

Sequencing of Gyrase and Topoisomerase IV Quinolone-Resistance-Determining Regions of *Chlamydia trachomatis* and Characterization of Quinolone-Resistant Mutants Obtained In Vitro

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The L2 reference strain of *Chlamydia trachomatis* was exposed to subinhibitory concentrations of ofloxacin (0.5 µg/ml) and sparfloxacin (0.015 µg/ml) to select fluoroquinolone-resistant mutants. In this study, two resistant strains were isolated after four rounds of selection. The *C. trachomatis* mutants presented with high-level resistance to various fluoroquinolones, particularly to sparfloxacin, for which a 1,000-fold increase in the MICs for the mutant strains compared to the MIC for the susceptible strain was found. The MICs of unrelated antibiotics (doxycycline and erythromycin) for the mutant strains were identical to those for the reference strain. The gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*parC*, *parE*) genes of the susceptible and resistant strains of *C. trachomatis* were partially sequenced. A point mutation was found in the *gyrA* quinolone-resistance-determining region (QRDR) of both resistant strains, leading to a Ser83→Ile substitution (*Escherichia coli* numbering) in the corresponding protein. The *gyrB*, *parC*, and *parE* QRDRs of the resistant strains were identical to those of the reference strain. These results suggest that in *C. trachomatis*, DNA gyrase is the primary target of ofloxacin and sparfloxacin.

Chlamydia trachomatis, an obligate intracellular bacterium, causes a wide spectrum of human diseases and is one of the most common sexually transmitted pathogens in the world (49). The antibiotic of choice for the treatment of chlamydial infection is doxycycline, but erythromycin and azithromycin are used as alternative therapies (41). Some fluoroquinolones also have good antichlamydial activity, and ofloxacin has been administered for the treatment of *C. trachomatis* urethritis and cervicitis and is proposed for use in the treatment of pelvic inflammatory diseases (41). Recent studies of the in vitro and in vivo activities of sparfloxacin against *C. trachomatis* have reported that it may provide a promising therapy for genital chlamydial infections (36, 38). The relative resistance of *C. trachomatis* isolates to tetracyclines and erythromycin has been reported previously (21, 30). Although fluoroquinolone resistance in *C. trachomatis* has never been described, the emergence of resistant isolates posttherapy could occur and could have dramatic clinical implications.

The principal targets of the quinolones are DNA gyrase and topoisomerase IV (Topo IV). DNA gyrase is composed of two pairs of subunits A and B encoded by the *gyrA* and *gyrB* genes, respectively. Gyrase catalyzes ATP-dependent negative supercoiling of DNA and is involved in DNA replication, recombination, and transcription (40, 51). Topo IV, recently described in *Escherichia coli* (22), is a tetramer consisting of two pairs of ParC (GrlA) and ParE (GrlB) subunits, which are homologous to the GyrA and GyrB subunits of DNA gyrase, respectively, and is involved in the partitioning of the replicated chromosome (1, 22). Fluoroquinolone resistance has been related to single point mutations which occur in the quinolone-resis-

tance-determining region (QRDR) of DNA gyrase and Topo IV subunit genes. The GyrA QRDR is located between amino acid residues equivalent to Ala-67 through Gln-106 in *E. coli* (54), and the most frequent mutations associated with quinolone resistance are located at Ser-83 and relatively fewer mutations associated with quinolone resistance are located at Asp-87 (5, 15, 34, 54). Recent studies have identified in the analogous QRDR of ParC similar point mutations that result in fluoroquinolone resistance (2, 24, 32). Furthermore, substitutions of the amino acids corresponding to residues 426, 447, and 463 in GyrB (*E. coli* numbering) (11, 20, 55) and residues 420, 445, and 458 in ParE (3, 9, 37) have also been determined to confer quinolone resistance.

Other mechanisms of resistance associated with a reduction of the level of intracellular accumulation of fluoroquinolones and involving active efflux systems or decreased permeability of the outer membrane in gram-negative bacteria have also been identified (39).

In this study, the sequences of the putative *gyrA* and *parC* QRDRs and of most of the *gyrB* and *parE* genes of the *C. trachomatis* L2/434/Bu (L2) reference strain are reported. In addition, two quinolone-resistant mutants of *C. trachomatis* were selected with ofloxacin and sparfloxacin by using a stepwise procedure and were characterized for gyrase (*gyrA*, *gyrB*) and Topo IV (*parC*, *parE*) QRDRs. A point mutation located in the *gyrA* QRDRs of these strains was associated with fluoroquinolone resistance.

MATERIALS AND METHODS

Bacterial strains and cells. The reference strain *C. trachomatis* L2 was used for the selection of quinolone-resistant mutants. Competent *E. coli* JM 109, which was used in the cloning experiments, was obtained from Promega (Charbonnières, France).

McCoy cells, which were used for chlamydial culture, were grown in minimal essential medium supplemented with 5% fetal bovine serum and 2 mM L-glutamine and were incubated at 37°C in 5% CO₂.

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Antibiotics. The antimicrobial agents tested were sparfloracin and pefloxacin (purchased from Rhône-Poulenc-Rorer, Vitry-sur-Seine, France), ciprofloxacin (Bayer-Pharma, Puteaux, France), ofloxacin and erythromycin (Roussel-Uclaf, Paris, France), norfloxacin (Marion-Merrell-Dow, Levallois-Perret, France), and doxycycline (Pfizer, Orsay, France).

