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Characterization of *Mycoplasma hominis* Mutations Involved in Resistance to Fluoroquinolones

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Fluoroquinolone-resistant mutants of *Mycoplasma hominis* were selected in vitro from the PG21 susceptible reference strain either by multistep selection on increasing concentrations of various fluoroquinolones or by one-step selection on agar medium with ofloxacin. The quinolone resistance-determining regions (QRDR) of the structural genes encoding the A and B subunits of DNA gyrase were amplified by PCR, and the nucleotide sequences of eight multistep-selected resistant strains were compared to those of susceptible strain PG21. Four high-level resistant mutants that were selected on norfloxacin or ofloxacin contained a C-to-T transition in the *gyrA* QRDR, leading to substitution of Ser-83 by Leu in the GyrA protein. Analysis of the sequence of the *gyrB* QRDR of the eight multistep-selected mutants did not reveal any difference compared to that of the *gyrB* QRDR of the reference strain *M. hominis* PG21. Similar analyses of eight one-step-selected mutants did not reveal any base change in the *gyrA* and *gyrB* QRDRs. These results suggest that in *M. hominis*, like in other bacterial species, a *gyrA* mutation at Ser-83 is associated with fluoroquinolone resistance.

Human mycoplasmas are responsible for respiratory and genital infections. *Mycoplasma hominis*, a genital mycoplasma, is often present at high titers in the vagina during bacterial vaginosis and is involved in endometritis, salpingitis, and postpartum septicemia (41). Furthermore, it has been recognized as being responsible for extragenital infections such as pyelonephritis, postoperative wound infections, and infections in immunosuppressed patients (26, 38).

Fluoroquinolones are broad-spectrum antibiotics active against species which are naturally resistant to nalidixic acid such as gram-positive bacteria, chlamydia, and mycoplasmas. Fluoroquinolones block DNA replication by inhibiting DNA gyrase activity. DNA gyrase, a type II DNA topoisomerase, is a tetrameric enzyme made up of two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively (32). Recently, it has been shown that a second type II topoisomerase, topoisomerase IV, also is a target of quinolones (3, 5, 13, 22, 24, 31). Topoisomerase IV, like DNA gyrase, has a tetrameric structure of the type A2B2. The A and B subunits of topoisomerase IV are encoded by the *parC* and *parE* genes, respectively (*grlA* and *grlB*, respectively, in *Staphylococcus aureus*).

The quinolone resistance mechanisms that have been described fall into two classes: (i) mutations in the target enzyme genes *gyrA* (8, 18, 47), *gyrB* (45, 48), or *parC* (5, 14, 29) and (ii) reductions in the level of quinolone accumulation inside the cells (19). In many gram-positive and gram-negative bacteria, high-level resistance to fluoroquinolones has been related to mutations in the *gyrA* gene. These mutations cluster within a conserved region of *gyrA*, referred to as the quinolone resistance-determining region (QRDR) (6, 47). In *Escherichia coli*, this region is located between nucleotides 199 and 318 of the *E. coli gyrA* gene (corresponding to amino acids 67 to 106 of the GyrA protein). In the *gyrB* gene, only two quinolone resistance-determining sites (amino acids 426 and 447) have been described (48). They represent the QRDR of the *gyrB* gene.

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The antimicrobial agents used to infections caused by mycoplasmas include tetracyclines, macrolides, and fluoroquinolones. For treating genital mycoplasma infections, tetracyclines have been considered the drug of choice. However, tetracycline resistance mediated by the *tetM* determinant has been reported in *M. hominis* (9), as well as in *Ureaplasma urealyticum* (10). In addition, *M. hominis* is naturally resistant to erythromycin. In this respect, fluoroquinolones which are frequently used for the treatment of upper genital tract infections represent an effective alternative because they are the only drugs bactericidal against mycoplasmas. However, the use of fluoroquinolones in the treatment of infections in which mycoplasmas could be involved could lead to the appearance of quinolone resistance in these microorganisms. Recently, quinolone-resistant mycoplasmas have been identified in contaminated cell cultures treated with fluoroquinolones (1, 11). In mycoplasmas the mechanisms of resistance to fluoroquinolones have not yet been documented. In this study we have characterized the putative QRDRs of the *gyrA* and *gyrB* genes from strain PG21 of *M. hominis* and from several fluoroquinolone-resistant strains selected in vitro.

