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Cloning and Nucleotide Sequence of the DNA Gyrase (*gyrA*) Gene from *Mycoplasma hominis* and Characterization of Quinolone-Resistant Mutants Selected In Vitro with Trovafloxacin

C. M. BÉBÉAR,* O. GRAU, A. CHARRON, H. RENAUDIN, D. GRUSON, AND C. BÉBÉAR
Laboratoire de Bactériologie, Université Victor Segalen Bordeaux 2, 33076 Bordeaux, France

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We report the cloning and characterization of the *gyrA* gene of the *Mycoplasma hominis* DNA gyrase, which was previously shown to be associated with quinolone resistance in this organism. The 2,733-bp *gyrA* gene encodes a protein of 911 amino acids with a calculated molecular mass of 102.5 kDa. As expected, *M. hominis* GyrA exhibits higher homology with the GyrA subunits of the gram-positive bacteria *Clostridium acetobutylicum*, *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* than with its *Escherichia coli* counterpart. Knowing the entire sequence of the *gyrA* gene of *M. hominis* could be very useful for confirming the role of the GyrA subunit in fluoroquinolone resistance. Twenty-nine mutants of *M. hominis* were selected stepwise for resistance to trovafloxacin, a new potent fluoroquinolone, and their *gyrA*, *gyrB*, *parC*, and *parE* quinolone resistance-determining regions were characterized. Three rounds of selection yielded 3 first-step, 12 second-step, and 14 third-step mutants. The first-step mutants harbored a single substitution, Glu460→Lys (*E. coli* coordinates), in ParE. GyrA changes, Ser83→Leu, Glu87→Lys, and Ala119→Glu or Val, were found only in the second round of selection. At the third step, additional substitutions, at ParC Ser80, Ser81, and Glu84 and ParE Leu440, associated with high-level resistance to fluoroquinolones, appeared. Thus, high-level resistance to trovafloxacin required three steps and was associated with alterations in both fluoroquinolone targets. According to these genetic data, in *M. hominis*, as in *Staphylococcus aureus* and *Streptococcus pneumoniae*, topoisomerase IV seems to be the primary target of trovafloxacin.

The intracellular targets of fluoroquinolones in bacteria are considered to be the type II topoisomerases, DNA gyrase and topoisomerase IV (23). DNA gyrase is composed of two A and two B subunits, encoded by the *gyrA* and the *gyrB* genes, respectively. This tetrameric enzyme catalyzes ATP-dependent negative supercoiling of DNA. Topoisomerase IV, a C_2E_2 tetramer encoded by the *parC* and *parE* genes, is essential for chromosome partitioning. Mutations in the quinolone resistance-determining regions (QRDRs) of GyrA and ParC mainly and GyrB and ParE less frequently have been described as the major mechanism for quinolone resistance (10, 23).

Mycoplasma hominis is a cause of urogenital tract infections and has been implicated in extragenital infections as well, especially in immunocompromised patients (46). We recently reported in vitro and in vivo fluoroquinolone-resistant mutants of *M. hominis* associated with alterations in GyrA, ParC, and ParE QRDRs (3, 5, 7). Furthermore, previous genetic studies showed that topoisomerase IV was the primary target of pefloxacin, ofloxacin, and ciprofloxacin, whereas DNA gyrase was the primary target of sparfloxacin (5, 25).

Concerning the target genes of fluoroquinolones in *M. hominis*, the *gyrB*, *parC*, and *parE* genes and only the QRDR sequence of *gyrA* have been cloned and sequenced (3, 4, 28). Here we report the cloning, sequencing, and organization of the complete *M. hominis gyrA* gene, as well as a detailed analysis of the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs from *M. hominis* mutants selected in a stepwise-manner for resistance to trovafloxacin.

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Université Victor Segalen Bordeaux 2, 146 Rue Léo Saignat, 33076 Bordeaux Cedex, France. Phone: (33) 5.57.57.16.25. Fax: (33) 5.56.93.29.40. E-mail: cecile.bebear@u-bordeaux2.fr.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA manipulations. The *M. hominis* reference strain PG21 (ATCC 23114) was grown in Hayflick modified broth medium supplemented with arginine (17). The *Escherichia coli* strain JM109 and the vector pGEM3zf(+) (Promega) were used to construct libraries and to subclone DNA inserts. Chromosomal DNA from *M. hominis* PG21 was obtained as previously described (47). Manipulations of DNA, including electrophoresis, Southern blotting, and in situ colony hybridization, were carried out by standard procedures (40). For Southern and colony hybridization, DNA was radiolabeled with 50 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol) using a NonaPrimer kit from Appligene. Plasmid DNA was amplified in *E. coli* by using a QIAprep spin miniprep kit (Qiagen).

Restriction mapping and cloning procedures for the *gyrA* locus. Genomic DNA was singly or doubly digested with various restriction enzymes. Restriction fragments were separated by electrophoresis, blotted to nylon membranes, and hybridized to α-^{32}P-labeled probes under standard stringent conditions. Two DNA probes, MH3-MH4 and 321-322, corresponding to the 5′ and 3′ regions of *gyrA*, respectively, were generated by PCR amplification of the *M. hominis* genomic DNA with primers MH3 and MH4 (3) and with primers 321 and 322 (this study; see below), respectively. A restriction map was constructed from the hybridization patterns of genomic DNA obtained with each of the two probes.

M. hominis PG21 genomic DNA was then digested with *Eco*RI, *Bgl*II, or *Bgl*II-*Hin*dIII. The fragments were ligated to the linearized vector dephosphorylated and digested with *Eco*RI, *Bam*HI, or *Bam*HI-*Hin*dIII, respectively. After transformation of *E. coli* by ligation mixtures, recombinant clones containing the *gyrA* sequences were selected by colony hybridization with the [α-^{32}P]dCTP-labeled MH3-MH4 or 321-322 DNA fragments. Hybridization-positive clones were selected, and their plasmid content was determined. Three recombinant plasmids, pMH3.1, pMH14.1, and pMH2.20, containing *M. hominis* DNA inserts of 4.2, 3.7, and 0.95 kbp, respectively, were selected for sequencing studies.

