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parC Mutations in Fluoroquinolone-Resistant *Borrelia burgdorferi*

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We have isolated in vitro fluoroquinolone-resistant mutants of the Lyme disease agent, *Borrelia burgdorferi*. Mutations in *parC*, which encodes a subunit of topoisomerase IV, were associated with loss of susceptibility to sparfloxacin, moxifloxacin, and Bay-Y3118, but not ciprofloxacin. This is the first description of fluoroquinolone resistance in the spirochete phylum.

*Borrelia burgdorferi*, a bacterium in the spirochete phylum, is the causative agent of Lyme disease (27, 33, 34). It has an unusual genome comprised of a small linear chromosome and a large complement of both linear and circular plasmids (1, 11). Both DNA gyrase and topoisomerase IV map to the linear chromosome in *B. burgdorferi* (11, 15, 19, 31).

DNA gyrase and topoisomerase IV are prokaryotic type II topoisomerases, a group of enzymes that alter DNA topology by breaking and resealing both strands of the double helix. DNA gyrase maintains negative supercoiling in the cell, and topoisomerase IV relaxes supercoiled DNA and decatenates daughter DNA after replication (8, 9, 26, 41). Both DNA gyrase and topoisomerase IV are tetramers comprised of two A subunits (GyrA or ParC) and two B subunits (GyrB or ParE). The A subunits are involved in the double-stranded nicking and resealing reactions, while the B subunits are responsible for providing energy through ATP hydrolysis (26).

Fluoroquinolones are chemotherapeutic agents that target type II topoisomerases by preventing the resealing step in the topoisomerase mechanism (7, 8, 13, 22, 35). Fluoroquinolone treatment results in ternary DNA-topoisomerase-fluoroquinolone complexes that cause lethal double-stranded DNA breaks (3, 17, 40) and block transcription and replication (36, 37). Resistance to fluoroquinolones usually maps to fluoroquinolone complexes that cause double-stranded DNA breaks, which can inhibit cell growth.

Fluoroquinolone resistance-determining regions (QRDRs) that are found in the A subunits of DNA gyrase and topoisomerase IV, encoded by *gyrA* and *parC*, respectively (4, 25, 39). Mapping a first-step mutation to one of these genes indicates that the respective gene encodes the primary target of fluoroquinolones, which is the primary target of DNA gyrase; however, the primary target also depends on the particular fluoroquinolone (3, 10, 18, 24, 39).

We have isolated fluoroquinolone-resistant first-step mutants of *B. burgdorferi* by selection in increasing doses of three different fluoroquinolones in vitro. Although *B. burgdorferi* is not susceptible to many fluoroquinolones (5, 12, 20, 21, 30), recently developed fluoroquinolones demonstrate some therapeutic potential (16). This study provides the first description of fluoroquinolone resistance in the spirochete phylum and is only the second report, to our knowledge, in which genomic mutations have been associated with antibiotic resistance in *B. burgdorferi* (32).

Selection of fluoroquinolone-resistant mutants. High-passage *B. burgdorferi* strain B31 was grown at 34°C in Barbour-Stoenner-Kelly (BSK)-H medium (Sigma). The fluoroquinolones moxifloxacin, ciprofloxacin, sparfloxacin, and Bay-Y3118 were generously provided by Peter Heisig (Abteilung Pharmazeutische Biologie und Mikrobiologie, Institut für Pharmazie, Universität Hamburg). The concentration at which 50% of growth is inhibited (IC50) of each fluoroquinolone was determined for wild-type strain B31 (Table 1) using susceptibility assays as previously described (30). Fluoroquinolones at the IC50 were added to B31 cultures. Each culture was evaluated by dark-field microscopy for growth after 7 days. Non-growing cultures were continually passaged 1:10 into the identical antibiotic concentration until growth was observed by dark-field microscopy, and growing cultures were diluted 10-fold into medium containing twice the concentration of the respective fluoroquinolone. Cultures growing in 16-fold the wild-type IC50 of a particular fluoroquinolone were plated for isolation in semisolid medium containing 10-fold the IC50, and cultures growing in 18-fold the wild-type IC50 were plated on 100-fold the IC50 as previously described (29). Five to 10 colonies were selected from each plate; no mutant was identified from ciprofloxacin-treated cultures despite exhaustive efforts.

