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Molecular Evaluation of Antibiotic Susceptibility: *Tropheryma whipplei* Paradigm

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***Tropheryma whipplei*, the agent of Whipple's disease, grows fastidiously only in cell cultures without plaque production, and only three strains have been passaged. The formation of bacterial clumps in the supernatant precludes enumeration of viable bacteria and MIC determination. We evaluated the bacteriostatic effects of fluoroquinolones against two *T. whipplei* isolates by measuring the inhibition of the DNA copy number increase by real-time quantitative PCR. The analysis of the *T. whipplei* genome database allowed the identification not only of the *gyrA* gene but also the *parC* gene encoding the alpha subunit of the natural fluoroquinolone targets DNA gyrase (GyrA) and topoisomerase IV (ParC), respectively. The *parC* gene was detected in actinobacteria for the first time. High ciprofloxacin MICs (4 and 8 μg/ml) were correlated with the presence in *T. whipplei* GyrA and ParC sequences with an alanine residue at positions 83 and 80 (*Escherichia coli* numbering), respectively. Alanines at these positions have previously been associated with increased fluoroquinolone resistance in *E. coli* and mycobacteria. However, the MIC of levofloxacin was low (0.25 μg/ml). The same *T. whipplei* GyrA and ParC sequences were found in two other cultured strains and in nine uncultured tissue samples from Whipple's disease patients, allowing one to speculate that *T. whipplei* is naturally relatively resistant to fluoroquinolones.**

Tropheryma whipplei is the agent of Whipple's disease. Because *T. whipplei* cannot be grown in axenic medium, the present methodologies for antibiotic susceptibility testing of extracellular bacteria were not applicable. *T. whipplei* is extremely fastidious and resisted reproducible culture until 2000, when it was propagated in human fibroblasts (25). To the best of our knowledge, only three strains have been passaged more than five times (unpublished data). In the fibroblast cell system, it does not produce cytopathic effects such as plaque formation, and bacterial growth results in the formation of ropes of cells in the cell culture supernatant (18). These ropes consist of large clumps of bacteria that are very difficult to disperse without altering cell viability. The formation of similar cords has been described for *Mycobacterium tuberculosis* (2, 33), which, together with *T. whipplei*, belongs to the phylum *Actinobacterium*, the members of which are gram-positive organisms with high G+C contents.

To overcome these technical difficulties, the antibiotic susceptibilities of three human strains of *T. whipplei* were determined by a strict molecular approach. The MICs of two fluoroquinolone compounds (which were used as examples) for *T. whipplei* were determined by a quantitative PCR-based method that measured the inhibition of the increase in the number of bacterial DNA copies, as reported for rickettsiae (27).

At the same time, two type II topoisomerase genes were identified in the *T. whipplei* genome These corresponded to putative *gyrA* and *parC* genes. We tentatively correlated fluoroquinolone susceptibility with specific target gene sequences and detected *T. whipplei gyrA* and *parC* in uncultured samples from patients with Whipple's disease by PCR and sequencing. Specific mutations in DNA sequences leading to amino acid substitutions have been associated with resistance both by experimental mutagenesis techniques and in clinical strains. A well-characterized example includes the fluoroquinolones and type II topoisomerase-mediated resistance. Type II topoisomerases, including DNA gyrase and topoisomerase IV, are natural targets for fluoroquinolones (14). Fluoroquinolone resistance has been associated in many bacterial species with the presence of specific amino acids at critical positions in the quinolone resistance-determining region (QRDR) of GyrA and ParC, the proteins encoded by *gyrA* and *parC*, respectively (4, 6, 14, 21, 24, 34). This allows MIC determination by genomic detection of resistance to be replaced by techniques such as DNA hybridization, PCR sequencing, and PCR-restriction fragment length polymorphism analysis (20). The purpose of this work was to evaluate the susceptibilities of *T. whipplei* to quinolones and to determine the correlation of resistance with a DNA target enzyme. The detection of these target DNAs was later performed with uncultured samples from patients with Whipple's disease.

MATERIALS AND METHODS

***T. whipplei* strains.** Twelve *T. whipplei* strains were studied (Table 1), including three isolates obtained in our laboratory in an HEL cell culture system and nine uncultured strains whose DNA was extracted from biopsy samples from Whipple's disease patients. The *T. whipplei* Twist strain, isolated from a cardiac valve sample (18, 25), was cocultivated with MRC5 fibroblast cells until its 35th passage before antibiotic susceptibility testing. *T. whipplei* Endo2, grown from a cardiac valve sample from another endocarditis patient, was tested after 11 passages in cell culture. *T. whipplei* Slow2, grown from a duodenal biopsy specimen, was tested after 16 passages. The absence of *Mycoplasma* sp. contamination of cell cultures was checked weekly by using the Mycoplasma detection kit (Boehringer Mannheim GmbH, Mannheim, Germany).