PCR amplification. PCRs were carried out with a Perkin-Elmer Cetus thermal cycler with 100 ng of template DNA for *M. hominis* PG21 or with 2 μl of a broth culture for trovafloxacin-resistant mutants and 1 μM each primer as described elsewhere (5). Primer sets MH3 and MH4, MH6 and MH7, MH11 and MH13, and MH27 and MH28, previously described (5), were used to amplify the *gyrA*, *gyrB*, *parC*, and *parE* QRDRs, respectively. The *gyrA* 3′-end probe from *M.*

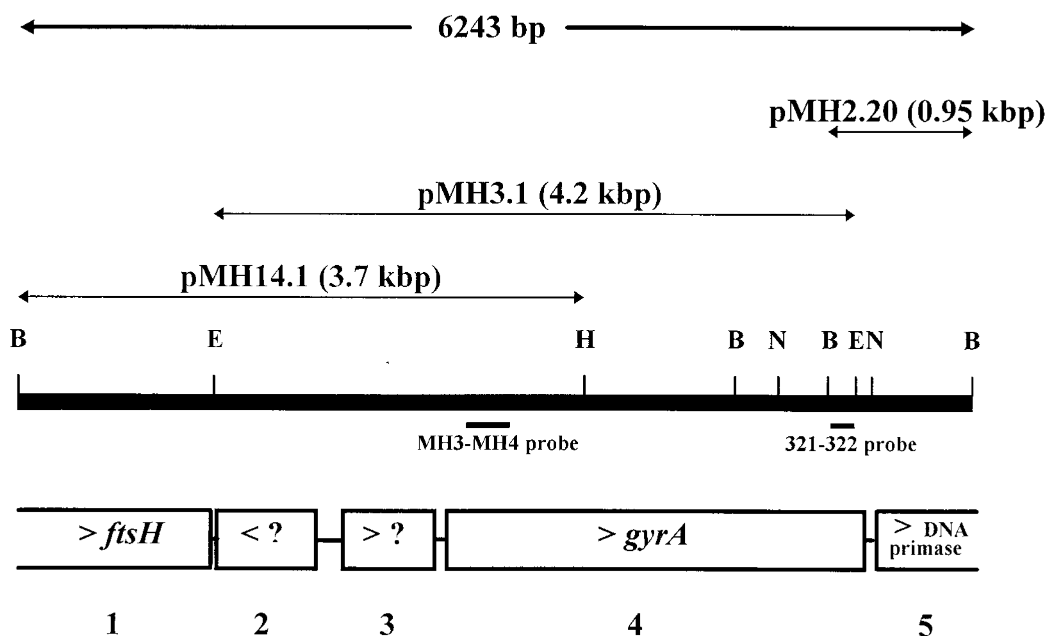

FIG. 1. Restriction map and organization of the *M. hominis* PG21 *gyrA* locus. E, *Eco*RI; H, *Hin*dIII; B, *Bgl*II; N, *Nsi*I. ?, hypothetical gene. ORFs are numbered 1 to 5. Arrowheads indicate the transcription sense of the ORFs. Sizes of DNA inserts are indicated in parentheses.

hominis PG21 was generated with primers 321 (5′-TTAACAAGCGATGGTG TTGC-3′) and 322 (5′-GATAATTTTCTGTCATTGTCTTC-3′). Amplification was achieved with an initial denaturation step of 10 min at 94°C; 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final 10-min extension step at 72°C.

DNA sequence analysis. Double-stranded DNA was sequenced on both strands by using an ABI PRISM dRhodamine terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS and an ABI PRISM 377 sequencer (Perkin-Elmer Applied Biosystems) according to the manufacturer's instructions. Forward and reverse primers, flanking the multiple-cloning-site polylinker of the pGEM3zf(+) vector, as well as internal primers were used to obtain the complete sequences of the DNA inserts of the three recombinant plasmids. PCR products of the quinolone-resistant strains were directly sequenced after purification with a Wizard PCR Preps DNA purification system (Promega). Pairwise and multiple sequence alignments were done with ALIGNp, CLUSTAL W (Infobiogen), and BLAST (National Center for Biotechnology Information) software.

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed as previously described by using restriction endonucleases *Bam*HI, *Sal*I, *Sma*I, and *Xho*I (4, 28). Fragments containing the *gyrA* gene were identified by using the [α-^{32}P]dCTP-labeled MH3-MH4 DNA fragment as a probe.

Antimicrobial agents and determination of MICs. Antibiotics were purchased from the following manufacturers: norfloxacin, Merck Sharp & Dohme, Roma, Italy; pefloxacin and sparfloxacin, Rhône-Poulenc-Rorer, Vitry-sur-Seine, France; ofloxacin, Hoechst Marion Roussel, Romainville, France; ciprofloxacin, Bayer-Pharma, Puteaux, France; and trovafloxacin, Pfizer, Orsay, France. The MICs of the fluoroquinolones were determined by the agar dilution method as previously described (2).

In vitro selection of trovafloxacin-resistant mutants. Stepwise selection of trovafloxacin-resistant mutants was performed by plating approximately 2×10^7 color-changing units of strain PG21 onto Hayflick modified agar medium containing increasing inhibitory concentrations of trovafloxacin. After 48 h of incubation at 37°C, resistant colonies were grown in broth medium without antibiotic and used for the next round of selection. The frequency of mutation was determined as the number of colonies appearing on the plate with antibiotic divided by the number of colonies in the inoculum.

Nucleotide sequence accession number. The DNA sequence corresponding to the *gyrA*-encompassing fragment has been assigned GenBank accession no. AF242654.

RESULTS AND DISCUSSION

Cloning and organization of the *gyrA* locus. Southern blot hybridization of *M. hominis* genomic DNA with probes MH3-MH4 and 321-322 revealed that most of the *gyrA* gene was contained in a 4.2-kbp *Eco*RI fragment. By using combinations of single and double digests of the DNA with the enzymes *Bgl*II, *Eco*RI, *Hin*dIII, and *Nsi*I, we established the restriction map of the *gyrA* gene region. As indicated in Fig. 1, the 3.7-kbp *Bgl*II-*Hin*dIII fragment overlapping the 4.2-kbp *Eco*RI fragment was found to contain the 5′ end of *gyrA*. These two fragments were recovered from genomic libraries of *M. hominis* by in situ colony hybridization, and the respective recombinant plasmids, pMH3.1 and pMH14.1, obtained were sequenced (Fig. 1). DNA probe 321-322, corresponding to the 3′ end of the *M. hominis* sequenced fragment, was chosen to clone the 3′ end of the *gyrA* gene and its flanking regions. The 0.95-kbp *Bgl*II fragment containing the *gyrA* 3′ end was cloned in *E. coli*, and recombinant plasmid pMH2.20 (Fig. 1) was sequenced.