Mutations in *parC* of fluoroquinolone-resistant *B. burgdorferi*. DNA was isolated from fluoroquinolone-resistant *B. burgdorferi* as previously described (31). The region of the *gyrA* gene (BB0435) encoding the QRDR was amplified by PCR with primers gyrB 1885F (5'-GTAATTAATCTTGATGTTGGAATTGG-3') and gyrA 538R (5'-TTCCAACAGCAATTCCCAAAATCA-3'). The region of the *parC* gene (BB0355) encoding the QRDR of topoisomerase IV was amplified with parC 68F (5'-CTAGTGTTTGTATATGGAATTGG-3') and parC 311R (5'-CTAGAAGCAGCAATTGAC-3').
The sequences of the 570-bp gyrA region in all fluoroquinolone-resistant mutants were identical to that of the wild-type strain B31, but all nine strains sequenced had mutations in the 244-bp parC region (Table 2). Mutants selected in Bay-Y3118, an experimental fluoroquinolone not used clinically, contained one of two mutations, T69K or S70P. Mutant KG1R2, which is ninefold more resistant to Bay-Y3118 than parental B31, had a C-to-A transversion at nucleotide 206 in parC that resulted in a Thr-69-to-Lys change. Mutant KG1R1, which is eightfold more resistant than the wild type to Bay-Y3118, had a T-to-C transition at nucleotide 208 in parC that encoded a Ser-70-to-Pro change. Mutant KG1S3, selected in sparfloxacin, had a C-to-G transversion at nucleotide 206 in parC that resulted in a Thr-69-to-Arg change.

Mutants KG1S1 and KG1M1, which resulted from independent selection in sparfloxacin and moxifloxacin, respectively, had an A-to-G transition at nucleotide 218 of parC that encoded a Gln-73-to-Gly change. This E73G mutation was associated with 14-fold resistance to moxifloxacin and 11-fold resistance to sparfloxacin. Selection in a higher level of resistance to sparfloxacin resulted in isolation of mutant KG2S5 with a Glu-73-to-Gly change. This E73G mutation was associated with 14-fold resistance to moxifloxacin and 11-fold resistance to sparfloxacin. Selection in a higher level of susceptibility and the frequency of resistance (6). Interestingly, the mutants are not necessarily most resistant to the other fluoroquinolones are not resistant to the other fluoroquinolones (Table 1). The reason for the intractability is not clear, although it is likely due to subtle structural differences among the antibiotics. Furthermore, this result suggests that the choice of antibiotic therapy should consider both the level of susceptibility and the frequency of resistance (6). Interestingly, the mutants are not necessarily most resistant to the fluoroquinolone used to select them: KG1R2 and KG1R1 were isolated in Bay-Y3118 but are more resistant to sparfloxacin and moxifloxacin, respectively (Tables 1 and 2). We hypothesize that all four classes of mutant can be selected by each of the three fluoroquinolones; our failure to identify each class of mutation under selective pressure from each of the antibiotics is likely a result of limited sample size. However, different mutants have different resistance profiles. KG1R2

Conclusions. Fluoroquinolones are potent chemotherapeutic agents that target the A subunit of both DNA gyrase and topoisomerase IV. We have isolated fluoroquinolone-resistant mutants of B. burgdorferi and have mapped single point mutations to the QRDR of the parC gene encoding the A subunit of topoisomerase IV. This indicates that the primary target of the fluoroquinolones moxifloxacin, sparfloxacin, and Bay-Y3118 in B. burgdorferi is topoisomerase IV. This is the first example of mutations that confer resistance to fluoroquinolones in the Lyme disease agent or any spirochete.

A previous study by Kraiczy et al. (20) determined the fluoroquinolone susceptibility of B. burgdorferi. Although the collection of fluoroquinolones studied was broader and the method of assaying susceptibility differed from our study, the data are consistent. Their MICs for strain B31 are about twice our IC50 values. We and others have also previously reported similar susceptibilities to ciprofloxacin, moxifloxacin, or sparfloxacin (5, 12, 21, 30). Susceptibility of B. burgdorferi to newer fluoroquinolones (20) suggests that these antibiotics have potential as therapeutic agents for Lyme disease (16).