MATERIALS AND METHODS

Bacterial strains and vectors. *E. coli* TG1 was used to amplify M13mp18 recombinant bacteriophages. Reference strain *M. hominis* PG21 was used to select quinolone-resistant mutants. Mycoplasmas were grown at 37°C in Hayflick modified agar or broth medium supplemented with arginine (15).

Antibiotics. The following antibiotics were purchased from the indicated manufacturers: nalidixic acid, Sterling-Winthrop, Clichy, France; norfloxacin, Marion-Merrell-Dow, Levallois-Perret, France; pefloxacin and sparfloxacin, Rhône-Poulenc-Rorer, Vitry-sur-Seine, France; ofloxacin and chloramphenicol, Roussel Uclaf, Paris, France; ciprofloxacin, Bayer-Pharma, Puteaux, France; doxycycline, Pfizer, Orsay, France; and josamycin, Bellon, Neuilly-sur-Seine, France.

Determination of MICs. The MICs of various antibiotics were determined by the metabolic inhibition method performed in 96-well microtiter plates (4). Increasing (twofold) concentrations of each antibiotic ranging from 0.01 to 128 μg/ml were tested. Each well of the microtiter plate contained an initial inoculum of 10^6 color-changing units per ml in 200 μl of Hayflick modified medium supplemented with arginine. After incubation at 37°C, the MIC was defined as the lowest concentration of antibiotic that inhibited the color change of the medium at the time when a color change could be observed in the control without antibiotics. The method of Steers et al. (39) was also used to determine MICs by the agar dilution method on Hayflick modified medium.

TABLE 1. Nucleotide sequences of the primers used for PCR

Primer (size [bp])	Sequence
MH1 (21)	5′-GATGG(A,T)TT(A,G)AA(A,G)CC(A,T)GT(T,A)CA(T,C)-3′
MH2 (21)	5′-TTG(T,A)ATATT(A,T)GT(A,T)GCCAT(A,T)CC-3′
MH3 (20)	5′-TATGGTATGAGTGAACTTGG-3′
MH4 (20)	5′-AATTAGGGAAACGTGATGGC-3′
MH6 (21)	5′-CTTCCTGGAAAATTAGCAGAC-3′
MH7 (21)	5′-CTGTGCCTAAGGCGTGAATCA-3′

Selection of fluoroquinolone-resistant mutants of *M. hominis* PG21. Selection of quinolone-resistant mutants was performed by serial transfers of *M. hominis* PG21 in Hayflick modified broth medium containing subinhibitory concentrations of norfloxacin, pefloxacin, ofloxacin, and ciprofloxacin. In the first experiment, the MICs of the four drugs listed for *M. hominis* PG21 were determined in microtitration plates. After recording the MICs, a volume of 200 μl of the culture containing the highest antibiotic concentration with visible growth was transferred to 5 ml of Hayflick medium, and the culture was incubated at 37°C for 24 h. This suspension was then used to inoculate a new microdilution panel. The whole process was repeated 12 consecutive times.

One-step selection of ofloxacin-resistant mutants was performed by plating 200 μl of an *M. hominis* PG21 culture (MIC of ofloxacin, 0.2 μg/ml) onto Hayflick modified agar medium containing 10-fold the MIC of ofloxacin (2 μg/ml). The frequency of mutation was determined as the number of colonies appearing on the plate with antibiotic divided by the number of colonies obtained in the inoculum.

DNA isolation. Propagation of M13 recombinant phages in *E. coli* and isolation of phage DNA were done by standard procedures (36). Large- and small-scale preparations of mycoplasmal genomic DNAs were made as described previously (33, 43).