Sequencing of the inserts of the three recombinant plasmids allowed the characterization of a 6,243-bp genomic DNA fragment of *M. hominis* (Fig. 1). This sequence was found to contain five putative open reading frames (ORFs) (ORF1 to ORF5). Three ORFs (ORF1, ORF4, and ORF5) were functionally assigned, based on significant sequence similarities to genes encoding proteins with known functions from other organisms (Fig. 1). ORF4, nucleotides 2798 to 5530, was assigned as the *gyrA* gene of *M. hominis* (see below).

Sequence analysis of the *M. hominis* GyrA subunit. The predicted GyrA polypeptide contains 911 amino acids (aa) and has a calculated molecular mass of 102.5 kDa. It contains only one UGA-encoded tryptophan residue and has a G+C content of 31%. AT-rich sequences characteristic of putative −10 and −35 promoter sequences and a putative ribosome binding site were found upstream of the ATG initiation codon. The *M. hominis* GyrA subunit, with 911 residues, seems to be the largest GyrA subunit sequenced so far among organisms related to gram-positive bacteria. Only three gram-negative bacteria have a larger GyrA protein, *Neisseria gonorrhoeae* (916 aa) (8), *Aeromonas salmonicida* (922 aa) (33), and *Pseudomonas aeruginosa* (923 aa) (27). It is noteworthy that *M. hominis*

ParC, 866 aa long, is also the largest topoisomerase IV ParC subunit known (4). Compared to other GyrA subunits, the *M. hominis* protein contains an additional stretch of 58 aa at the N-terminal end (see Fig. 3).

Compared to the GyrA and ParC proteins of *M. genitalium* (16), *M. pneumoniae* (22), *Ureaplasma urealyticum* (accession no. AF222894), *Bacillus subtilis* (26), *Staphylococcus aureus* (13, 30), *Streptococcus pneumoniae* (1, 34), *Clostridium acetobutylicum* (44), and *E. coli* (38, 42), the *M. hominis* GyrA polypeptide exhibits a higher percentage of identity with the GyrA subunits than with the ParC subunits. The identity of *M. hominis* GyrA with the other GyrA proteins varies between 39.2% (*E. coli*) and 47.8% (*U. urealyticum*), while its identity with the ParC proteins ranges from 28.3% (*M. pneumoniae*) to 35.4% (*B. subtilis*). Like the topoisomerase IV ParC and ParE subunits (4), *M. hominis* GyrA shows higher homology with its counterpart in the gram-positive bacteria *B. subtilis* (45% identity), *S. pneumoniae* (44.1%), and *S. aureus* (42.9%) than with that in the gram-negative bacterium *E. coli* (39.2%).

Among the eubacteria, the best identity score, found with *C. acetobutylicum* GyrA, is in agreement with the phylogenetic origin of the *Mollicutes*, believed to have arisen from ancestors of low-G+C-content gram-positive bacteria, such as *Clostridium* (48). In a comparison with other human mycoplasmas, *M. hominis* GyrA was found to share 47.8, 45.3, and 43.9% identical amino acids with the GyrA subunits of *U. urealyticum*, *M. genitalium*, and *M. pneumoniae*, respectively. From these data, we assigned the ORF4-encoded polypeptide as the GyrA subunit of *M. hominis*. An overall identity of 33.3% was found between the GyrA and ParC peptide sequences of *M. hominis*. This percentage is lower than that of the GyrB-ParE comparison (44.2%) (4).

A protein tree was constructed from the GyrA and ParC sequences of the nine bacteria listed above. As shown in Fig. 2, the GyrA and ParC sequences clearly clustered in two groups. The topoisomerase II sequences of the gram-positive bacteria with low G+C contents (*B. subtilis*, *S. aureus*, *S. pneumoniae*, and *C. acetobutylicum*) and of the class *Mollicutes* (*M. pneumoniae*, *M. genitalium*, *U. urealyticum*, and *M. hominis*) formed differentiated clusters, as previously shown by Huang (24). For mycoplasmas, phylogenetic data obtained with topoisomerase II are in good agreement with those obtained with 16S rRNA (29). Indeed, *M. pneumoniae* GyrA and *M. genitalium* GyrA belong to the same phylogenetic group, while *M. hominis* GyrA and *U. urealyticum* GyrA form two distinct groups. As in the 16S rRNA tree, *U. urealyticum* formed on group and *M. pneumoniae* and *M. genitalium* formed another group arising from the same branch.

In Fig. 3, the GyrA amino acid sequence of *M. hominis* was compared to those of *M. genitalium*, *M. pneumoniae*, *B. subtilis*, *S. aureus*, *S. pneumoniae*, *C. acetobutylicum*, and *E. coli*. The highest homology among the GyrA proteins of all eight bacteria is located at the N-terminal moiety, while the C-terminal region is much less conserved. Amino acid residues Ser153, Ser154, and Glu157 of *M. hominis* are the equivalents of Ser83, Ser84, and Glu87 of *E. coli* GyrA, which have been shown to be hot spots for quinolone resistance. Indeed, we have reported substitutions of these three amino acids in fluoroquinolone-resistant mutants of *M. hominis* selected in vivo and in vitro (3, 5, 7).

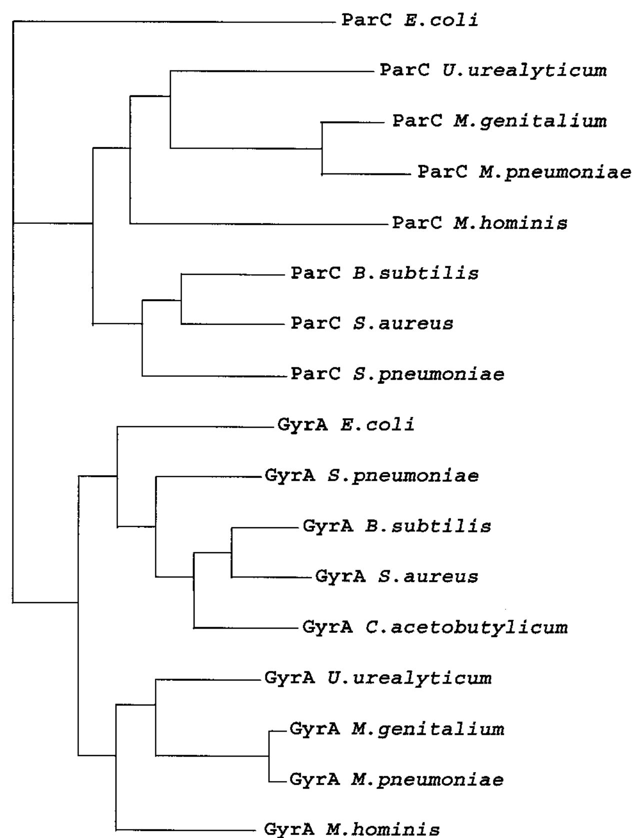

FIG. 2. Protein tree for full-length GyrA and ParC subunits from nine bacteria: *M. hominis* (4; this study), *M. genitalium* (16), *M. pneumoniae* (22), *U. urealyticum*, *B. subtilis* (26), *S. aureus* (13, 30), *S. pneumoniae* (1, 34), *C. acetobulylicum* (44), and *E. coli* (38, 42). The tree was compiled by using the CLUSTAL W multiple-alignment program.

