAAG</td>
<td>gyrB</td>
</tr>
<tr>
<td>7</td>
<td>U178F + BglII</td>
<td>ACCAGATGTTGTGTTTATGACTATA</td>
<td>gyrB</td>
</tr>
<tr>
<td>8</td>
<td>1905R + BclI</td>
<td>ACCTTATTATCTATACCAAGATTACTAC</td>
<td>gyrB</td>
</tr>
<tr>
<td>9</td>
<td>24R</td>
<td>CCATATATGGTTATTTTCCAT</td>
<td>dnaA</td>
</tr>
<tr>
<td>10</td>
<td>IIR</td>
<td>AATCTCTATTGGAACAGAG</td>
<td>gyrA</td>
</tr>
</tbody>
</table>

* The relative positions and orientations of primers in chromosomal or cp26 genes are indicated by these numbers in Fig. 3A.
dorferi colonies was done in a 9600 DNA Thermal Cycler (Perkin Elmer/Applied Biosystems). Reaction volume was 20 µl per sample, and PCR conditions were 30 cycles of 94°C for 0.5 min, 50°C for 0.5 min, and 68°C for 2 min. Individual B. burgdorferi colonies were picked with sterile toothpicks directly to tubes with PCR mix. There was no additional boiling or lysis step before PCR amplification. PCR products were analyzed by agarose gel electrophoresis and visualized with ethidium bromide. Colonia identified by PCR as having a gyrB insertion on cp26 were subcultured in liquid medium.

Southern blot. Total genomic DNA was isolated from B. burgdorferi as previously described (37). Total plasmid DNA, including both linear and circular molecules, was isolated from B. burgdorferi with QiaGen (Chatsworth, Calif.) columns as instructed by the manufacturer. The plasmid content of QiaGen column-purified borrelial DNA is similar to that of total genomic DNA, whereas chromosomal DNA is undetectable (34a). Southern blot analyses (51) were performed with the following electrophoretic conditions: DNA was separated on a 0.8% agarose gel by field inversion electrophoresis for 24 h at 7 V/cm with program 3 of a PPI-200 programmable power inverter (the forward pulse ranges between 0.15 and 4.803 s, the reverse pulse ranges between 0.05 and 1.601 s, and one complete cycle takes 2 min 3.9 s) (MJ Research, Watertown, Mass.). DNA was transferred bidirectionally to Biotrans nylon membranes (ICN, Irvine, Calif.) and hybridized with a radiolabeled probe at 55°C in rotating bottles in a hybridization oven (Bellco, Vineland, N.J.) (37). Probe fragments were generated by PCR amplification, and the purified PCR products were radiolabeled with [α-32P]dATP (Du Pont, Boston, Mass.) by random priming (Life Technologies, Gaithersburg, Md.).

RESULTS

Construction of a gyrB'/cp26 DNA fragment for allelic exchange. To generate a DNA fragment for pilot gene inactivation experiments, the complete gyrB' gene, including the promoter, of Cou' B. burgdorferi clone B31-NGR was amplified by PCR and inserted into a previously cloned fragment that spanned the oppA and guaB genes from cp26. The gyrB' gene was cloned into the intergenic region between the 3' ends of the converging genes; the resulting recombinant, pKK58, has greater than 1 kb of cp26 sequence flanking either side of gyrB' (Fig. 1A). A linearized version of this plasmid, in which the gene conferring ampicillin resistance was inactivated by digestion with PvuI, was the DNA fragment used in the initial transformations. The relative positions on cp26 of oppA, guaB, guaA, ospC, and the targeted insertion site for gyrB' are shown in Fig. 1B.

Screening of Cou' transformants by PCR analysis. B. burgdorferi B31 was transformed by electroporation with linearized pKK58. Sham electroporations without DNA, or in which DNA was added after electroporation, were done to control for spontaneous Cou' B. burgdorferi. Approximately 1 in 105 bacteria were Cou' following transformation with 1 µg of linearized pKK58 DNA. Spontaneous resistance arose at a frequency of about 5 in 108 bacteria. Colonies were screened for targeted insertion on cp26 by PCR (Fig. 2), using opposing

FIG. 1. pKK58 and the 26-kb circular plasmid of B. burgdorferi. (A) The positions of relevant restriction sites and genes on pKK58 and the extent of the cp26 genomic fragment with the gyrB' insertion are as indicated. (B) The positions of relevant restriction sites and genes on cp26 and the site of the targeted gyrB' insertion are as indicated. Arrows beneath genes point in the direction of transcription.