MIC determinations. The antibiotic susceptibilities of the L2 reference strain of *C. trachomatis* and in vitro-selected quinolone-resistant mutants were determined by the standard method. Briefly, McCoy cells that had been seeded into 24-well plastic plates with glass coverslips and incubated for 24 h at 37°C in 5% CO₂ were inoculated with 10³ inclusion-forming units of the L2 strain per ml. The plates were centrifuged at 1,200 × g at 37°C for 1 h and were incubated for 2 h at 37°C in 5% CO₂. Then, the cell monolayers were washed with 0.5 ml of culture medium and were incubated with twofold dilution series of antibiotics in complete medium containing culture medium supplemented with 0.5% (wt/vol) glucose and 1 µg of cycloheximide per ml. The drug concentrations tested ranged from 0.25 to 256 µg/ml for ciprofloxacin, pefloxacin, and ofloxacin, from 3 to 384 µg/ml for norfloxacin, from 0.008 to 64 µg/ml for sparfloracin, from 0.05 to 1.6 µg/ml for erythromycin, and from 0.0125 to 0.4 µg/ml for doxycycline. Moreover, the titer of *C. trachomatis* was verified in each MIC determination experiment with McCoy cells inoculated with 10-fold dilutions of the chlamydial strain and incubated in antibiotic-free complete medium. The plates were incubated for 48 h at 37°C in 5% CO₂. Then, the MICs and strain titers were determined by fluorescence microscopy. Cell monolayers were fixed in methanol, stained for chlamydial inclusions with an anti-*Chlamydia* major outer membrane protein fluorescein-conjugated monoclonal antibody (Syva Microtrak; Behring-Syva, Rueil-Malmaison, France), and observed at ×400 magnification. The MIC was defined as the lowest antibiotic concentration at which no inclusion was observed.

Selection of ofloxacin- and sparfloracin-resistant mutants. The quinolone-resistant mutants of the L2 strain of *C. trachomatis* were selected by successive passages in the presence of subinhibitory concentrations of ofloxacin and sparfloracin. The protocol used for the stepwise selection of resistant mutants of *C. trachomatis* was derived from those previously described by Tipples and McClarty (50) and Wang et al. (52). First, confluent McCoy cell monolayers in 75-cm² flasks were inoculated with approximately 10⁸ inclusion-forming units of *C. trachomatis* L2, and complete medium supplemented with 0.5 and 0.015 µg of ofloxacin and sparfloracin per ml, respectively, was immediately added. The infected cells were incubated at 37°C in 5% CO₂ for 48 h. Then, the supernatant was removed and a freeze-thaw cycle at -80°C was performed to disrupt the cells and liberate the intracellular bacteria. After thawing, sterile glass beads and 5 ml of complete medium plus 0.5 and 0.015 µg of ofloxacin and sparfloracin per ml, respectively, were added. The cellular suspension (i.e., elementary bodies [EBs] and cell debris) was centrifuged at 400 × g for 5 min. Then, 4 ml of the supernatant, which contained EBs, was used to inoculate fresh confluent McCoy cell monolayers in the presence of subinhibitory concentrations of ofloxacin and sparfloracin (0.5 and 0.015 µg/ml, respectively). The flasks were incubated for 48 h at 37°C in 5% CO₂, and the presence of inclusions was observed by inverted light microscopy. The passages were repeated with the same fluoroquinolone concentrations until a highly infectious inoculum was obtained, and the MICs for these selected strains were determined. An aliquot (1 ml) from each passage was stored at -80°C. To attempt to increase the level of resistance of the mutants selected with subinhibitory concentrations of ofloxacin and sparfloracin, several passages were performed with these fluoroquinolones at 16, 32, and 64 µg/ml.

DNA isolation. Genomic DNA was extracted from three chlamydial strains, the L2 reference strain and the in vitro-selected ofloxacin- and sparfloracin-resistant strains L2-OFXR and L2-SPXR, respectively. Each strain was grown on confluent McCoy cell monolayers in three flasks (75 cm²) with antibiotic-free complete medium for the reference strain or in the presence of 8 µg of ofloxacin per ml or 4 µg of sparfloracin per ml for the resistant strains L2-OFXR and L2-SPXR, respectively, to maintain selection pressure. EBs were purified by using a previously described protocol (42, 46) that included a DNase I treatment to digest the eukaryotic DNA. Then, the EBs, which were resuspended in phosphate-buffered saline (pH 7.2), were mixed with a lysis buffer (10 mM Tris HCl [pH 8.3], 50 mM KCl, 4.5 mM MgCl₂, 0.45% Nonidet P-40, 0.45% Tween 20) and were treated with 200 µg of proteinase K per ml. They were heated at 56°C for 90 min and at 95°C for 15 min. Then, the DNA was purified by phenol-chloroform-isoamyl alcohol extractions and ethanol precipitation as described previously (44, 46). Control DNA, isolated from uninfected McCoy cells, was extracted by the same protocol. Moreover, a simplified procedure consisting of direct lysis after culture (i.e., without EB purification and DNA extraction and precipitation) was also used for some PCR experiments. Lysates were prepared from the three chlamydial strains and from uninfected control McCoy cells.

Amplification of the QRDRs of the *gyrA* and *gyrB* genes. PCR amplification of the *gyrA* QRDR of *C. trachomatis* L2 was carried out with the degenerate primers CTA1 and CTA2 (Table 1), whose nucleotide sequences were deduced from highly conserved motifs of the *Chlamydia psittaci* (19), *Helicobacter pylori* (28), and *Campylobacter jejuni* (53) GyrA proteins. From the sequence of the CTA1-CTA2 fragment, two specific primers, primers CTA3 plus CTA4 (Table 1; see Fig. 1), were chosen to amplify a 362-bp DNA fragment from the resistant mutant strains of *C. trachomatis*.

The nucleotide sequences of the degenerate primers CTB1 and CTB2 (Table 1) were deduced from the sequence of the two conserved motifs PGKLDAC

TABLE 1. Nucleotide sequences of the primers used for PCR

Gene	Primer	Nucleotide sequence (5'→3')
<i>gyrA</i>	CTA1	GA(T,C)GG(T,C)TT(G,A)AA(G,A)CC(T,C)GT (G,T)CAT
	CTA2	(G,C)GCCAT(G,C)CC(T,C)AC(G,A)GCGAT(A,C) CC
	CTA3	TTAAAACCTTCTCAGCGACG
	CTA4	GAAGGAAAAACTACAGGTTTC
	CTA5	TCCTTCATTTCCTCTTCAAG
<i>gyrB</i>	CTB1	CC(T,C)GG(T,C)AA(G,A)TT(G,A)GC(G,C)GA (T,C)TG(T,C)
	CTB2	(G,A)TC(A,C)AC(G,A)TC(G,C)GC(G,A)TC(G,A) GTCAT
	CTB3	AGGAATCCCTATTCAGATTC
	CTB4	AACACCTTGCTACAACCGTT
	CTB5	TAAGTTAATTGACTGCTTGG
	CTB6	ACGTTTATAACGCAATTTGC
	CTB7	TGGATGGCTATTTGCTGAAC
<i>parC</i>	CTC1	GA(T,C)GG(T,C)CT(G,A)AA(G,A)CC(T,C)GT (G,T)CA(G,A)
	CTC2	(A,C)GT(T,C)AG(T,C)GG(G,A)TT(G,T)CC(G,A) AA(G,A)TT
	CTC3	GATGGCTCAAGCCTGTTC
	CTC4	CAGTGGATTGCCAAGTTC
	CTC5	CATTTACCATCAACATAC
<i>parE</i>	CTE1	AA(T,C)GC(A,G)AT(A,T)GA(T,C)GA(A,G)TT (T,C)GT
	CTE2	AAGGCATTCTCTTACACACA
	CTE3	ACAACCTGATGCGGAAACCCAT
	CTE4	GCTGTGCTCTCTTTTAA
	CTE5	TCAACATCCGCATCTGTTGC
	CTE6	CACAGACAATTCTCTGTATG