Location of the DNA gyrase (*gyrA*) gene on the genomic map of *M. hominis* PG21. PFGE and Southern blot hybridization with probe MH3-MH4 containing the *gyrA* QRDR of *M. hominis* confirmed that the *gyrA* gene was located within *Sma*I, *Bam*HI, *Xho*I, and *Sal*I genomic DNA fragments of 100, 84.5, 124, and 410 kbp, respectively (4, 28). Hence, the *gyrA* gene is located within the 74-kpb region where these restriction fragments overlap. It is noteworthy that this region is quite distant from the topoisomerase II genes *gyrB* and *parC-parE*.

In many bacteria, *gyrB* lies close to the origin of replication, where genes are organized in the following order: *dnaA*, *dnaN*, *recF*, *gyrB*, and *gyrA*. A similar gene organization has been described for *M. pneumoniae* (22) and *M. genitalium* (16). In *U. urealyticum*, for which the complete genomic sequence is now available (http://genome.microbio.uab.edu/uu/), *gyrB* and *gyrA* are contiguous and are located between *dnaN* and *recA* but about 100 kbp downstream from *dnaA*. However, in *M. hominis*, *gyrA* and *gyrB* were shown not to be coupled. Instead, *gyrA* mapped at least 35 kbp downstream of *gyrB* (28). These results were confirmed by our PFGE data showing a *gyrA* gene 47 and 31 kbp distant from *gyrB* and *parE-parC*, respectively (data not shown). Furthermore, we found the following gene organization around the *gyrA* gene; *ftsH* homolog, hypothetical MG347-ORF homolog, *gyrA* homolog, and *dnaE* homolog. In *M. genitalium* and *M. pneumoniae*, *ftsH* and *dnaE* homologs are located within 30- and 40-kbp regions, respectively, surrounding the origin of replication. In addition, the *M. hominis* DNA primase motif homolog found downstream from *gyrA* shared homology with only the 3′-end parts of other bacterial *dnaE* or *dnaG* genes. These genes encode DNA primases that synthesize small RNA primers at replication forks during DNA synthesis. In *M. genitalium* (16) and *M. pneumoniae* (22), they are located close to the origin of replication. Such a situation in *M.*

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Mh  MLFGEDDNNKKENKNFEEDNSHDLDEYTNFDDRIKKIFSDDDETKAKKIDEEDEEEIPQDKEGYRVEPQLLDKEINGLKPANLSKVMK   88
Mg  ----------------------------------------------------------MAKQQDQVDKIRENLDNSTVKSISLANELE   30
Mp  ----------------------------------------------------------MAKQQDQIDKIRQELAQSAIKNISLSSELE   30
Bs  ---------------------------------------------------------------------MSEQNTPQVREINISQEMR   19
Sa  ---------------------------------------------------------------------MAELPQSRINERNITSEMR   19
Sp  ------------------------------------------------------------------------MQDKNLVNVNLTKEMK   16
Ca  -----------------------------------------------------------------------MLNEGKVLPVDISSEMK   17
Ec  ----------------------------------------------------------------------MSDLAREITPVNIEEELK   18