FIG. 2. Screening Cou' transformants for gyrB' insertion on cp26 following electroporation with pKK58 DNA. Individual B. burgdorferi colonies were PCR amplified with primers 1 and 2 (Table 2 and Fig. 3A). PCR products from 96 samples were analyzed by agarose gel electrophoresis and visualized with ethidium bromide. The positions of the sample wells (four sets) are indicated on the left. The first and last samples in each row are size standards. Open and closed arrows on the right indicate the mobilities of the cp26 PCR fragment with and without the gyrB' insertion, respectively. Control samples amplified at the same time as the colonies were pKK58 (lane 1), wt B31 (lane 2), a blank spot on the plate (lane 3), and the reagent blank (lane 4).
primers in the \textit{oppA} and \textit{guaB} genes (primers 1 and 2 [Fig. 3A]); the primer-binding sites were also present in the \textit{pKK58} DNA fragment that was used in the transformation. Depending on where the donor DNA fragment recombined, at least three different classes of \textit{Cou} transformants should be identifiable by PCR with these primers: (i) the desired event, homologous recombination with flanking \textit{cp26} sequences, would generate a single PCR fragment that is approximately 2 kb larger than the wt \textit{cp26} fragment as a result of the insertion of \textit{gyrB}'; (ii) homologous recombination between \textit{gyrB} sequences on \textit{pKK58} DNA and the chromosomal \textit{gyrB} gene would leave only a wt PCR target on \textit{cp26}; and (iii) illegitimate recombination at a nonhomologous site would give rise to two PCR fragments, one from the unaltered \textit{cp26} locus and a second, larger fragment from the \textit{pKK58} insertion elsewhere.

The results of one such experiment are shown in Fig. 2. Ninety-two colonies were tested by PCR; 91 colonies had a PCR fragment that was the same size as the wt fragment from untransformed spirochetes, and one colony had a PCR fragment that was consistent with the insertion of \textit{gyrB} on \textit{cp26}. No amplified products were obtained when a blank spot on the plate instead of a colony was picked (Fig. 2, lane 3, controls), indicating that neither the DNA used in the electrophoresis nor that of coumermycin \textit{A1}-susceptible dead bacteria gave rise to the amplified fragments. In total, 2 of 461 (0.4\%) of the \textit{Cou} colonies screened in this experiment appeared to have \textit{gyrB} inserted at the targeted site on \textit{cp26}. The other 459 colonies had a PCR fragment identical to the wt \textit{cp26} locus; these represent transformants in which the donor DNA recombined via \textit{gyrB} sequences at the chromosomal locus rather than via flanking \textit{oppA} and \textit{guaB} sequences on \textit{cp26}. There were no \textit{Cou} clones with both PCR fragments, suggesting that illegitimate recombination of the transforming DNA into a nonhomologous site in the genome is rare.

\textbf{Confirmation of \textit{gyrB} insertion on \textit{cp26} by PCR and Southern blot analysis.} To verify the insertion of \textit{gyrB} on \textit{cp26} in candidate transformants, we used PCR primers within \textit{gyrB} in combination with various primers in \textit{oppA} and \textit{guaB}. The approximate locations of these \textit{cp26} and \textit{gyrB} primers are shown in Fig. 3A. The results with five primer pairs and three template DNA preparations (wt \textit{B31}, transformant clone \textit{B31-67}, and plasmid \textit{pKK58}) are shown in Fig. 3B. Amplification products between all \textit{gyrB} and \textit{cp26} primers (primer pairs 1-6, 3-6, 5-2, and 5-4) were obtained with \textit{B31-67} DNA (lanes b). No amplified products were obtained with these primers and wt \textit{B31} DNA (lanes a). No amplification products were obtained with \textit{pKK58} plasmid DNA when distal \textit{cp26} primers outside the cloned segment were used with \textit{gyrB} primers (primer pairs 3-6 and 5-4 [lanes c]). These data are entirely consistent with the targeted insertion of \textit{gyrB} between \textit{guaB} and \textit{oppA} on \textit{cp26} in \textit{B31-67}. Identical results were obtained with transformant \textit{B31-3} (data not shown).