Amplification of the QRDR region of the *gyrA* and *gyrB* genes. Amplification of the QRDR of the *gyrA* gene was initially carried out with degenerated primers MH1 and MH2 (Table 1). The nucleotide sequences of these primers were deduced from the amino acid sequences of two highly conserved regions of bacterial GyrA proteins. After the nucleotide sequence around the *gyrA* QRDR of *M. hominis* was determined, amplification was performed with primers MH3 and MH4 (Table 1), located upstream (MH3) and downstream (MH4) of the *gyrA* QRDR. Amplification of the QRDR of the *gyrB* gene was performed with primers MH6 and MH7 (Table 1), designed from the nucleotide sequence of the *M. hominis gyrB* gene (23). PCR amplification was performed with a Perkin-Elmer Cetus thermal cycler in a final volume of 50 μl containing approximately 100 ng of template DNA, 0.2 μM (each) primer, 200 μM (each) deoxynucleoside triphosphate (Boehringer), 1 mM $MgCl_2$, 25 μl of *Taq* buffer, and 5 U of *Taq* polymerase (Gibco-BRL). After a denaturation step of 10 min at 92°C, amplification was performed over 40 cycles, each one of which consisted of 1 min at 92°C, 1 min at 57°C, and 2 min at 72°C, with a final extension step of 10 min at 72°C. A negative control in which DNA was replaced by distilled water was introduced in each run of the experiments. PCR products were analyzed by electrophoresis in 2% agarose gels as described previously (36).

DNA sequencing and sequence analysis. The nucleotide sequences of PCR-amplified DNA fragments were determined by the dideoxy-chain termination technique (37). Prior to sequencing, the amplification products of *M. hominis* PG21 were first subcloned in *E. coli* by using the M13mp18 vector, and the nucleotide sequences of the individual clones were determined. PCR products of quinolone-resistant strains were purified by using the Wizard PCR Preps DNA Purification System (Promega). Some of them were directly sequenced with the *fmol* DNA Sequencing System kit (Promega) by using MH5 (5′-CTGTATAAC GCATTGCAGC-3′) as the radiolabeled primer and were sequenced according to the manufacturer's recommendations. Sequencing of the other PCR products 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 number. The nucleotide sequence data reported in this paper will appear in the GenBank nucleotide sequence databases with the following accession number: U59880.

RESULTS

Selection of fluoroquinolone-resistant *M. hominis* PG21 mutants. Spontaneous norfloxacin-, pefloxacin-, ofloxacin-, and ciprofloxacin-resistant mutants were isolated by serial passaging of *M. hominis* PG21 in broth culture. After 12 successive passages, *M. hominis* PG21 cultures were plated onto agar plates. Eight multistep resistant mutants were isolated and triply cloned. Mutants C1 and C6 were selected on ciprofloxacin, mutants N3 and N7 were selected on norfloxacin, mutants O7 and O9 were selected on ofloxacin, and mutants P1 and P4 were selected on pefloxacin. The two mutants of each pair arose from the same plating.

The susceptibilities of the eight mutants to these antibiotics as well as sparfloxacin are summarized in Table 2. For each mutant, the MICs of all five quinolones were significantly higher than those for reference strain PG21. For example, the increase in the MIC of sparfloxacin ranged from a factor of 5 for the P1 and P4 mutants to a factor of 400 for mutants O7 and O9. The highest level of resistance was obtained with strains N3 and N7, selected in the presence of norfloxacin, and O7 and O9, selected in the presence of ofloxacin. For these mutants, it is noteworthy that the MIC of sparfloxacin, the most active fluoroquinolone against mycoplasmas, increased 100- to 400-fold compared to that for the PG21 wild-type strain (MICs, 1 to 4 μg/ml versus ≤0.01 μg/ml). The resistant strains selected with pefloxacin (P1 and P4) and ciprofloxacin (C1 and C6) showed a lower level of resistance to the quinolones. MICs were determined in broth and on agar medium. However, no significant difference (no more than 1 dilution) was observed between the results obtained by either one of the two methods.

Interestingly, no differences in MICs were observed between PG21 and the mutant strains when nonrelated antibiotics such as doxycycline, josamycin, and chloramphenicol were tested.

One-step selection on solid medium with 2 μg of ofloxacin per ml yielded spontaneous resistant mutants at a frequency of 3×10^{-6}. The eight selected mutants (mutants IO1 to IO8) all shared the same fluoroquinolone susceptibilities. When compared to the multistep mutants (mutants O7 and O9) obtained with ofloxacin, the one-step-selected mutants were characterized by a lower level of resistance to ofloxacin (MIC, 8 μg/ml), pefloxacin (MIC, 16 μg/ml), norfloxacin (MIC, 16 μg/ml), ciprofloxacin (MIC, 1 μg/ml), and, particularly, sparfloxacin (MICs, 0.02 to 0.05 μg/ml).