(residues 404 to 410 by the *E. coli* GyrB numbering) and MTDADVD (residues 496 to 502) flanking the GyrB QRDRs of various bacteria (26). An unexpected 1,521-bp PCR product was amplified and was completely sequenced with two internal primers, primers CTB3 and CTB4 (Table 1; see Fig. 1). From the sequence of this large fragment, two specific primers, primers CTB5 and CTB6 (Table 1; see Fig. 1), were chosen to amplify a 262-bp *gyrB* fragment including the QRDRs from the resistant strains of *C. trachomatis*.

The *gyrB* and *gyrA* genes were recently described to be contiguous in *Chlamydia* (19), and so a PCR amplification was performed with the specific primers CTB5 (*gyrB*) and CTA4 (*gyrA*). The amplified fragment was directly sequenced with the internal primers CTB7 and CTA5 (Table 1; see Fig. 1), whose sequences were chosen from the sequences of the *gyrB* and *gyrA* genes, respectively.

All PCRs were performed in a final volume of 50 µl containing each primer at a concentration of 1 µM, 200 µM deoxynucleoside triphosphates, 1.5 mM MgCl₂, 1× Taq buffer, 2 U of Taq polymerase (Perkin-Elmer Applied Biosystems, Roissy, France), and 100 ng of purified strain L2 DNA. After a denaturation step of 10 min at 95°C, amplification with the degenerate primers was performed over 40 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C. With the pairs of specific primers CTA3 plus CTA4, CTB5 plus CTB6, and CTB5 plus CTA4, PCR conditions differed from those described above only by the annealing temperature (60°C). Two negative controls containing DNA extracted from uninfected McCoy cells or water were included in each PCR experiment.

Amplification of the QRDRs of the *parC* and *parE* genes. PCR amplification of the *parC* QRDR of the *C. trachomatis* L2 reference strain was carried out with the degenerate primers CTC1 and CTC2 (Table 1), whose sequences were deduced from the amino acid sequence of the *C. psittaci* ParC protein (19). From the nucleotide sequence of the CTC1-CTC2 product, two specific primers, primers CTC3 and CTC4 (Table 1; see Fig. 2), were selected to amplify a 201-bp fragment, including the QRDRs from the resistant strains of *C. trachomatis*, under the conditions described above for CTA3-CTA4 amplification.

The degenerate primer CTE1 (Table 1), whose sequence was deduced from one of the two GyrB-like partial sequences of *C. trachomatis* published by Huang (18), was chosen to amplify the *parE* gene of this organism. The *parE* and *parC* genes were recently found to be contiguous in *Chlamydia* (19), and so primer CTE1 was associated with the specific primer CTC4 (*parC*) for PCR amplification. The amplified product was directly sequenced with four internal primers, primers CTE2, CTE3, CTE6 (in the *parE* gene), and CTC5 (in the *parC* gene) (Table 1; see Fig. 2). Then, from the nucleotide sequence of the *parE* gene of *C. trachomatis*, two specific primers, primers CTE4 and CTE5 (Table 1; see Fig.

TABLE 2. Antibiotic susceptibilities of the reference strain and fluoroquinolone-resistant mutants of *C. trachomatis* L2

Strain	Selecting agents	MIC ($\mu\text{g/ml}$) ^a						
		OFX	SPX	PFX	CFX	NFX	ERY	DOX
Reference	None	1	0.03	2	1	12	0.4	0.05
L2-OFXR	Ofloxacin	64	32	32	32	96	0.4	0.05
L2-SPXR	Sparfloxacin	32	32	32	16	48	0.4	0.05

^a OFX, ofloxacin; SPX, sparfloxacin; PFX, pefloxacin; CFX, ciprofloxacin; NFX, norfloxacin; ERY, erythromycin; DOX, doxycycline.

2), were selected to amplify the QRDRs from the fluoroquinolone-resistant strains as described above.

DNA sequencing and sequence analysis. The amplification products of *C. trachomatis* L2 and quinolone-resistant strains were purified with the Wizard PCR Preps DNA Purification System (Promega). The PCR products of *C. trachomatis* L2 were cloned in *E. coli* by using the pGEM-T Easy Vector System II (Promega) and were then sequenced, whereas those of the quinolone-resistant strains were directly sequenced. Sequencing was carried out with an AmpliTaq DNA polymerase FS Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI-Prism 377 sequencer (Applied Biosystems Division, Perkin-Elmer) according to the manufacturer's instructions.

Nucleotide sequence accession numbers. The nucleotide sequence data reported here will appear in the GenBank nucleotide sequence databases with the following accession numbers: AF044267 for the *gyrB* and *gyrA* sequences and AF044268 for the *parE* and *parC* sequences.

RESULTS

In vitro selection of ofloxacin- and sparfloxacin-resistant mutants of *C. trachomatis*. Two fluoroquinolone-resistant mutants of *C. trachomatis* were isolated by repeated exposures of the L2 reference strain to subinhibitory concentrations of ofloxacin and sparfloxacin. Spontaneous mutants L2-OFXR and L2-SPXR were obtained after four passages of the reference strain in the presence of 0.5 μg of ofloxacin per ml and 0.015 μg of sparfloxacin per ml, respectively. After the first passage with subinhibitory concentrations of ofloxacin and sparfloxacin, a few small inclusions could be seen. The inclusion size and number increased during the subsequent steps with the same antibiotic concentration until about 80 to 100% of the McCoy cells were infected after four passages. These fluoroquinolone-resistant mutants were expanded, and their *gyrA*, *gyrB*, *parC*, and *parE* QRDR sequences were analyzed. Passages in the presence of higher concentrations (16, 32, and 64 $\mu\text{g/ml}$) of ofloxacin and sparfloxacin were unsuccessful in generating higher levels of resistance.