We did Southern blot analyses to directly demonstrate the presence of \textit{gyrB} on \textit{cp26}, adjacent to \textit{guaB}, in those transformants that appeared to have a targeted insertion. Previous sequence analyses had identified a \textit{BamH}I site in \textit{guaA} (30); mapping studies indicated that this was the only \textit{BamH}I site on \textit{cp26} (9a). The \textit{B31} chromosomal \textit{gyrB} locus also contains a single \textit{BamH}I site (46b). Figure 1B shows the anticipated positions of the \textit{BamH}I sites and genes on \textit{cp26} relative to the \textit{gyrB} insertion. Duplicate blots, hybridized with either a \textit{guaB} or a \textit{gyrB} probe, were made from a field inversion gel on which were electrophoresed uncut and \textit{BamH}I-digested DNA preparations from representative \textit{Cou} transformants, one in which \textit{gyrB} was inserted into \textit{cp26} (\textit{B31-3}) and one in which \textit{gyrB} was recombined with the chromosomal \textit{gyrB} gene (\textit{B31-9}) (Fig. 4).

Digestion of \textit{B31-9} DNA with \textit{BamH}I yielded a large (approximately 26-kb) fragment that hybridized with the \textit{guaB} probe (Fig. 4, lane 9, cut), consistent with the presence of a single \textit{BamH}I site in \textit{cp26}. Insertion of \textit{gyrB} adjacent to \textit{guaB} in clone \textit{B31-3} introduced another \textit{BamH}I site and consequently yielded a much smaller (approximately 3-kb) fragment that hybridized with the \textit{guaB} probe (lane 3, cut). Hybridization of the \textit{gyrB} probe to uncut \textit{cp26} from clone \textit{B31-3} confirmed that \textit{gyrB} is present on the plasmid in \textit{B31-3} (lane 3, uncut) and linked with \textit{guaB} on the 3-kb \textit{BamH}I fragment (lane 3, cut). Circular plasmids are retarded and not well resolved, relative to linear DNA, under these field inversion electrophoresis conditions (54); therefore, uncut DNA yields multiple slowly migrating \textit{cp26} bands that hybridize to the \textit{guaB} probe (lanes 3 and 9, uncut). As shown in Fig. 4, we have noted an unanticipated but consistent difference in the mobility of uncut \textit{cp26} between \textit{B31-3} and \textit{B31-9}, possibly reflecting a difference in DNA supercoiling. As predicted, clones \textit{B31-3} and \textit{B31-9} each contain a chromosomal \textit{gyrB} allele, and the \textit{BamH}I fragment from this gene was the same size (approximately 50 kb) in both clones. Similar results were obtained by Southern analysis of clone \textit{B31-67} (data not shown). We conclude that \textit{Cou} transformants \textit{B31-3} and \textit{B31-67} contain \textit{gyrB}'}
at the targeted site on cp26 in addition to the gyrB gene at the wt chromosomal locus. There was no evidence for insertion of gyrB
t at any other site in these bacteria or for any chromosomal or plasmid rearrangement.

**Analysis of chromosomal and cp26 gyrB alleles.** Although we anticipated that the DNA introduced by electroporation could generate CouB. burgdorferi by recombination with homologous sequences on either cp26 or the chromosome, most (459 of 461) of the transformants resulted from recombination with the chromosomal gyrB gene. One possible explanation for this finding is that the gyrB allele on cp26 does not confer CouB, and those rare transformants with gyrB inserted on the plasmid must also have a gyrB allele conferring CouB at the chromosomal locus. Another possibility is that the normal gyrB promoter is not efficiently transcribed on cp26, perhaps because of differences in DNA supercoiling or topology between the linear chromosome and a circular plasmid, and the plasmid gyrB gene must acquire some additional mutation for adequate expression at this site. To address these possibilities, the gyrB genes from both loci of transformant B31-3 were amplified with primers in flanking sequences and the PCR fragments were subjected to partial sequence analysis. These studies indicated that the promoter region and residues critical for CouB of the cp26 gyrB allele of B31-3 were identical in sequence to the B31-NGR gyrB DNA with which it was transformed and that the chromosomal gyrB allele of B31-3 was wt at those residues that are mutated in B31-NGR or other CouB B. burgdorferi strains (not shown).