                                                                     ◆◆ ◆
Mh  TSFIEYAMSVIVSRALPDARDGLKPVHRRILYGMSELGMFYTAPHKKSARIVGDVLGKYHPHGDSSVYEAMVRMAQDFSLRYPLIDGH  176
Mg  RSFMEYAMSVIVARALPDARDGLKPVHRRVLYGAYIGGMHHDRPFKKSARIVGDVMSKFHPHGDMAIYDTMSRMAQDFSLRYLLIDGH  118
Mp  RSFMEYAMSVIVARALPDARDGLKPVHRRVLYGAYTGGMHHDRPFKKSARIVGDVMSKFHPHGDMAIYDTMSRMAQDFSLRYLLIDGH  118
Bs  TSFLDYAMSVIVSRALPDVRDGLKPVHRRILYAMNDLGMTSDKPYKKSARIVGEVIGKYHPHGDSAVYESMVRMAQDFNYRYMLVDGH  107
Sa  ESFLDYAMSVIVARALPDVRDGLKPVHRRILYGLNEQGMTPDKSYKKSARIVGDVMGKYHPHGDSSIYEAMVRMAQDFSYRYPLVDGQ  107
Sp  ASFIDYAMSVIVARALPDVRDGLKPVHRRILYGMNELGVTPDKPHKKSARITGDVMGKYHPHGDSSIYEAMVRMAQWWSYRYMLVDGH  104
Ca  KCYIDYAMSVIVSRALPDVRDGLKPVHRRILYSMHELGLTPEKGYRKCARIVGDVLGKYHPHGDSSVYGALVRLAQDFNLRYTVVDGH  105
Ec  SSYLDYAMSVIVGRALPDVRDGLKPVHRRVLYAMNVLGNDWNKAYKKSARVVGDVIGKYHPHGDSAVYDTIVRMAQPFSLRYMLVDGQ  106
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            ◆
Mh  GNFGSVDGDEAAAMRYTEARMSKIAGAMVDGIKKNTVDFMDNYDATEKEPVVLPSRFPNLLVSGSSGIAVGMATNIPPHNLGETIDAV  264
Mg  GNFGSIDGDRPAAQRYTEARLSKLAAELLKDIDKDTVDFIANYDGEEKEPTVLPAAFPNLLANGSSGIAVGMSTSIPSHNLSELIAGL  206
Mp  GNFGSIDGDRPAAQRYTEARLSKLAGELLRDIDKDTVDFVANYDGEEQEPTVLPAAFPNLLANGSSGIAVGMSTSIPSHNLSELIQGL  206
Bs  GNFGSVDGDSAAAMRYTEARMSKISMEILRDITKDTIDYQDNYDGSEREPVVMPSRFPNLLVNGAAGIAVGMATNIPPHQLGEIIDGV  195
Sa  GNFGSMDGDGAAAMRYTEARMTKITLELLRDINKDTIDFIDNYDGNEREPSVLPARFPNLLANGASGIAVGMATNIPPHNLTELINGV  195
Sp  GNFGSMDGDSAAAQRYTEARMSKIALEMLRDINKNTVDFVDNYDANEREPLVLPARFPNLLVNGATGIAVGMATNIPPHNLGETIDAV  192
Ca  GNFGSVDGDSAAAMRYTEAKMNKIALEMVRDIGKNTVDFIPNFDGEEKEPVVLPSRFPNLLVNGSAGIAVGMATNIPPHNLGEVIDGI  193
Ec  GNFGSIDGDSAAAMRYTEIRLAKIAHELMADLEKETVDFVDNYDGTEKIPDVMPTKIPNLLVNGSSGIAVGMATNIPPHNLTEVINGC  194
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Mh  CALAKNPEINVFELMEYIQAPDFPTGGIIFNKKGLIEAYSTGRGSITIRSKAHIQELSN-GKSKIIVTEIPYEVKKTEIMEKISELIK  351
Mg  IMLIDNPQCTFQELLTVIKGPDFPTGANIIYTKGIESYFETGKGNVVIRSKVEIEQLQT--RSALVVTEIPYMVNKTTLIEKIVELVK  292
Mp  ILLIDNPDCTINDLLGVIKGPDFPTGANIIYTKGIESYFETGKGNVVIRSKVSIEQLPT--RAALVVTEIPYMVNKTSLIEKIVELVK  292
Bs  LAVSENPDITIPELMEVIPGPDFPTAGQILGRSGIRKAYESGRGSITIRAKAEIEQTSS-GKERIIVTELPYQVNKAKLIEKIADLVR  282
Sa  LSLSKNPDISIAELMEDIEGPDFPTAGLILGKSGIRRAYETGRGSIQMRSRAVIEERGG-GRQRIVVTEIPFQVNKARMIEKIAELVR  282
Sp  KLVMDNPEVTTKDLMEVLPGPDFPTGALVMGKSGIHKAYETGKGSIVLRSRTEIETTKT-GRERIVVTEFPYMVNKTKVHEHIVRLVQ  279
Ca  TMLIDNPEATILELMAQIKGPDFPTAGIIMGKSGIRAAYETGRGKITVRAKSEIEVEDN-GKQKIIITEIPYQVNKARLVESIADLVK  280
Ec  LAYIDDEDISIEGLMEHIPGPDFPTAAIINGRRGIEEAYRTGRGKVYIRARAEVEVDAKTGRETIIVHEIPYQVNKARLIEKIAELVK  282
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Mh  DKKIDGIADFRDES-NRDGIRIVIDIKKSFVPEIVLNNLFKLTKLQSNYSFNMIALVNNEPKLLNLKACLEVYLKHQIEVVSRRLKFD  438
Mg  AEEISGIADIRDES-SREGIRLVIEVKRDTVPEVLLNQLFKSTRLQVRFPVNMLALVKGAPVLLNMKQALEVYLDHQIDVLVRKTKFV  379
Mp  AEEITGIADIRDES-SREGIRLVIEVKRDTVPEVLLNQLFKSTRLQVRFPVNMLALVKGAPKLLNMKQALTVYLEHQLDVLIRKTQFN  379
Bs  DKKIEGITDLRDES-DRTGMRIVIEIRRDANANVILNNLYKQTALQTSFGINLLALVDGQPKVLTLKQCLEHYLDHQKVVIRRRTAYE  369
Sa  DKKIDGITDLRDETSLRTGVRVVIDVRKDANASVILNNLYKQTPLQTSFGVNMIALVNGRPKLINLKEALVHYLEHQKTVVRRRTQYN  370
Sp  EKRIEGITAVRDES-NREGVRFVIEVKRDASANVILNNLFKMTQMQTNFGFNMLAIQNGIPKILSLRQILDAYIEHQKEVVVRRTRFD  366
Ca  DKRIVGISDLRDES-DRDGMRIVIEIKKDANSNIILNQLYKHTRLQDTFGINMLALVDNRPEVLNLKQILQHYIKFQEQVIRRRTEFD  367
Ec  EKRVEGISALRDES-DKDGMRIVIEVKRDAVGEVVLNNLYSQTQLQVSFGINMVALHHGQPKIMNLKDIIAAFVRHRREVVTRRTIFE  369
         **   ***     * * **           ** *    *  *        *   *     *                   *  *

Mh  LEKDRARAHILEGLKICIENIDAVIKIIKESKTDQEAQSKLSQAFN---------------------------------LSEIQTKA  492
Mg  LNKQQERYHILSGLLIAALNIDEVVAIIKKSANNQEAINTLNTK----------------------------------FKLDEIQAKA  433
Mp  LKKYQERFHILSGLLIAALNIDEVIAIIKKSANNQVAMEALHER----------------------------------FGLDEIQARA  433
Bs  LRKAEARAHILEGLRVALDHLDAVISLIRNSQTAEIARTGLIEQ----------------------------------FSLTEKQAQA  423
Sa  LRKAKDRAHILEGLRIALDHIDEIISTIRESDTDKVAMESLQQR----------------------------------FKLSEKQAQA  424
Sp  KEKAEARAHILEGLLIALDHIDEVIRIIRASETDAEAQAELMSK----------------------------------FKLSERQSQA  420
Ca  LEKASARAHILEGLKIALDHIDEVISLIRGSKTAQEAKLGLMD--------------------------KFG--------LSEKQAQA  421
Ec  LRKARDRAHILEALAVALANIDPIIELIRHAPTPAEAKTALVANPWQLGNVAAMLERAGDDAARPEWLEPEFGVRDGLYYLTEQQAQA  457
      *   * ***  *        *     *        *    *                                   * * *  *