The patterns of susceptibility to fluoroquinolones and unrelated antibiotics of the L2 reference strain and its two derivative mutants, L2-OFXR and L2-SPXR, are shown in Table 2. The MICs of the five fluoroquinolones tested increased for both mutants compared with those for the reference strain, particularly the MICs of sparfloxacin, for which a 1,000-fold increase in the MIC was obtained (MIC, 32 $\mu\text{g/ml}$). Both mutants showed high-level resistance to ofloxacin, pefloxacin, and ciprofloxacin, and the increase in the MICs for the mutants varied from 16- to 64-fold compared with the MIC for the susceptible reference strain. Eight- and fourfold increases in the MICs of norfloxacin were found for strains L2-OFXR and L2-SPXR, respectively. The MICs of ofloxacin, ciprofloxacin, and norfloxacin were slightly lower for strain L2-SPXR, selected on sparfloxacin, than for the ofloxacin-selected L2-OFXR mutant, with at most a difference of 1 dilution. This difference is probably due to the imprecision of the MIC reading method, and it is not significant. Interestingly, the MICs of unrelated antibiotics, such as doxycycline and erythromycin,

were identical for the reference strain and its derivative mutants (Table 2).

Amplification and sequence analysis of the *gyrA* QRDR and most of the *gyrB* gene of *C. trachomatis*. The degenerate primer pairs CTA1-CTA2 and CTB1-CTB2 were chosen to amplify the *gyrA* and *gyrB* QRDRs of *C. trachomatis* L2, respectively. As expected, a 441-bp DNA fragment was obtained with primer pair CTA1-CTA2, whereas an unexpected large product of 1,521 bp was amplified with primers CTB1 and CTB2. Analysis of the sequences of these two fragments revealed the presence of highly conserved motifs in the bacterial GyrA- and GyrB-like proteins, respectively. With the specific primers CTA4 (*gyrA*) and CTB5 (*gyrB*), a 1,657-bp DNA fragment was amplified, confirming that the *gyrB* and *gyrA* genes of *C. trachomatis* are contiguous, with the *gyrB* gene being located upstream of the *gyrA* gene (19). The nucleotide and amino acid sequences of most of the *gyrB* gene and the 5' end of the *gyrA* gene of *C. trachomatis* are presented in Fig. 1. The sequences of the chlamydial GyrA and GyrB proteins were compared to the sequences of other bacterial GyrA-like (Table 3) and GyrB-like (Table 4) proteins, respectively. The amino acid sequence of the GyrA fragment of *C. trachomatis* was closely related to that of *C. psittaci* (19), with 91% identity. When compared to other GyrA QRDRs, the *C. trachomatis* GyrA QRDR sequence was more related to those of *E. coli* (47) and *Staphylococcus aureus* (17). The GyrA QRDR of *C. trachomatis* showed a higher percentage of identity with the GyrA sequences than with the ParC sequences of other bacteria, particularly for *Borrelia burgdorferi* (19) (Table 3). The sequence of the N-terminal region of the chlamydial GyrB protein was identical to one of the two GyrB-like partial sequences of *C. trachomatis* (sequence CT1) published by Huang (18) (data not shown). The GyrB protein of *C. trachomatis* shared 42, 46, 48, and 48.5% identities with those of *B. burgdorferi* (45), *S. aureus* (17), *Bacillus subtilis* (29), and *E. coli* (4), respectively (Table 4), and appeared to be more closely related to the GyrB proteins than to the ParE proteins of these bacteria (Table 4).

Furthermore, no terminator-like structure was found between the *gyrB* and *gyrA* genes (Fig. 1).

From these data, we concluded that the fragments amplified with the primers pairs CTA1-CTA2 and CTB1-CTB2 belonged to the *gyrA* and *gyrB* genes of *C. trachomatis*, respectively.

Amplification and sequence analysis of the *parC* QRDR and most of the *parE* gene of *C. trachomatis*. With the degenerate primers CTC1 and CTC2, whose sequences were deduced from the regions flanking the QRDR of the *C. psittaci* ParC protein (19), an expected 204-bp DNA fragment was amplified from *C. trachomatis*. When the specific primer CTC4 (*parC*) was associated with the degenerate primer CTE1, whose sequence was deduced from the partial GyrB-like sequence CT2 of *C. trachomatis* described by Huang (18), a 1,967-bp DNA fragment was amplified. This result suggested that the *parE* and *parC* genes are contiguous in *C. trachomatis*, as described recently (19), with the *parE* gene being located upstream of the *parC* gene on the chlamydial chromosome. The nucleotide and deduced amino acid sequences of most of the *parE* gene and the 5' end of the *parC* gene of *C. trachomatis* are presented in Fig. 2. Partial sequences of the ParC and ParE proteins of *C. trachomatis* were compared to those other bacterial GyrA-like (Table 3) and GyrB-like (Table 4) protein sequences. The amino acid sequence of the CTC1-CTC2 fragment amplified from *C. trachomatis* shared 93.5% identity with the ParC QRDR of *C. psittaci* (19). When compared to the ParC sequences of unrelated bacterial species, the ParC QRDR of *C. trachomatis* was more closely related to that of *B. burgdorferi* (19), with 68.5% identity. The ParC QRDR of