PCR amplification and sequence analysis of the QRDR of the *gyrA* gene of *M. hominis* PG21. Two consensus degenerated primers, MH1 and MH2, were chosen from the alignment of known amino acid sequences of the GyrA proteins of *E. coli* (40), *Bacillus subtilis* (27), *S. aureus* (25), *Spiroplasma citri* (46), and *Mycoplasma pneumoniae* (7). The nucleotide sequences of

TABLE 2. Susceptibility to fluoroquinolones of *M. hominis* PG21 and the mutants selected with various fluoroquinolones

M. hominis PG21 passage	Mutant	MIC (μg/ml)[a]					
		NAL	NFX	PFX	OFX	CFX	SPX
Before passage		≥256	8	2	0.2	0.2	≤0.01
After passage in:							
Ciprofloxacin	C1	≥256	64	32	16	16	0.2
	C6	≥256	64	32	16	16	0.5
Norfloxacin	N3	≥256	128	32	32	32	1
	N7	≥256	128	32	32	32	1
Ofloxacin	O7	≥256	128	64	64	64	4
	O9	≥256	128	64	64	64	4
Pefloxacin	P1	≥256	128	32	16	4	0.05
	P4	≥256	128	32	16	4	0.05

[a] NAL, nalidixic acid; NFX, norfloxacin; PFX, pefloxacin; OFX, ofloxacin; CFX, ciprofloxacin; SPX, sparfloxacin.

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1   CGTAGAATTTTATATGGTATGAGTGAACTTGGAATGTTTTATACAGCGCC
    R  R  I  L  Y  G  M  S  E  L  G  M  F  Y  T  A  P

51  ACATAAAAAATCGGCAAGAATCGTCGGAGATGTTTTAGGTAAATATCACC
     H  K  K  S  A  R  I  V  G  D  V  L  G  K  Y  H  P

                T*
101 CACATGGAGATTCATCAGTATATGAAGCTATGGTAAGAATGGCTCAAGAT
      H  G  D  S  S  V  Y  E  A  M  V  R  M  A  Q  D

151 TTTTCACTTCGCTATCCTTTAATTGATGGACACGGTAACTTTGGATCTGT
    F  S  L  A  Y  P  L  I  D  G  H  G  N  F  G  S  V

201 TGACGGAGACGAAGCTGCTGCAATGCGTTATACAGAAGCTAGAATGAGCA
     D  G  D  E  A  A  A  M  R  Y  T  E  A  R  M  S  K

251 AGATTGCTGGTGCAATGGTTGATGGTATTAAGAAAAATACTGTTGACTTT
       I  A  G  A  M  V  D  G  I  K  K  N  T  V  D  F

300 ATGGACAACTATGACGCAACTGAAAAAGAACCAGTTGTCTTGCCATCACG
    M  D  N  Y  D  A  T  E  K  E  P  V  V  L  P  S  R

351 TTTCCCTAATTTATTAGTTTCTGGTTCAAGTGGTATTGCTGTT
     F  P  N  L  L  V  S  G  S  S  G  I  A  V
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FIG. 1. Nucleotide and amino acid sequences of the MH1-MH2-amplified fragment of the *gyrA* gene of *M. hominis* PG21. The QRDR is underlined. Italic type indicates the *Hin*fI restriction site. T*, nucleotide mutation (C→T) leading to the Ser→Leu substitution.