Mh  IVDMKLGRLTGLAIEKMNEELNEVHERIANYIKILGDHNLLINLIIEELQEIKNAYGDARRSDINWDELSSIDNEDLIPKRDIVITLT  580
Mg  VLDMRLRSLSVLEVNKLQTEQKELKDSIEFCKKVLADQKLQLKIIKEELQKINDQFGDERRSEILYDISEEIDDESLIKVENVVITMS  521
Mp  VLDMRLRSLSVLEVNKLQTEQQELKALIEFCQQVLADKQLQLKLIKEQLTKINEQFGDPRRSEILYGISEDIDDEDLITQENVVITMS  521
Bs  ILDMRLQRLTGLEREKIEEEYQSLVKLIAELKDILANEYKVLEIIREELTEIKERFNDERRTEIVTSGLETIEDEDLIERENIVVTLT  511
Sa  ILDMRLRRLTGLERDKIEAEYNELLNYISELEAILADEEVLLQLVRDELTEIRDRFGDDRRTEIQLGGFEDLEDEDLIPEEQIVITLS  512
Sp  ILDMRLRRLTGLERDKIQSEYDDLLALIADLADILAKPERVSQIIKDELDEVKRKFSDKRRTELMVGQVLSLEDEDLIEESDVLITLS  508
Ca  ILDLKLQRLTGLEREKIEDEYNELMKTIAELKSILADENKILAIIRDELNEIKAKYGDERKTAIER-AENDIDIEDLIQEENVVITLT  508
Ec  ILDLRLQKLTGLEHEKLLDEYKELLDQIAELLRILGSADRLMEVIREELELVREQFGDKRRTEITA-NSADINLEDLITQEDVVVTLS  544
      *  *  *  *   *   *       *      *             *        * *              * **       *
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FIG. 3. Alignment of the *M. hominis* (Mh) GyrA amino acid sequence with those of its counterparts in *M. genitalium* (Mg) (16), *M. pneumoniae* (Mp) (22), *B. subtilis* (Bs) (26), *S. aureus* (Sa) (30), *S. pneumoniae* (Sp) (1), *C. acetobutylicum* (Ca) (44), and *E. coli* (Ec) (42). An asterisk indicates a residue identical in all eight proteins. Residues involved in quinolone resistance in *M. hominis* are indicated by diamonds. Dashes indicate gaps.

hominis, with *gyrA* being found between *ftsH* and *dnaE* homologs but not downstream from the *gyrB* and *dnaA* regions, could be explained by chromosomal rearrangement. Thus, it is tempting to speculate that *M. hominis gyrB* and *gyrA* were first contiguous and located in the vicinity of the initiation site of replication, before being separated by chromosomal rearrangement during evolution. It should be noted that *M. hominis oriC* has not yet been identified.

Characterization of trovafloxacin-resistant mutants selected in a stepwise manner. Trovafloxacin-resistant mutants were selected stepwise in vitro to further examine the role of topoisomerase IV and DNA gyrase in the development of resistance. The scheme used for the selection of trovafloxacin-resistant mutants is summarized in Fig. 4. Following this procedure, three independent sets of first- and second-step mutants and four independent sets of third-step mutants were obtained with mutation frequencies ranging from 10^{-7} to 10^{-4}. Except for the 10^{-4} frequency obtained with mutant IT3 on a trovafloxacin concentration corresponding to the MIC, mutation frequencies were similar for all steps and all concentrations used for selection (one to four times the MIC). These frequencies were relatively high (1×10^{-7} to 2.5×10^{-6}) and were equivalent to those found for the selection of ofloxacin- and sparfloxacin-resistant mutants (5, 25), confirming the high rate of mutation of *M. hominis* (12). The 29 trovafloxacin-resistant strains obtained were characterized for their susceptibilities to six fluoroquinolones and for the QRDR status of their *gyrA*, *parC*, *gyrB*, and *parE* genes (Table 1).

For each of the first-step mutants, IT1 to IT3, there was only a ParE Glu466→Lys change associated with a fourfold significant increase in the MICs of trovafloxacin, ciprofloxacin, and norfloxacin, and there was no significant increase (one- to

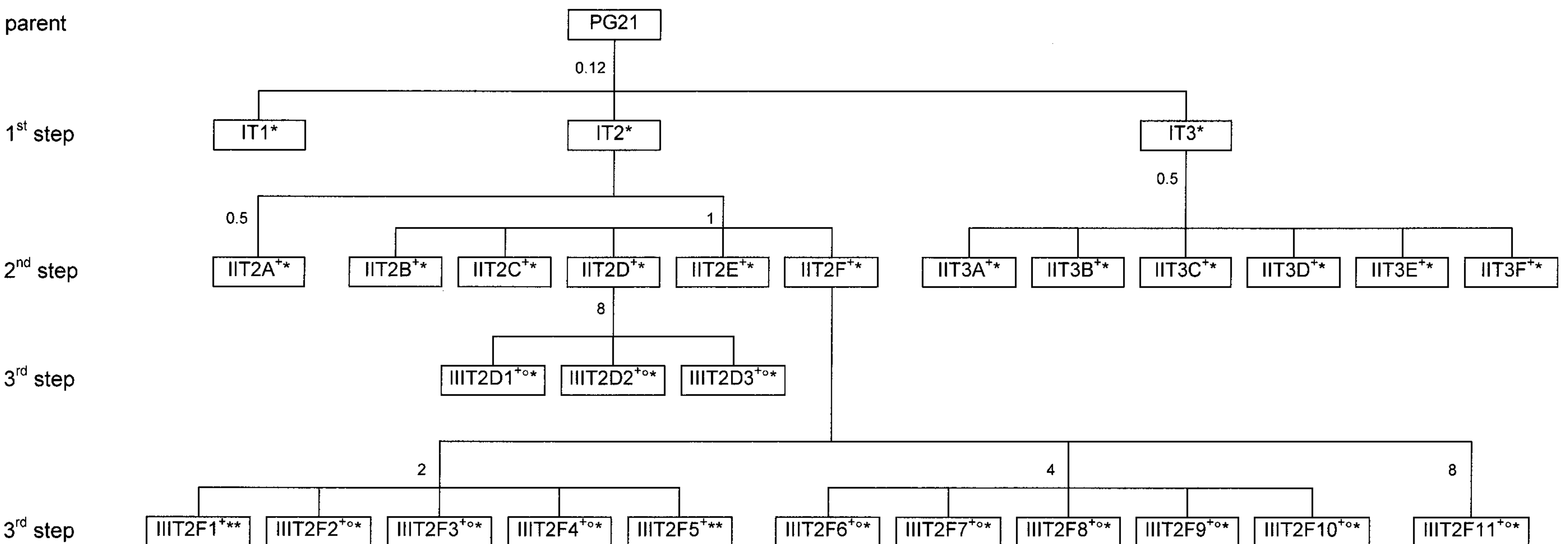

FIG. 4. Relationships between *M. hominis* PG21 and fluoroquinolone-resistant mutants IT1 to IIIT2F11 selected by stepwise exposure to trovafloxacin. The numbers outside the boxes indicate the trovafloxacin concentrations (in micrograms per milliliter) used in the selection steps. The superscripts +, °, and * indicate the presence of mutations in GyrA, ParC, and ParE, respectively (Table 1).