To directly test the ability of the cp26 gyrB allele of B31-3 to confer CouB, the PCR fragments described above were used to transform B. burgdorferi by electroporation and the number of CouB transformants was determined by plating (Table 3). The chromosomal gyrB genes from B31-NGR and B31-9 were amplified as positive controls, and the unaltered cp26 site from B31-9 (in which there was no gyrB insertion) was amplified as a negative control. The transformation frequency following electroporation of DNA from the cp26 gyrB allele of B31-3 (10⁻³) was comparable to the transformation frequencies with DNA from the chromosomal gyrB genes of B31-9 (3 × 10⁻⁶) and B31-NGR (6 × 10⁻⁶). The transformation frequency following electroporation of DNA from the chromosomal gyrB allele of B31-3 was the same as the background level of spontaneous Cou (9 × 10⁻⁶), approximately 100-fold less than that of the cp26 gyrB allele. We conclude that the gyrB insertions on cp26 confer CouB, that no additional mutations were necessary for adequate gene expression at this site, and that some other factor dictated the low frequency of integration at this site.

The transformations described in Table 3 were done with a cp26 gyrB fragment that contained very little cp26 flanking sequence (166 nucleotides 5' and 244 nucleotides 3'). We repeated this experiment with more distal cp26 primers, located approximately 1 kb away on either side of the gyrB insertion, to amplify cp26 fragments from B31-3 and B31-9. In this experiment, the transformation frequency was 2 × 10⁻⁵/µg of the B31-3 cp26 gyrB fragment, compared with a spontaneous Cou frequency of less than 10⁻⁷ (B31-9 cp26 fragment, lacking gyrB). PCR screening of 89 CouB transformants obtained with the B31-3 cp26 gyrB fragment indicated that all had recombined into the chromosomal gyrB locus. Therefore, even when gyrB was derived from cp26, it still preferentially recombined into the chromosomal locus. This result again indicated that the low frequency of gyrB insertion on cp26 following transformation with pKK58 DNA was not due to the need to acquire additional mutations that allow a dominant phenotype and adequate expression of gyrB on cp26.

**Transformation of B. burgdorferi with borrelial plasmid DNA.** We wished to determine if cp26 containing gyrB could be used to transform wt spirochetes to a resistant phenotype,
TABLE 4. Transformation with borrelial plasmid DNA

<table>
<thead>
<tr>
<th>Expt</th>
<th>Plasmid DNAa</th>
<th>No. of Coub coloniesb</th>
<th>gyrBc on dcp26f</th>
<th>Transformation frequencyg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 ml</td>
<td>0.5 ml</td>
<td>1.0 ml</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>B31-3</td>
<td>3</td>
<td>11</td>
<td>17</td>
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<td></td>
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<td></td>
<td>B31-67</td>
<td>1</td>
<td>8</td>
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<td>ND²</td>
<td>9</td>
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<tr>
<td>II</td>
<td>B31-67, uncet</td>
<td>5</td>
<td>4, 5, 6</td>
<td>1/20</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>B31-67, cutw with BglIV</td>
<td>27, 37, 27</td>
<td>16/80</td>
<td>6.0 × 10⁻⁷</td>
</tr>
<tr>
<td>No DNA</td>
<td>5, 2, 7, 5</td>
<td>ND</td>
<td>1.0 × 10⁻⁷</td>
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a Plasmid DNA isolated from B. burgdorferi by using Qiagen columns included all linear and circular plasmids. B31-9 cp26 does not contain gyrB and thus represents a control for spontaneous Cour.
b The final volume of each electroporation was 10 ml. Of this, the indicated volume was plated, and the number of Cour colonies per plate is given.
c Colonies were screened for gyrB insertion on cp26 by PCR with primers 1 and 2, as indicated in Fig. 3A. The fraction given represents the number of colonies positive for gyrB on cp26 per total number of colonies screened.
d Fraction of viable bacteria in the transformation that are Cour per microgram of donor DNA.
² ND, not done.
w Plasmid DNA from B31-67 was digested with BglI before electroporation. Not all Cour colonies were screened by PCR.