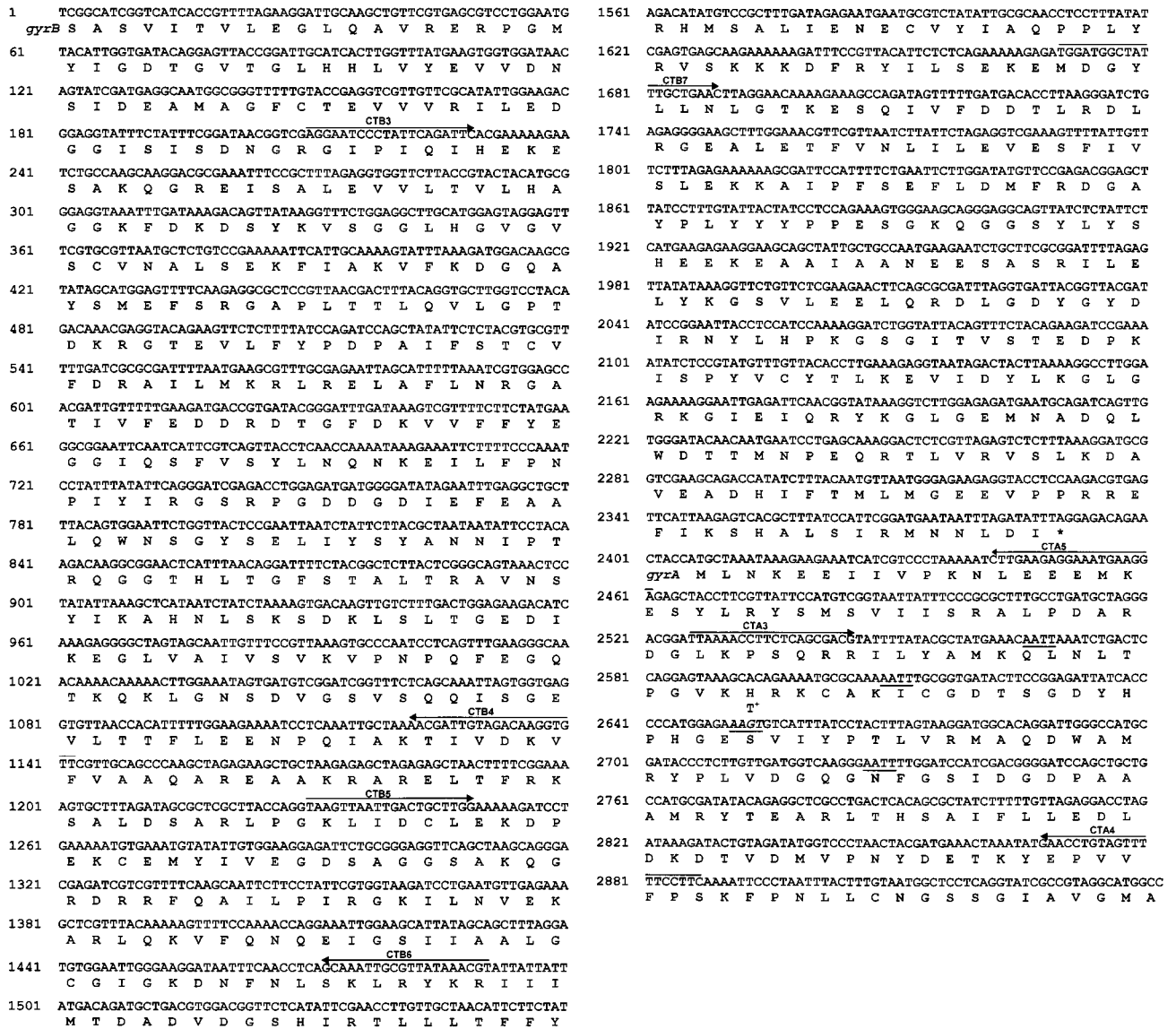


FIG. 1. Nucleotide and amino acid sequences of a 2,942-bp fragment which contains most of the *gyrB* gene and the 5' end of the *gyrA* gene of the *C. trachomatis* L2 reference strain. Specific primers are indicated by arrows. +, nucleotide mutation (G→T) in the *gyrA* gene leading to the Ser→Ile substitution in the GyrA protein. *Tsp*5091 restriction sites (AATT) are underlined; the site indicated in italics corresponds to the site created by the mutation (G→T).

C. trachomatis showed higher percentages of identity with the ParC sequences than with the GyrA sequences of other bacteria such as *B. burgdorferi* (19), *E. coli* (22, 47), and *B. subtilis* (29, 43) but, surprisingly, not with that of *S. aureus* (Table 3). Furthermore, the GyrA and ParC QRDR sequences of *C. trachomatis* shared 38% identity.

When compared to other ParE sequences, the chlamydial ParE protein appeared to be quite distant from the other known bacterial ParE sequences except that of *B. burgdorferi* (10) (50% identity) (Table 4). The ParE protein of *C. trachomatis* showed a significantly higher percentage of identity with the ParE sequences than with the GyrB sequences of *B. burgdorferi*

TABLE 3. Identities between the amino acid sequences of the GyrA and ParC QRDRs from various species^a

<i>C. trachomatis</i> sequence compared	% Sequence identity								
	Ec GyrA	Sa GyrA	Bs GyrA	Bb GyrA	Ec ParC	Sa GrIA	BsGrIA	BbParC	Ct ParC
GyrA	63	63	60.5	57	54	58	59.5	41.5	38
ParC	33	42	35.5	39.5	37	37	37	68.5	100

^a Portions of GyrA from *E. coli* (Ec) (47), *S. aureus* (Sa) (17), *B. subtilis* (Bs) (29), *B. burgdorferi* (Bb) (19), and *C. trachomatis* and of ParC from *E. coli* (Ec) (22), *S. aureus* (Sa) (6), *B. subtilis* (Bs) (43), *B. burgdorferi* (Bb) (19), and *C. trachomatis* (Ct) were compared. All sequences compared corresponded to amino acids 39 to 179 and 36 to 111 of *E. coli* GyrA and ParC, respectively.

TABLE 4. Identities between the amino acid sequences of most of the GyrB and ParE proteins from various species^a

<i>C. trachomatis</i> sequence compared	% Sequence identity								
	Ec GyrB	Sa GyrB	Bs GyrB	Bb GyrB	Ec ParE	Sa GrlB	Bs GrlB	Bb ParE	Ct ParE
GyrB	48.5	46	48	42	33	38.5	39.5	23.5	22
ParE	21	27	27	27	28	25	26.5	50	100

^a Portions of GyrB from *E. coli* (Ec) (4), *S. aureus* (Sa) (17), *B. subtilis* (Bs) (29), *B. burgdorferi* (Bb) (45), and *C. trachomatis* and of ParE from *E. coli* (Ec) (22), *S. aureus* (Sa) (6), *B. subtilis* (Bs) (43), *B. burgdorferi* (Bb) (10), and *C. trachomatis* (Ct) were compared. All the sequences compared corresponded to amino acids 6 to 804 and 42 to 555 of *E. coli* GyrB and ParE, respectively.