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TABLE 1. *T. whipplei* strain and patient characteristics

Patients sex/age (yr)	Strain (genotype)[a]	Symptoms	Biopsy sample	Antibiotic(s) taken before sampling
Male/42	Twist (2A)	Endocarditis	Cardiac valve	Gentamicin + ampicillin for 4 wk, ciprofloxacin several for 4 weeks
Female/74	Slow2 (1A)	Diarrhea, arthralgia	Duodenum	None
Male/63	Endo2 (1A)	Endocarditis	Cardiac valve	Gentamicin + amoxicillin for 3 wk
Male/70	Unc (ND)	Fever, weight loss, lymphadenopathy, encephalitis	Duodenum	None
Male/63	Unc (1A)	Diarrhea, weight loss	Duodenum	None
Male/69	Unc (1A)	Diarrhea, arthralgia	Duodenum	None
Female/33	Unc (1A)	Fever, weight loss, lymphadenopathy	Duodenum	Co-trimoxazole for 1 year
Male/47	Unc (1A)	Fever, diarrhea, weight loss	Duodenum	Co-trimoxazole for 1 year
Male/60	Unc (2A)	Diarrhea, arthralgia	Duodenum	None
Female/69	Unc (ND)	Arthralgia, pulmonary embolism	Duodenum	None
Male/58	Unc (2A)	Diarrhea, arthralgia	Duodenum	None
Male/64	Unc (2A)	Endocarditis	Cardiac valve	None

[a] ND, not determined; Unc, uncultured.

Antibiotic solutions. The antibiotics tested were ciprofloxacin (Bayer Pharma, Sens, France) and levofloxacin (Hoechst-Marion-Roussel, Romainville, France) at twofold serial concentrations ranging from 0.25 to 8 μg/ml. Stock solutions were prepared according to the instructions of the manufacturers and stored at −80°C until use. Working solutions were prepared by dilution of stock solutions in minimum essential medium.

Growth kinetics of *T. whipplei* Twist, Slow2, and Endo2. *T. whipplei*-infected MRC5 cells were grown in a 150-cm^2 culture flask (Falcon; Beckman) by incubation at 37°C in a 5% CO_2-enriched atmosphere. Minimum essential medium (Life Technologies, GIBCO BRL, Cergy Pontoise, France) supplemented with 10% fetal calf serum and 2 mmol of L-glutamine (Gibco) was used as the incubation medium. Cellular infection was monitored twice a week by scraping infected cells from the culture flasks and microscopic examination of cell smears stained by the Gimenez technique. When heavy infection was detected (usually after ~1 month of incubation), the cell supernatant was discarded and infected MRC5 cells were detached by using sterile glass beads with 5 ml of fresh medium. The cells were lysed by sonication (three times for 30 s each time on ice at 60 mV), and the resulting bacterial inoculum was diluted 1:100 in culture medium and used to infect confluent MRC5 cell monolayers in 24-well microtiter plates (D. Dutcher, Brumath, France) (0.2 ml of inoculum in 2 ml of medium per well). The plates were incubated at 37°C in 5% CO_2. Infected cells in three different wells were harvested every 3 days from day 0 to day 21 postinfection. The growth kinetics of *T. whipplei* in MRC5 cells were determined by enumeration of the genome copies in each cell suspension by quantitative PCR.

Antibiotic susceptibility testing of *T. whipplei* by quantitative PCR. Confluent MRC5 cell monolayers in 24-well microtiter plates were infected with a *T. whipplei* inoculum as described above. *T. whipplei* strains Twist, Slow2, and Endo2 were tested for their fluoroquinolone susceptibilities. After incubation of the cultures for 48 h, antibiotics at various concentrations were added to the culture medium (50 μl of 40 times the desired final concentration in 2 ml of medium). Antibiotic-free wells served as growth controls, whereas uninfected MRC5 cells served as negative controls. During antibiotic susceptibility testing, cell cultures were harvested every 3 days (three wells per antibiotic concentration tested) for a total of 12 days, and cell suspensions were frozen at −80°C until DNA extraction for quantitative PCR assays. The lack of toxicity of antibiotics to MRC5 cells was controlled by examination of cell monolayers under an inverted microscope at the time that the cell cultures were harvested. MICs were defined as the minimal antibiotic concentrations that allowed complete inhibition of DNA growth, as measured by quantitative PCR assay.