these primers were optimized at the level of the third base of each codon according to the biased codon usage in the *M. hominis* genome. Primers MH1 and MH2 were used to amplify *M. hominis* PG21 genomic DNA sequences containing the putative QRDR of the *gyrA* gene. As expected, an amplification product of 435 bp was obtained. The DNA fragment was cloned into *E. coli* and sequenced. The nucleotide and the deduced amino acid sequences are presented in Fig. 1, in which the 40 amino acids of the GyrA QRDR are underlined. In Fig. 2, the GyrA QRDR of *M. hominis* was compared to the GyrA QRDR sequences of *E. coli* (40), *B. subtilis* (27), *S. aureus* (25), *S. citri* (46), *M. pneumoniae* (7), and *Mycoplasma genitalium* (2) and the corresponding sequences of ParC of *E. coli* (22), ParC of *M. genitalium* (3), and GrlA of *S. aureus* (13). The amino acid sequence of the *M. hominis* GyrA QRDR shared 72.5, 77.5, and 85% identity with those of the GyrA proteins of *E. coli*, *B. subtilis*, and *S. aureus*, respectively. When compared to other mollicutes, the GyrA QRDR of *M. hominis* was found to share 85% identical amino acids with those of *S. citri*, a mollicute pathogenic for plants, and 72.5% with those of the mycoplasmas pathogenic for humans, *M. pneumoniae* and *M. genitalium*. The GyrA QRDR of *M. hominis* also showed significant amino acid sequence homology with the ParC proteins of

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          67              83                106
Mh GyrA   ARIVGDVLGKYHPHGDSSVYEAMVRMAQDFSLAYPLIDGH
Ec GyrA   --V----I---------A--DTI-----P---R-M-V--Q  (72.5%)*
Bs GyrA   -----E-I---------A---S--------NYR-M-V---  (77.5%)
Sa GyrA   -------M----------I------------YR---V--Q  (85%)
Sc GyrA   -----E-I---------TA-------------YR-------  (85%)
Mp GyrA   -------MS-F-----MAI-DT-S--------R-L-----  (72.5%)
Mg GyrA   -------MS-F-----MAI-DT-S--------R-L-----  (72.5%)
Ec ParC   --T--------------AC-----L---P--YR---V--Q  (77.5%)
Sa GrlA   -KT----I-Q---------------LS--WK-RHV--EM-  (67.5%)
Mg ParC   --A--EIM----------I-D-II--S-SWKNNWTTVSI-  (50%)
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FIG. 2. Alignment of the amino acid sequence of the GyrA QRDR of *M. hominis* PG21 (Mh GyrA) with those of *E. coli* (Ec GyrA), *B. subtilis* (Bs GyrA), *S. aureus* (Sa GyrA), *S. citri* (Sc GyrA), *M. pneumoniae* (Mp GyrA), and *M. genitalium* (Mg GyrA) and with those of *E. coli* ParC (Ec ParC), *S. aureus* GrlA (Sa GrlA), and *M. genitalium* ParC (Mg ParC). –, identical amino acids; *, percent identical amino acids.

E. coli (77.5% identity) and *M. genitalium* (50% identity) and with the GrlA protein of *S. aureus* (67.5% identity). These sequence comparisons showed that the fragment amplified from *M. hominis* was more similar to the GyrA sequences than to the GrlA or ParC sequences of *S. aureus* or *M. genitalium*. The *M. hominis* sequence, however, was equally similar to the GyrA and ParC peptidic sequences of *E. coli*. Taken together, these results indicate that although the reported amino acid sequence of *M. hominis* PG21 has some similarity to the amino acid sequences of ParC proteins, it has significantly higher homology with the amino acid sequences of GyrA proteins.

Characterization of the *gyrA* and the *gyrB* QRDRs of fluoroquinolone-resistant strains of *M. hominis*. Nondegenerated primers MH3 and MH4 were chosen from the nucleotide sequence of the 435-bp DNA fragment of *M. hominis* containing the *gyrA* gene QRDR. These primers allowed for the amplification of a 350-bp DNA fragment from the *gyrA* genes of the eight fluoroquinolone-resistant strains selected in vitro (strains C1, C6, N3, N7, O7, O9, P1, and P4). The purified amplification products were sequenced directly, and their nucleotide sequences were compared to that of the *gyrA* QRDR from the wild-type strain PG21. For the four most resistant strains, strains N3, N7, O7, and O9, a C→T base change was found at nucleotide 113 (Fig. 1). This mutation led to a Ser→Leu substitution at the position corresponding to amino acid 83 in the *E. coli* GyrA QRDR (Fig. 1). For the four other resistant strains (strains C1, C6, P1, and P4), sequence comparison did not reveal any base changes.