(10, 45) and *E. coli* (4, 22). The GyrB and ParE sequences of *C. trachomatis* shared only 22% identity and presented a difference in size of about 150 amino acids in the C-terminal region, with ParE being smaller than GyrB.

Furthermore, no terminator-like structure was detected between the *parE* and *parC* genes.

Characterization of ofloxacin- and sparfloxacin-resistant strains of *C. trachomatis*. The *gyrA*, *gyrB*, *parC*, and *parE* QRDRs of the L2-OFXR and L2-SPXR strains were amplified by PCR and were directly sequenced. The sequences of the specific primers CTA3 and CTA4 were chosen from the nucleotide sequence of the 441-bp DNA fragment of *C. trachomatis* containing the *gyrA* QRDR. These primers amplified an expected 362-bp fragment of the *C. trachomatis gyrA* gene. A single base change (G→T) was detected in the sequence of the *gyrA* QRDR of resistant strains L2-OFXR and L2-SPXR compared to the sequence of the L2 strain. This mutation resulted in a Ser (AGT)-to-Ile (ATT) substitution at the position corresponding to amino acid 83 in the *E. coli* GyrA protein (Fig. 1). Interestingly, the G→T mutation led to the creation of a *Tsp509I* restriction site (AATT). After digestion with *Tsp509I*, the 362-bp PCR product of *C. trachomatis* L2 yielded four fragments of 161, 118, 43, and 40 bp, suggesting the presence of three *Tsp509I* sites. In contrast, the digested CTA3-CTA4 PCR product of resistant strains L2-OFXR and L2-SPXR produced five fragments of 161, 76, 43, 42, and 40 bp, indicating the presence of one additional *Tsp509I* site (data not shown).

The specific primer pairs CTB5-CTB6, CTC3-CTC4, and CTE4-CTE5, corresponding to the *gyrB*, *parC*, and *parE* QRDR of *C. trachomatis*, respectively, amplified 262-, 201-, and 443-bp fragments, respectively. No mutation was detected in the QRDRs of the resistant strains L2-OFXR and L2-SPXR.

Furthermore, similar results were obtained when purified DNA and lysates were used as DNA templates for PCR amplifications. Thus, lysates, the preparation of which is easier than that of extracted DNA, could be used as the DNA template for PCR amplification for the characterization of a large number of *C. trachomatis* strains.

To summarize, the Ser83→Ile substitution was found in the GyrA QRDRs of both mutants of *C. trachomatis* selected on ofloxacin and sparfloxacin, whereas no base change was found in the ParC, GyrB, and ParE QRDRs of these mutants compared to those of the L2 reference strain.

DISCUSSION

The sequences of the *gyrA* and *parC* QRDRs and of most of the *gyrB* and *parE* genes of *C. trachomatis* L2 are reported here. By using primers corresponding to highly conserved regions in each of the topoisomerase II subunits, these sequences were amplified and characterized. Sequence analysis of the CTA1-CTA2 PCR product amplified from *C. trachomatis* showed that it shares higher percentages of identity with the

GyrA QRDRs than with the ParC sequences of other bacteria, and it was assigned as a GyrA fragment of this organism.

The sequence of most of the GyrB protein of *C. trachomatis* was compared to those of other bacterial GyrB-like proteins, and it shared higher homology scores with the GyrB proteins than with the ParE proteins of other bacteria. Comparison of the protein sequence of the *C. trachomatis* GyrB with those of some gram-negative and gram-positive bacteria revealed that the chlamydial protein falls in the same class as the proteobacterial GyrB proteins, which are about 800 amino acids in length (19). Moreover, it is noteworthy that all known ParE proteins are about 650 amino acids in length; this seems to confirm that the sequence assigned as the GyrB of *C. trachomatis* belonged to DNA gyrase subunit B. Moreover, the *gyrB* and *gyrA* genes of *C. trachomatis* were found to be contiguous, as described recently (19). In *Chlamydia* (19), gram-positive bacteria such as *S. aureus* (27) and *B. subtilis* (29), mycoplasmas (except *Mycoplasma hominis*), and spirochetes (19), the *gyrB* and *gyrA* genes are contiguous, whereas in gram-negative proteobacteria such as *E. coli* or *Haemophilus influenzae*, the *gyrA* and *gyrB* genes have different chromosomal locations (19). Furthermore, since no terminator-like structure was found between the *gyrB* and *gyrA* genes of *C. trachomatis*, it is likely that the DNA gyrase genes are transcribed as a single message.

Partial sequences of the Topo IV genes of *C. trachomatis* were also determined. When compared to the ParC proteins of other bacterial genera, the ParC sequence of *C. trachomatis* was more related to that of *B. burgdorferi*, confirming the recent observations of Huang (19).

The sequence of the ParE protein of *C. trachomatis*, which shares a low degree of homology with those of the ParE proteins of gram-negative and gram-positive bacteria, is closely related to the *B. burgdorferi* ParE sequence and shares some common motifs with it; this is in agreement with a recent comparative study of the bacterial type II topoisomerase subunit genes (19). Moreover, as found for the other bacterial ParE sequences that are known, the C-terminal region of the chlamydial ParE protein is about 150 amino acids shorter than those of the proteobacterial and chlamydial GyrB proteins. Like the DNA gyrase genes, the *parC* and *parE* genes of *C. trachomatis* were found to be contiguous, as has been found in gram-positive bacteria such as *S. aureus* (6) and *B. subtilis* (43). Furthermore, as found for the gyrase genes, no terminator-like structure was detected between the *parE* and *parC* genes of *C. trachomatis*, suggesting that the Topo IV genes are also co-transcribed.