TABLE 1. Characteristics of trovafloxacin-selected mutants of *M. hominis*

Strain	Amino acid change at the indicated position in the QRDR of[a]:								MIC (μg/ml)[b] of:					
	GyrA			ParC			ParE		TVA	SPX	CIP	OFX	NOR	PEF
	153	157	189	91	92	95	446	466						
PG21 (reference strain)	Ser	Glu	Ala	Ser	Ser	Glu	Leu	Glu	0.06	0.06	2	1	32	4
First-step mutants														
IT1	—[c]	—	—	—	—	—	—	Lys	0.25	0.12	8	2	128	4
IT2	—	—	—	—	—	—	—	Lys	0.25	0.12	8	2	128	4
IT3	—	—	—	—	—	—	—	Lys	0.25	0.12	8	2	128	4
Second-step mutants														
IIT2A	—	—	Val	—	—	—	—	Lys	0.5	0.12	8	1	128	4
IIT2B	Leu	—	—	—	—	—	—	Lys	2	1	32	2	128	4
IIT2C	Leu	—	—	—	—	—	—	Lys	2	1	32	2	128	4
IIT2D	Leu	—	—	—	—	—	—	Lys	2	1	32	2	128	4
IIT2E	Leu	—	—	—	—	—	—	Lys	2	1	32	2	128	4
IIT2F	Leu	—	—	—	—	—	—	Lys	2	1	32	2	128	4
IIT3A	—	—	Glu	—	—	—	—	Lys	1	0.5	16	2	128	4
IIT3B	—	—	Glu	—	—	—	—	Lys	1	0.5	16	2	128	4
IIT3C	—	Lys	—	—	—	—	—	Lys	1	1	16	2	128	4
IIT3D	—	—	Glu	—	—	—	—	Lys	1	0.5	16	2	128	4
IIT3E	—	—	Glu	—	—	—	—	Lys	1	0.5	16	2	128	4
IIT3F	—	—	Glu	—	—	—	—	Lys	1	0.5	16	2	128	4
Third-step mutants														
IIIT2D1	Leu	—	—	—	Pro	—	—	Lys	16	64	>128	64	>128	32
IIIT2D2	Leu	—	—	Ile	—	—	—	Lys	16	32	128	64	>128	32
IIIT2D3	Leu	—	—	—	—	Gln	—	Lys	8	8	32	16	>128	16
IIIT2F1	Leu	—	—	—	—	—	Phe	Lys	16	32	128	64	>128	32
IIIT2F2	Leu	—	—	Ile	—	—	—	Lys	16	64	128	64	>128	32
IIIT2F3	Leu	—	—	Ile	—	—	—	Lys	16	64	>128	64	>128	32
IIIT2F4	Leu	—	—	Ile	—	—	—	Lys	16	64	>128	64	>128	32
IIIT2F5	Leu	—	—	—	—	—	Phe	Lys	16	64	128	64	>128	32
IIIT2F6	Leu	—	—	Ile	—	—	—	Lys	16	64	>128	64	>128	32
IIIT2F7	Leu	—	—	Ile	—	—	—	Lys	16	64	>128	64	>128	32
IIIT2F8	Leu	—	—	Ile	—	—	—	Lys	16	64	>128	64	>128	32
IIIT2F9	Leu	—	—	Ile	—	—	—	Lys	16	64	>128	64	>128	32
IIIT2F10	Leu	—	—	Ile	—	—	—	Lys	16	64	>128	64	>128	32
IIIT2F11	Leu	—	—	Ile	—	—	—	Lys	16	64	128	64	>128	32

[a] GyrA, ParC, and ParE residue positions are based on the respective gene sequences for *M. hominis* (4; this study). GyrA positions 153, 157, and 189 correspond to *E. coli* coordinates 83, 87, and 119, respectively. ParC positions 91, 92, and 95 correspond to *E. coli* coordinates 80, 81, and 84, respectively. ParE positions 446 and 466 correspond to *E. coli* coordinates 440 and 460, respectively.
[b] TVA, trovafloxacin; SPX, sparfloxacin; CIP, ciprofloxacin; OFX, ofloxacin; NOR, norfloxacin; PEF, pefloxacin.
[c] —, identical to that in the reference strain, PG21.

twofold) in the MICs of sparfloxacin, ofloxacin, and pefloxacin. A twofold increase usually is considered not significant within experimental error for the twofold dilution method used for MIC determinations.

When first-step mutants IT2 and IT3 were used as parental strains, 12 second-step mutants were selected; all had acquired an additional GyrA substitution. Six of them, IIT2B to IIT2F, bearing a Ser153→Leu substitution, and IIT3C, bearing a Glu157→Lys substitution, showed four- to eightfold increases in the MICs of trovafloxacin, sparfloxacin, and ciprofloxacin but no changes in the MICs of ofloxacin, norfloxacin, and pefloxacin. The six remaining mutants, IIT2A, IIT3A, IIT3B, and IIT3D to IIT3F, all carried an amino acid change at Ala189 in GyrA. For second-step mutants harboring the Ala→Glu change, the MICs of trovafloxacin, sparfloxacin, and ciprofloxacin were two- to fourfold higher than those for their parental strains. Surprisingly, mutant IIT2A did not shown any significant fluoroquinolone MIC increase even though it had acquired a Ala→Val substitution at the same position, 189.

Finally, 14 third-step mutants generated from two different parental strains, IIT2D and IIT2F (ParE Glu466→Lys and GyrA Ser153→Leu), all had acquired an additional alteration in topoisomerase IV subunits, either ParC or ParE. Ten of them, IIIT2D2, IIIT2F2 to IIIT2F4, and IIIT2F6 to IIIT2F11, were found to carry the ParC Ser91→Ile substitution, while mutants IIIT2D1 and IIIT2D3 had Ser92→Pro and Glu95→Gln changes, respectively. Except for strain IIIT2D3, all of these third-step mutants were characterized by significant increases in the MICs of trovafloxacin (8-fold), sparfloxacin (32- to 64-fold), and ciprofloxacin (≥4-fold) and especially by dramatic increases in the MICs of ofloxacin (32-fold and pefloxacin (8-fold). Strain IIIT2D3, bearing the Glu95→Gln substitution, exhibited globally two- to eightfold smaller increases in the MICs of the quinolones tested, compared to the other third-step *parC* mutant strains. Two mutants, IIIT2F1 and IIIT2F5, harbored an additional substitution in ParE, corresponding to a Leu446→Phe change and associated with the same fluoroquinolone MIC increases as those seen with the ParC Ser91-mutated third-step mutants. It should be noted that the increased ofloxacin and pefloxacin MICs were found

associated only with ParC and ParE amino acid changes at position 446 and not with the ParE substitution at position 466, contained in first-step mutants.