should reduce the number of transformants with gyrB⁰ on cp26 if the donor plasmid was displacing the resident plasmid, because a second event, religation, would be necessary to stably maintain the transforming plasmid DNA. Conversely, if transformants containing gyrB⁰ on cp26 arose by recombination between donor and resident plasmids, linearizing cp26 might stimulate this event because a discontinuity would already exist in the transforming DNA. We subsequently compared transformation efficiencies of uncut and linearized total plasmid DNA from B31-67. Mapping studies of cp26 have demonstrated a single BglI site, approximately 13 kb away in both directions from the gyrB⁰ insertion site (Fig. 1B) (9a). Transformation with BglI-digested plasmid DNA resulted in approximately sixfold more Cour colonies than with uncut plasmid per microgram of DNA (6 × 10⁻⁷, and 1 × 10⁻⁷, respectively) (Table 4, experiment II). Again, the frequency of Cour transformants following electroporation with uncut plasmid DNA was not different from that of spontaneous Cour colonies in the sham electroporation without DNA (10⁻⁷). PCR screening of the transformants demonstrated the presence of gyrB⁰ on cp26 in 16 of 80 (20%) of the Cour colonies following electroporation with BglI-digested plasmid DNA (Table 4, experiment II). Since the overall frequency of transformation was increased with linearized cp26 and the fraction of transformants with gyrB⁰ on cp26 did not decrease, this experiment suggests that recombination between donor DNA and resident cp26, rather than displacement of the wt plasmid, probably generated the Cour transformants carrying gyrB⁰ on cp26 in these experiments with borrelial plasmid DNA.

DISCUSSION

We have carried out important steps toward the goal of gene inactivation by allelic exchange in B. burgdorferi, using as a selectable marker a mutated borrelial gyrB gene that confers Cour. For this strategy to work, gyrB must confer a dominant phenotype because the wt (coumermycin A₁-susceptible) gyrB gene will still be present on the chromosome. Although gyrB⁰ is a dominant mutation in other bacteria (31), our initial transformation results were not entirely convincing that this was true in B. burgdorferi: only 0.4% of the Cour colonies following electroporation with linearized pKK58 DNA were generated by targeted insertion of gyrB⁰ on cp26. However, the frequency of gyrB⁰ insertion on cp26 (4 × 10⁻⁷) was still much greater than what would be anticipated for two independent events, in which transforming DNA recombined at both chromosomal and cp26 sites (10⁻⁻⁷⁻). Subsequent experiments demonstrated that Cour borreliae with the gyrB⁰ allele inserted on cp26 were heterozygous for the gyrB alleles, with a wt copy of the gene on the chromosome (Table 3). Therefore, gyrB⁰ is dominant and can be used as a selectable marker for gene inactivation in B. burgdorferi. We conclude, however, that some aspect of the transformation with pKK58 DNA favored recombination at the chromosomal gyrB locus rather than via cp26 sequences. A strong possibility is that the amount of cp26 flanking sequence in pKK58 (1.75 kb ⁵ and 1.4 kb ³ of gyrB⁰) was insufficient to efficiently mediate homologous recombination on either side of a 2-kb segment of heterologous DNA (gyrB⁰). There is precedent in other transformation systems for preferential incorporation of small mismatches rather than large regions of heterologous sequences (33). It is also possible that the biochemistry of homologous recombination with the linear donor DNA is different if the substrate is a supercoiled plasmid rather than the linear chromosome. In E. coli, homologous recombination with plasmids can be mediated by a RecA-independent pathway (23). In addition, targeted insertion of
gyrB\(^{-}\) into cp26 requires a double crossover, whereas recombination into gyrB on the chromosome could occur by gene conversion (requiring only homologous pairing spanning the mutation and mismatch repair), which might be more frequent.

When uncut, total borrelial plasmid DNA from a Coul\(^{-}\) transformant with gyrB\(^{+}\) inserted on cp26 was transformed back into coumermycin A\(_1\) susceptible borreliae, the transformation frequency per microgram of DNA was comparable to the level of spontaneous Coul\(^{-}\) (Table 4, experiment I). Transformation with total borrelial plasmid DNA that had been digested with Bgl\(_2\), which linearizes cp26, increased the transformation frequency about sixfold over the uncut plasmid level (Table 4, experiment II); approximately 20% of these Coul\(^{-}\) colonies carried cp26 with gyrB\(^{+}\). These experiments indicate that Coul\(^{-}\) transformants with gyrB on cp26 are generated at a 50-fold higher frequency with linearized cp26 plasmid DNA than with the smaller linear pKK58 DNA fragments used in the initial transformation experiments (20% versus 0.4%). This finding is consistent with the interpretation that increasing the amount of cp26 flanking sequence on either side of gyrB (approximately 13 kb versus 1.5 kb) facilitated homologous recombination with cp26. Additional experiments are necessary to determine what stimulates recombination at the targeted site relative to the chromosomal gyrB locus. A different selectable marker that did not have a wt borrelial counterpart would obviously circumvent this problem. We are currently investigating the utility of a gene conferring chloramphenicol resistance (18), which has been used for gene inactivation by allelic exchange in the spirochete Serpulina hyodysenteriae (38).