In summary, this study revealed that in *C. trachomatis*, the genes encoding the two subunits of the DNA gyrase, on the one hand, and Topo IV, on the other hand, are contiguous, as is found in gram-positive bacteria and spirochetes. The GyrB protein of *C. trachomatis* is similar in length to that of the gram-negative bacteria, and the chlamydial Topo IV proteins appeared to be more closely related to that of the spirochete *B. burgdorferi*. The sequences reported here for the L2 strain of

1 AACCGAATTGATGAGTTCGTCATGGGATATGGACATACCATCCACATAACAGGAGACGCA
 parE N A I D E F V M G Y G H T I H I T G D A
 61 CACGAACTGTCTATTTCGTGATGAAGGCCGCGCATTCCCTGGGGAAGGTGATTGATTGT
 H E L S I R D E G R G I P L G K V I D C
 121 GTTCTAAATCAACTGGCCGAAATACACTCAGGATGTTTCCATTTTCTGTGGGA
 V S K I N T G A A K Y T Q D V F H F S V G
 181 TTAATGGCTGGGACTGAAAGCCGTTAATGCCTATCGCAACATTTCTGTGACGTTCT
 L N G V G L K A V N A L S Q H F S V R S
 241 GTGCGGAACAAAATTCCTCAAAGCTTCTTCCAAAGGCATTCTTACACACAGAA
 V R N K K F L K A S F S K G I L L H T E
 301 CAGGGCGCTACCCAAGATCCCGAGGTCAGAAAGTCTTTCTCCTCCGATCATGAACATA
 Q G A T Q D P D D G T E V V F S P D H E L
 361 TTCGAGAATTTTCTTCCAAAGTGGAGTTTCTAAAAAGAAAATCCGCTCAATACACCTAT
 F E N F S F Q V E F L K K K I R Q Y T Y
 421 CTCCATCCCGACTGACAATTTATAACGGACAGCTATGTTTCCACCCGTGCTT
 L H F G L T I I Y N G E R I V S T R G L
 481 CTGTGATCTTTGAAGAAAGTGCACAAACCCCTCTCTGATTTCCCTTACGTTTCAA
 L D L F Q E E V Q T P L L Y S P I T F T F
 541 TACTCCGATCTCGCATTTCTTCTCCCATACAGAAACGCTTCCGAAACAATTTTTCC
 Y S D L A F L F S H T E T S S E Q Y F S
 601 TTTGTAATGGCAAGAAACAATGAGCGGAAACCCATCTCGTTCGCTTTAAAGAGGGT
 F V N G Q E T T D G T H L V A F K E G
 661 ATAGTCAAGGGCGTAAATGAGTTTTTGGAAAAAATTTTCTCTCAAGATATTCGTGAG
 I V K G V N E F F G K N F S S Q D I R E
 721 GGAATGCTGGCTGATTCGCCATTAATAATGCTTCTCCTATTTTGAATCCCAACCAAA
 G L A G C I A I K I A S P I F E S Q T K
 781 AATAAATAGGGAATACGAATATCCCGCGCAGAGTTAGCCAAACGCGTAAAGAAAGCTGTG
 N K L G N T N I R A E L A K R V K E A V
 841 CTCTCTCTTTGAAGAAGAATCCTTCCAGCGGGAACGGATCCAAGAGAAAATCAAATC
 L S S L K K N P S S A E R I Q E K I K L
 901 AACGAAAAATCCGGAAGAATGCGCAATTTCTCAAGCAAGAGCTCAAAGATAAACAAAA
 N E K T R K N A Q F L K Q E L K D K Q K
 961 AAATCCACTATAAAATCCCTAAACTTCGGGATGTAATTTTCACTCACAGCAATTTCT
 K L H Y K I P K L R D C K F H L T D N S
 1021 CTGTATGGTAAAAATCCTCTATTTTCTTACTGAGGAGAATCGGCTCCGCTCCGAT
 L Y G K N S S I F I F I E S A S A S I
 1081 CTAGTCTCGGGAATCCGCTCACCAAGCAGTGTCTCTTTCGAGAGGAAACCTATGAAC
 L A S R N P L T Q A V F S L R G K P M N
 1141 GTCTTTCCCAAGAAACCAATCTATAAAATGACGAACCTTTTACCTTGCATCA
 V F S S K E E T I Y K N D E L F Y L A T
 1201 GCCTCCGCTGCACAAAGACTCTCACAAATCTCGATACACCAGGTGATCTTAGCA
 A L G L H N K D S L Q N L R K V I A
 1261 ACAGATCGGGATGTTGATGATGATATTCGTAATCTTATGATTACTTTTCTCTAAAA
 T D A D V D G M H I R N L M I T F F L K
 1321 ACCTTCTTCTCTTGTGCAAGCAACCCATTTTCTAGAACCCCTCTTTTAA
 T F L P L V A S N H L F I L E T P L F K
 1381 GTATGCCAAGATGCGCACTTCTTATGCTACTCAGAAGAAGAAAATCTCCGACCAT
 V C H K D A T F Y C Y S E E E K L S T I
 1441 GAACACTCGGTAAAAAAGATCGTCTTAGAAATTAATCGTTTCAAGGCTTAGGAGAA
 E H I G K N S S I F I F I E I T R F K G L G E
 1501 ATTTCTCCTAAAGAATCAAATCTTTTTCGCGCGCAGACATCGCCTTAACCTCAGTTTCT
 I S P K E F K S F I G A D M R L T P V S
 1561 CTTCGTGATCAGAAACCCCTGGACACACTTTTACAATTTACATGGGGAAACACAAAA
 L R D T E T L D L L Q F Y M G K N T K
 1621 GAGAGAAGCATTTATTTATGAGAACTCTTGTACCTACCTTACCTATAACGAACITTA
 E R R L F I I E N L V T Y L * parC
 1681 TGAGCAGCTCTCGGACCTATTTAAACATTTTACACAGATGCGCTTACGTCATT
 M S D L S D L F K T H F T Q Y A S Y V I
 1741 TGGAACTGCAATCCCTCATGTTTATGATGGCCTCAAGCCTGTTTAAAGAGGCTTCTT
 L E R A I P H V L D G L K P V Q R R L L
 1801 GGACCTTATCCGATGATGGTAAATGCAATAAGTGCTAATATCGCAGGACGTA
 W T L F R M D D G K M H K V A N I A G R
 1861 CGATGGCGCTGCACCCGATGGTATCGGCTATCGTGAAGCTCTTGTGCTTTGGCAA
 T M A L H P H G D A P I V E A L V V L A
 1921 AATAAAGGTTCTGATGAGACACAAAGGAACTTTGGCAATCCACTGACT
 N K G F L I E T Q G N F G N P L T

FIG. 2. Nucleotide and amino acid sequences of a 1,970-bp fragment which contains most of the *parE* gene and the 5' end of the *parC* gene of the *C. trachomatis* L2 reference strain. Specific primers are indicated by arrows.