In summary, the development of a high level of resistance to trovafloxacin (MIC, ≥16 μg/ml) in *M. hominis* occurred in three steps, each associated with a mutation in the topoisomerase gene, beginning with a ParE alteration and involving alternating changes in DNA gyrase and topoisomerase IV.

Recent studies with the gram-positive bacteria *S. aureus* and *S. pneumoniae* indicated that different quinolones can have different preferential targets, depending on the bacterial species and on whether the studies are based on genetic or biochemical enzymatic data (9, 13, 15, 18, 20, 31, 32, 35–37, 43, 45). Trovafloxacin was reported to initially target topoisomerase IV by several genetic and enzymatic data for both *S. aureus* (14, 20) and *S. pneumoniae* (18, 19, 39, 45). For *M. hominis*, a low-G+C-content organism related to gram-positive bacteria, we and others showed by genetic studies that the primacy for fluoroquinolones of the target enzyme seemed to be drug specific (5, 25). DNA gyrase is the primary target of sparfloxacin, whereas topoisomerase IV is the primary target of pefloxacin, ofloxacin, and ciprofloxacin. In this study, we have determined the target specificity of trovafloxacin in *M. hominis* through analysis of mutants selected in a stepwise manner. All the first-step trovafloxacin-resistant mutants harbored a change in the ParE QRDR, while *gyrA* mutations were detected only in second-step mutants. These results indicate that, in *M. hominis*, as in *S. pneumoniae* or *S. aureus*, topoisomerase IV is the primary target of trovafloxacin.

GyrA and ParC mutations selected by trovafloxacin in *M. hominis* were predominantly those described previously for other fluoroquinolones. GyrA positions Ser153 (Ser83) and Glu157 (Asp87) (*E. coli* coordinates) and ParC positions Ser91 (Ser80) and Ser92 (Ala81) were found to be hot spots for quinolone resistance in many bacteria (23) and were already described as being mutated in *M. hominis* (5, 7, 25). In contrast, GyrA Ala189 (Ala119), ParC Glu95 (Glu84), and ParE Leu446 (Leu440) and Glu466 (Glu460) substitutions are novel. The GyrA Ala119→Val or Glu substitution was previously described for quinolone-resistant isolates of *Salmonella enterica* serovar Typhimurium (21). In *M. hominis*, only the Ala→Glu amino acid change was associated with significant increases in fluoroquinolone MICs. One explanation could be the charge difference induced by the amino acid change. Indeed, the Ala→Val substitution does not lead to a change in the residue charge (Ala and Val are both nonpolar), while the Ala→Glu change substitutes a nonpolar residue with a larger, negatively charged one. The new Glu84→Gln substitution in ParC has also been found to occur at the same position in the GyrA subunit of *S. pneumoniae* clinafloxacin-resistant mutants (36).

In contrast to the GyrA and ParC changes, to our knowledge, the ParE mutations acquired in the first- and third-step mutants have never been reported. First, the Glu466→Lys change in the first-step mutants does not occur in the EGDSA and PLRGK stretches designed as the GyrB QRDR (49). However, this position is located in a motif already associated with fluoroquinolone resistance. An Asn470→Asp mutation, 2 aa upstream from Glu466, has been described for *S. aureus* ParE (15). Moreover, the Glu474→Lys substitution recently described for *S. pneumoniae* GyrB (36) corresponds to the amino acid position just before Glu466 in *M. hominis*. The ParE Glu466 alteration, occurring in all *M. hominis* mutants, may be significant in quinolone resistance. A provocative experiment would be to point mutagenize back to wild type the ParE position 466 Lys mutants to see if the effect on the ParE subunit affects trovafloxacin resistance. The second mutation found in ParE, Leu446→Phe, lies in the second QRDR motif, PLRGK. Two ParE alterations have already been described for this motif; they concern the proline residue—Pro451→Ser or Gln in *S. aureus* (20, 41) and Pro454→Ser in *S. pneumoniae* (36). These data confirm that GyrB or ParE QRDR limits may require extension compared to the first QRDR, described for *E. coli* (49). It is interesting that, while the Glu466 substitution was associated with resistance to trovafloxacin, ciprofloxacin, and norfloxacin, only the substitution of Leu446 led to significant increases in the MICs of ofloxacin and pefloxacin. The functional role of the ParE QRDR is unknown, but it is tempting to speculate that some mutations could interfere with quinolone action, depending on the molecule and the mutated position.

The results described here indicate that only one mutation in *parE* or *parC* is necessary to reach a high level of resistance (MIC, ≥8 μg/ml) of *M. hominis* to ciprofloxacin, norfloxacin, and pefloxacin. In contrast, for sparfloxacin and trovafloxacin, at least three sequential mutational events, two in topoisomerase IV and one in DNA gyrase, are required to lead to high-level resistance in *M. hominis*, as previously described for trovafloxacin resistance in *S. aureus* (14) and coagulase-negative staphylococci (11). However, for sparfloxacin, the presence of GyrA Ser83 and ParC Ser80 or Glu84 mutations was shown to be associated with high-level resistance in sparfloxacin-selected mutants of *M. hominis* (5, 25). Furthermore, as previously reported for *M. hominis* in vitro and clinical isolates resistant to fluoroquinolones (6), our data suggest that trovafloxacin could retain activity against *parE* and *parE-gyrA* mutants (MICs, 0.12 to 2 μg/ml).

In conclusion, these data clearly confirm the enhanced activity of new fluoroquinolones, such as trovafloxacin, against mycoplasmas and indicate that susceptibility testing with ciprofloxacin or ofloxacin would not suffice to evaluate the activity of this antimicrobial class against these microorganisms. Furthermore, knowing the complete sequences of the four topoisomerase genes of *M. hominis* is the starting point for further enzymatic studies of DNA gyrase or topoisomerase IV preferential targeting of different fluoroquinolones.

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