B. burgdorferi is, to an extent, naturally resistant to fluoroquinolones, and we hypothesize that this is due to the presence of a glutamine at position 86 in GyrA, a highly conserved position occupied by a serine residue in almost all bacterial species, with the exception of mycobacteria, Helicobacter pylori, and spirochetes (Fig. 2) (7). The homolog of this serine is mutated in fluoroquinolone-resistant strains of several different species (7, 8, 14, 23, 28). In Escherichia coli, a single mutation of Ser-83 (homologous to Gln-86 of B. burgdorferi) to Trp in GyrA prevents binding of the fluoroquinolone norfloxacin (38) and results in 32-fold resistance to ciprofloxacin (39); other substitutions also confer resistance. We have been unable to isolate ciprofloxacin-resistant mutants, and the mutants that are resistant to the other fluoroquinolones are not resistant to ciprofloxacin (Table 1). The reason for the intractability is not clear, although it is likely due to subtle structural differences among the antibiotics. Furthermore, this result suggests that the choice of antibiotic therapy should consider both the level of susceptibility and the frequency of resistance (6). Interestingly, the mutants are not necessarily most resistant to the fluoroquinolone used to select them: KG1R2 and KG1R1 were isolated in Bay-Y3118 but are more resistant to sparfloxacin and moxifloxacin, respectively (Tables 1 and 2). We hypothesize that all four classes of mutant can be selected by each of the three fluoroquinolones; our failure to identify each class of mutation under selective pressure from each of the antibiotics is likely a result of limited sample size. However, different mutants have different resistance profiles. KG1R2

### Table 1. Fluoroquinolone susceptibility of B. burgdorferi wild-type strain B31 and mutants

<table>
<thead>
<tr>
<th>Fluoroquinolone</th>
<th>B31 (wild type)</th>
<th>KG1R2 (T69K)</th>
<th>KG1R1 (S70P)</th>
<th>KG1M1 (E73G)</th>
<th>KG2S5 (E73K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bay-Y3118</td>
<td>0.18</td>
<td>1.7 (9)</td>
<td>1.4 (8)</td>
<td>0.8 (4)</td>
<td>4.2 (23)</td>
</tr>
<tr>
<td>Sparfloxacin</td>
<td>0.23</td>
<td>9.2 (42)</td>
<td>10 (43)</td>
<td>2.4 (11)</td>
<td>15 (75)</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>0.38</td>
<td>9.8 (26)</td>
<td>17 (45)</td>
<td>5.2 (14)</td>
<td>5.8 (15)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.49</td>
<td>0.6 (1.2)</td>
<td>0.9 (1.8)</td>
<td>0.6 (1.2)</td>
<td>1.2 (2.5)</td>
</tr>
</tbody>
</table>

* a Values in parentheses represent fold resistance compared to wild-type B31.

### Table 2. Fluoroquinolone selection of ParC mutants in B. burgdorferi

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fluoroquinolone selection (µg ml⁻¹)</th>
<th>ParC mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG1R2</td>
<td>Bay-Y3118 (2)</td>
<td>Thr-69 to Lys (T69K)</td>
</tr>
<tr>
<td>KG1R1, KG1R3</td>
<td>Bay-Y3118 (2)</td>
<td>Ser-70 to Pro (S70P)</td>
</tr>
<tr>
<td>KG1M1, KG1M3</td>
<td>Moxifloxacin (4)</td>
<td>Glu-73 to Gly (E73G)</td>
</tr>
<tr>
<td>KG1S1</td>
<td>Sparfloxacin (2)</td>
<td>Glu-73 to Gly (E73G)</td>
</tr>
<tr>
<td>KG1S3</td>
<td>Sparfloxacin (2)</td>
<td>Thr-69 to Arg (T69R)</td>
</tr>
<tr>
<td>KG2S5, KG2S2</td>
<td>Sparfloxacin (20)</td>
<td>Glu-73 to Lys (E73K)</td>
</tr>
</tbody>
</table>

* a Strains in bold were further characterized in this study.
and KG2S5 are most resistant to sparflxacin, while KG1R1 and KG1M1 are most resistant to moxifloxacin. Again, this likely reflects slightly different interactions between each fluoroquinolone and topoisomerase IV. Two mutations are substitutions at Glu-73, which is homologous to a conserved acidic residue (Asp-87 in *E. coli* GyrA) that is commonly mutated in fluoroquinolone-resistant strains (7, 8, 28). KG2S5, with an E73K mutation, is more resistant to the fluoroquinolones, especially Bay-Y3118 and sparflxacin, than KG1M1, with an E73G mutation. E73G replaces the acidic residue with a neutral amino acid, and E73K replaces it with a basic amino acid. This suggests that these fluoroquinolones contact topoisomerase IV through an ionic interaction that is lost in E73G and replaced with repulsion in E73K. We are currently using fluoroquinolone-resistant *parC* as a counterselectable marker for genetic studies in *B. burgdorferi* (S. F. Bundle and D. S. Samuel, unpublished data).

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