C. trachomatis are very similar to those for the serovar D strain, whose complete genome sequence is available at <http://chlamydia-www.berkeley.edu:4231/>. The sequences of both the *gyrA* and the *gyrB* genes identified in that sequence cor-

respond to the *gyrA*-like (*gyrA* and *parC*) and *gyrB*-like (*gyrB* and *parE*) sequences, respectively, described here.

Here the isolation and characterization of in vitro-selected strains of *C. trachomatis* highly resistant to fluoroquinolones are reported. These mutants were selected from reference strain *C. trachomatis* L2 by stepwise selection with ofloxacin and sparfloxacin. The resistant chlamydial strains L2-OFXR and L2-SPXR showed high levels of resistance to all the fluoroquinolones tested and were susceptible to unrelated antibiotics (i.e., doxycycline and erythromycin).

Sequencing of the *gyrA* QRDRs of both resistant strains of *C. trachomatis* revealed only one point mutation (G→T) leading to the Ser83→Ile substitution. This single mutation was still present in the L2-OFXR and L2-SPXR strains cultivated without antibiotic for four passages, suggesting that the mutation is stable (data not shown). The amino acid at position 83 in the *E. coli* GyrA protein is the one most commonly associated with quinolone resistance (54). The Ser→Ile change is not the most prevalent substitution, but it has been described in clinical isolates of *Enterococcus faecalis* (23, 48) and *Aeromonas salmonicida* (33). A Thr83→Ile substitution in GyrA has also been described in clinical isolates of *C. jejuni* and *Pseudomonas aeruginosa*, which had high-level resistance to nalidixic acid and ciprofloxacin, respectively (25, 53). The Ser83→Ile change found in *C. trachomatis* is similar to the Ser83→Leu or Trp mutations in *E. coli* GyrA, leading to the replacement of a small polar amino acid by a nonpolar residue (5, 34). Interestingly, in resistant *C. trachomatis* strains, the G→T mutation in the *gyrA* QRDR led to the creation of a restriction site for the *Tsp509I* endonuclease and can be detected by restriction fragment length polymorphism analysis of the amplified fragment. The presence of the additional restriction site for *Tsp509I* could be used to identify the *gyrA* mutation in clinical isolates of *C. trachomatis* whose antibiotic susceptibilities are not systematically studied. Commonly, *gyrA* mutations at Ser83 led to the loss of the *HinfI* restriction enzyme site in resistant strains (8).

Recently, the first isolation of fluoroquinolone-resistant mutant from an intracellular bacterium, *Coxiella burnetii*, has been described (31). Sequence analysis of the *gyrA* QRDR of this strain with high-level resistance revealed a point mutation at position 87 (*E. coli* numbering). The selective agent was ciprofloxacin, whereas in our study, mutants were selected with ofloxacin and sparfloxacin.

The high level of resistance of the *C. trachomatis* mutants might not be explained by only one point mutation in their *gyrA* genes. Mutations in the *gyrB* (20, 55), *parC* (24, 32), and *parE* (3, 9, 37) genes which confer fluoroquinolone resistance in bacteria have been described. However, sequencing of the *parC*, *gyrB*, and *parE* QRDRs of the resistant strains *C. trachomatis* L2-OFXR and L2-SPXR revealed no mutations in their sequences compared with the sequence of the susceptible reference strain. The present results suggest that gyrase may be the primary target of fluoroquinolones in *C. trachomatis*, a gram-negative bacterium, which is in agreement with previous studies. Indeed, *gyrA* mutations have been described to occur primarily in gram-negative bacteria after exposure to fluoroquinolones, with *parC*-mediated resistance being detectable only in *gyrA* mutants (2, 16). In contrast, in the gram-positive bacterium *S. aureus*, mutations in the *parC* QRDR have been detected in strains with low-level quinolone resistance and precede those in *gyrA* (6, 7, 32), suggesting that in this species Topo IV is the primary target of quinolones, with the gyrase being altered secondarily. In *Streptococcus pneumoniae*, another gram-positive bacterium, the primary targets of ciprofloxacin and sparfloxacin are Topo IV and gyrase, respectively (35). Thus, in *S. pneumoniae*, the nature of the primary target

of quinolones seems to be dependent on the drug structure. In *C. trachomatis*, ofloxacin and sparfloxacin primarily target the same enzyme, DNA gyrase.

As found in *C. psittaci* (19), the ParC protein of *C. trachomatis* harbors an alanine residue at position 80 (*E. coli* numbering), a nonpolar amino acid. A comparative study of the intrinsic susceptibility to ofloxacin of reference strains of nine *Mycobacterium* species revealed that those harboring an Ala-83 in GyrA were 4- to 64-fold less susceptible than species harboring a serine residue at the same position (13). Moreover, an Ser→Ala substitution at position 83 in *E. coli* and *S. aureus* GyrA has been shown to confer relatively low-level resistance to quinolones (12, 14). Thus, the presence of an alanine at position 80 in ParC of *Chlamydia* may be responsible for the lower affinity of the ParC than the GyrA subunit for fluoroquinolones in this genus.

The possibility that other mechanisms of resistance involving drug permeation and/or drug efflux modifications may contribute to the high-level resistance to fluoroquinolones in *C. trachomatis* cannot be excluded. However, alteration of permeability or efflux mechanisms are usually responsible for low-level resistance and are associated with cross-resistance with different families of antibiotics. In summary, the present results suggest that high-level resistance to fluoroquinolones in *C. trachomatis* is associated with a Ser83→Ile substitution in the GyrA QRDR, whereas the other topoisomerase QRDRs were not found to be altered at this step of selection with ofloxacin and sparfloxacin. It will be interesting to continue stepwise selection with the same antibiotics to investigate the occurrence of *parC* mutations and to use other fluoroquinolones as selective agents to determine whether GyrA is the primary target of all quinolones in *C. trachomatis*. Selection of quinolone-resistant strains of *C. trachomatis* by repeated exposures of a susceptible strain to ofloxacin and sparfloxacin suggests that acquired resistance may occur in vivo during quinolone therapy, and therefore, surveillance of this situation will be necessary.

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