We are also simplifying our screen for targeted insertion of gyrB\(^{-}\) by incorporating a gene encoding a fluorescent marker between gyrB\(^{-}\) and flanking sequences. In the previous experiments with uncut borrelial plasmid DNA, Coul\(^{-}\) colonies containing gyrB\(^{+}\) on the plasmid could have arisen either from stable maintenance of cp26 DNA that was introduced by electroporation, following segregation of wt cp26, or from recombination between the transforming DNA and the resident cp26. We cannot determine which occurred because gyrB\(^{-}\) is the only marker that distinguishes the transforming and the resident forms of cp26. It is not known if borreliae exhibit plasmid incompatibility such that stable maintenance of a plasmid introduced by transformation would be inhibited by a homologous resident plasmid. These experiments point out one difficulty in attempting transformation with cp26 and investigating its potential utility as a borrelial plasmid vector: all Borrelia burgdorferi isolates analyzed contain cp26 (26, 28, 53). However, cp26 containing gyrB as a selectable marker might be used to transform other members of the genus, such as B. hermsii. Although B. hermsii contains a number of circular plasmids, it does not appear to have one that is closely related to cp26; guaA, guaB, oppA, and ospC homologs are present on linear plasmids or the chromosome in B. hermsii (9, 29, 30) (34a). Conversely, a good approach to developing a plasmid vector for B. burgdorferi may be to introduce a selectable marker such as gyrB\(^{-}\) into a B. hermsii plasmid. Alternatively, we have identified a family of related 32-kb circular plasmids in B. burgdorferi that can coexist within the same spirochete but are not present in all infectious clones (54). One of these plasmids may represent a more useful plasmid vector for B. burgdorferi.

A potential problem in inactivating borrelial genes, either chromosomal or plasmid borne, is the inherent polyplody of the genome: Kitten and Barbour have demonstrated that both the chromosome and the plasmids of B. hermsii are present at 8 to 16 copies per spirochete (21). Theoretically, heterozygotes should be generated by transformation in which the selectable marker had interrupted only some copies of the gene. However, we never found any examples of heterozygosity (with reference to cp26) following transformation with pKK58 or borrelial plasmid DNA. Therefore, the mechanisms of plasmid replication and segregation in borreliae must ensure homozygosity by the time a single spirochete has replicated to form a detectable colony. In vivo, B. hermsii must deal with this same problem in order to derive a clonal advantage from Vmp antigenic variation (3, 21), whereby the expressed vmp gene, present on a multicopy plasmid, is replaced with a copy of a previously silent vmp gene. B. hermsii must either suppress expression from some copies of this plasmid or coexpress Vmps from switched and unswitched plasmids until plasmid homozygosity is attained by segregation.

In this report, we demonstrated targeted insertion of an antibiotic resistance marker into a circular plasmid of B. burgdorferi via homologous recombination with flanking sequences. This is the first example in borreliae of a nonspontaneous mutation whereby the altered or inactivated targeted sequence does not by itself confer an intrinsic selectable advantage. Previously, B. burgdorferi variants have been selected by treatment with specific antiserum that permitted only growth of escape mutants lacking particular outer surface proteins (13, 14, 41, 42, 43, 44). Similarly, gyrB mutants were selected by the ability to grow in the presence of coumermycin A\(_1\) (47, 48). Such direct approaches to isolating specific mutations are powerful but restricted to a limited number of genes. The study presented in this report is an important step toward developing a convenient means of allelic exchange in B. burgdorferi with which to inactivate most genes. We are currently using gyrB\(^{-}\) to inactivate genes of interest on cp26 in order to study their roles in the infectious cycle of B. burgdorferi between ticks and mammals.

ACKNOWLEDGMENTS

We thank T. G. Schwan, J. Hinnebusch, R. Belland, and S. Hill for critical review of the manuscript, S. Smaus for assistance in manuscript preparation, and G. Hettrick and R. Evans for artwork and photography. We thank Van Tamplin, Joan Strange, and Holly Vestal for critical review of the manuscript. S. Smaus for assistance in manuscript preparation, and G. Hettrick and R. Evans for artwork and photography. We thank Van Tamplin, Joan Strange, and Holly Vestal for critical review of the manuscript.

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