Interestingly, in *M. hominis*, as in *E. coli*, the C→T mutation led to the loss of the *Hin*fI restriction site and could be detected as a restriction fragment length polymorphism. Upon digestion with *Hin*fI, the 350-bp amplification products of *M. hominis* PG21 and resistant strains C1, C6, P1, and P4 yielded three fragments of 252, 57, and 41 bp. In the absence of the *Hin*fI restriction site described above, the amplification products of *gyrA* mutants N3, N7, O7, and O9 yielded only two fragments of 293 and 57 bp (data not shown).

To determine whether base mutations in the *gyrB* gene could be involved in their resistance to fluoroquinolones, the *gyrB* QRDRs of the eight multistep-selected quinolone-resistant strains were amplified with primers MH6 and MH7 and were sequenced directly. For all eight mutants, the nucleotide sequence of the 223-bp-amplified fragment was found to be identical to that of the wild-type strain PG21 (23).

To summarize, the Ser-83→Leu substitution was found in the GyrA QRDRs of the mutants selected on norfloxacin and ofloxacin, whereas no mutations could be detected in the *gyrA* and *gyrB* QRDRs of those selected on pefloxacin or ciprofloxacin. Similar analyses of the eight one-step-selected mutants (mutants IO1 to IO8) did not reveal any base change in the *gyrA* and *gyrB* QRDRs compared to the QRDRs of the PG21 wild-type strain of *M. hominis.*

DISCUSSION

By using primers corresponding to highly conserved regions of GyrA proteins, the *gyrA* QRDR of *M. hominis* was amplified and characterized. The deduced amino acid sequence showed high homology with other GyrA-like proteins, ranging from 50% identity with that of the *M. genitalium* ParC protein to 85% identity with those of the *S. aureus* and *S. citri* GyrA proteins. From the higher percentage of similarity with the GyrA proteins than with the ParC proteins, it seems highly probable that the amplified DNA fragment of *M. hominis* represents the GyrA QRDR of this organism. In agreement with the phylogenetic origin of mollicutes, seen as having arisen

from ancestors that were gram-positive bacteria with low G+C contents (44), the *M. hominis* GyrA QRDR showed a higher percentage of identity with the GyrA proteins of gram-positive bacteria (85% identity with *S. aureus* and 77.5% identity with *B. subtilis*) than with the GyrA QRDR of gram-negative bacteria (72.5% identity with *E. coli*). Surprisingly, within the class *Mollicutes*, the sequence of the GyrA QRDR of *M. hominis* was closest to that of the GyrA QRDR of *S. citri*, a plant mollicute, than to those of the GyrA QRDRs of the human mycoplasmas *M. pneumoniae* and *M. genitalium*. However, this is in agreement with phylogenetic studies showing that the last two mycoplasmas belong to a phylogenetic group quite distant from that to which *M. hominis* belongs (42).

Among amino acid residues 67 to 106 referred as the GyrA QRDR of *E. coli*, Ser-83 seems to play a key role in resistance to fluoroquinolones (6). *M. hominis*, like *E. coli* and *S. aureus*, has the polar amino acid serine at position 83 in the GyrA QRDR, whereas *M. pneumoniae*, which is naturally less susceptible to fluoroquinolones than *M. hominis* (2- to 10-fold increased MICs), has a methionine, a nonpolar amino acid, at this same position. Thus, the intrinsic lower susceptibility of *M. pneumoniae* to quinolones seems to be due in part to the lack at this position of the hydroxyl residue carried by the side chain of Ser-83 in *M. hominis*. Such structural differences have been described in various bacteria (20), namely, in mycobacterial species (34).

After having characterized the *gyrA* QRDR of the wild-type strain *M. hominis* PG21, we looked for the presence of mutations in the *gyrA* QRDR of fluoroquinolone-resistant strains selected in vitro. These strains were selected from *M. hominis* PG21, either by serial-step selection with different fluoroquinolones or by one-step selection on agar medium containing ofloxacin.

The mutants obtained on ofloxacin in the one-step selection procedure appeared at a frequency close to 10^{-7}, similar to those reported for aminoglycoside-resistant mutants of *M. hominis* (35). This frequency is high enough for ofloxacin-resistant mutants to arise in and to be picked up from the lower genital tract or cell cultures, where the *M. hominis* titer can reach 10^7 to 10^8. It should be noted that the frequency of mutation of mycoplasmas obtained in this study is higher than that of many other bacteria. This could be related to the fact that *Mycoplasma* species are expected to have high mutation rates because of their small genome size and because their DNA polymerase reportedly lacks a 3′-to-5′ exonuclease activity (12). These one-step mutants displayed a low level of resistance to fluoroquinolones. Since we did not detect any base change in the *gyrA* and *gyrB* QRDRs of these mutants, fluoroquinolone resistance might be due to mutations in topoisomerase IV, the other target of quinolones. Also, the possibility of a decrease in drug permeation or an increase in drug efflux within mutants cannot be excluded.

In contrast, the mutants obtained by the multistep selection procedure (mutants C1, C6, N3, N7, O7, O9, P1, and P4) showed a high level of resistance to all fluoroquinolones tested. The two members of the four pairs, having been obtained from the same plating, can be considered siblings. Four of them (N3 and N7, selected on norfloxacin, and O7 and O9, selected on ofloxacin) were found to carry a nucleotide mutation (C→T) leading to a Ser→Leu substitution at position 83 in the GyrA QRDR. In the *E. coli* GyrA subunit, Ser-83 is the position most commonly associated with quinolone resistance (6). Mutations affecting the amino acid equivalent to Ser-83 in *E. coli* have been found in many other bacteria. In *E. coli*, substitution of Ser-83→Leu or Trp results in an important increase (40-fold) in the ciprofloxacin MIC (47). These four mutants showed the highest level of resistance to all fluoroquinolones tested, including the very effective fluoroquinolone sparfloxacin. However, it is only to sparfloxacin, ciprofloxacin, and ofloxacin that the four mutants with Ser-83 substitutions were substantially more resistant than the four others without *gyrA* mutations. These differences might be related to the intrinsic susceptibility of *M. hominis* to the five fluoroquinolones tested. Indeed, norfloxacin and pefloxacin are clearly less active against mycoplasmas than the three other quinolones. For *S. aureus*, it has been suggested that sparfloxacin resistance was not largely affected by mutations other than Ser-84 to Leu (corresponding to Ser-83 in the *E. coli* nomenclature) (21). A similar pattern was found with our multistep mutants. For the four mutants in which Ser-83 was replaced by Leu, 100- to 400-fold increases in the sparfloxacin MIC were found, whereas for the four other resistant strains, which did not present the mutation at Ser-83, the MICs of this drug were lower (only a 5- to 20-fold increase). As described previously for *E. coli* (34), the resistance associated with the mutation found in *M. hominis* is probably related to the loss of the hydroxyl residue and the bulkiness of the side chains associated with the replacement of Ser-83 by Leu. Recently, a new mutation was associated with quinolone resistance at codon 119 (*E. coli* nomenclature) of *Salmonella typhimurium* GyrA (17) and of *S. aureus* GrlA (28), near the residue tyrosine, known to be the active site of the A subunit. In our multistep and one-step mutants, no change was found at position 119, even in the mutants selected in multiple steps on pefloxacin and norfloxacin, which did not present any mutation in the *gyrA* QRDR.

However, according to various studies on resistance to quinolones described in the literature (6, 19), the very high level of resistance of the multistep mutants might not be explained only on the basis of these *gyrA* mutations. One possibility was the presence of additional mutations in the *gyrB* gene. Therefore, a region of the *gyrB* gene including the QRDR was amplified and directly sequenced for each one of the eight multistep mutants. No change was found either in amino acids 426 and 447 of the GyrB protein or in amino acid 463, which was recently described as a novel point mutation in the *gyrB* gene of a quinolone-resistant clinical isolate of *S. typhimurium* (16). These results strongly suggest the predominance of *gyrA* mutants over *gyrB* mutants.

Finally, the high level of fluoroquinolone resistance displayed not only by the four mutants with the GyrA Ser-83→Leu substitution but also by the four mutants without this substitution might also involve drug permeation and/or drug efflux modifications or mutations in topoisomerase IV, known to be a primary target of fluoroquinolones in the gram-positive species *S. aureus* (13, 14) and *Streptococcus pneumoniae* (29, 30). The homologs of topoisomerase IV genes *parC* and *parE* have recently been found in *M. genitalium* (3). A search for these genes in *M. hominis* is being conducted.

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