Escherichia coli ATCC 8739 and *Staphylococcus aureus* CIP ATCC 49976 were obtained from the Pasteur Institute (Institut Pasteur, Marnes La Coquette, France) and were used as controls for antibiotic susceptibility testing. The activities of ciprofloxacin and levofloxacin were determined on Mueller-Hinton agar (bioMérieux) incubated at 37°C for 18 h. The MICs were in the range of those reported by the Pasteur Institute. The antibiotic activities of the dilutions were checked after 30 days of incubation at 37°C by using *E. coli* and *S. aureus*.

Quantitative PCR assay with the LightCycler instrument. *T. whipplei* DNA was extracted from MRC5 cells infected in vitro by a previously described protocol (12). Infected MRC5 cell suspensions were centrifuged at 20,000 × g for 15 min at 4°C. After the supernatants were carefully removed, the cell pellets were resuspended in 200 μl of digestion buffer (50 mM Tris-HCl [pH 8.5], 1 mM EDTA, 0.5% sodium dodecyl sulfate, 200 μg of proteinase K per ml) and incubated for 90 min at 55°C. The DNA was than extracted by using QIAamp DNA binding columns (Qiagen, Courtaboeuf, France) and stored at −20°C until it was used for amplification by quantitative PCR assay.

DNA extracts were amplified by the LightCycler PCR assay (Roche Diagnostics) in glass capillaries (volume 20 μl) with primers that allowed amplification of the *T. whipplei* intergenic spacer (ITS) region (7). These primers were chosen to allow amplification of one copy of target gene per bacterium. The PCR mixture (20 μl) contained 2 μl of extracted DNA, 13.2 μl of H_2O, 1.6 μl of $MgCl_2$ (25 mmol), 1 μl (i.e., 10 pmol) of forward primer TwITSF (Table 2), 1 μl (i.e., 10 pmol) of reverse primer TwITSR (Table 2), and 2 μl of DNA-Master Hybridization Probes (Roche Diagnostics) containing *Taq* DNA polymerase, reaction buffer, a deoxynucleoside triphosphate mixture, and 10 mmol $MgCl_2$ (concentrated 10 times). Cycling conditions consisted of an initial denaturation at 95°C for 8 min, followed by 40 cycles with denaturation at 95°C for 15 s, annealing at 56°C for 5 s, and extension at 72°C for 8 s, with a ramping time of 20°C/s. A calibration curve for DNA quantification was determined by amplifying 10-fold serial dilutions of DNA extracted from the primary *T. whipplei* inoculum used to infect MRC5 cells.

Identification of *gyrA* and *parC* in the *T. whipplei* Twist genome. Two *gyrA*-like sequences were identified in the *T. whipplei* Twist genome by comparison (1) with known *gyrA* sequences deposited in GenBank with the BLAST program. Their open reading frames (ORFs) were determined by using the ORF finder program (28). Their G+C contents were determined by using the FREQBNK program (INFOBIOGEN, Evry, France). These ORFs were defined as putative *gyrA* and *parC* genes, which encode the A subunits of DNA gyrase and topoisomerase IV, respectively, by determination of the best sequence homologies of their protein counterparts (GyrA and ParC) with the same protein sequences from *Mycoplasma pneumoniae* and *M. tuberculosis*. *T. whipplei* GyrA was also

TABLE 2. Oligonucleotide primers used for PCR amplification and sequencing

Gene	Primers	Nucleotide sequence
its	TwITSF	5′-CCGAGGCTTATCGCAGATTG-3′
	TwITSR	5′-GGTGACTTAACCTTTTTGGAG-3′
gyrA QRDR	*gyr*AF1	5′-GATGGCTTAAAGCCCGTACA-3′
	*gyr*AR1	5′-CGACGGCTATTCCGC-3′
parC QRDR	*par*CF1	5′-GATGGCTTAAAGCCCGTACA-3′
	*par*CR1	5′-CGACGGCTATTCCGC-3′
gyrA QRDR	*Gyr*AF2	5′-CGGGCATTGCCAGACGC-3′
	*Gyr*AR2	5′-CGGGGGTATACTGGTTGCC-3′
parC QRDR	*Par*CF2	5′-CGGGCATTGCCAGACGC-3′
	*par*CR2	5′-CGGGGGTATACTGGTTGCC-3′

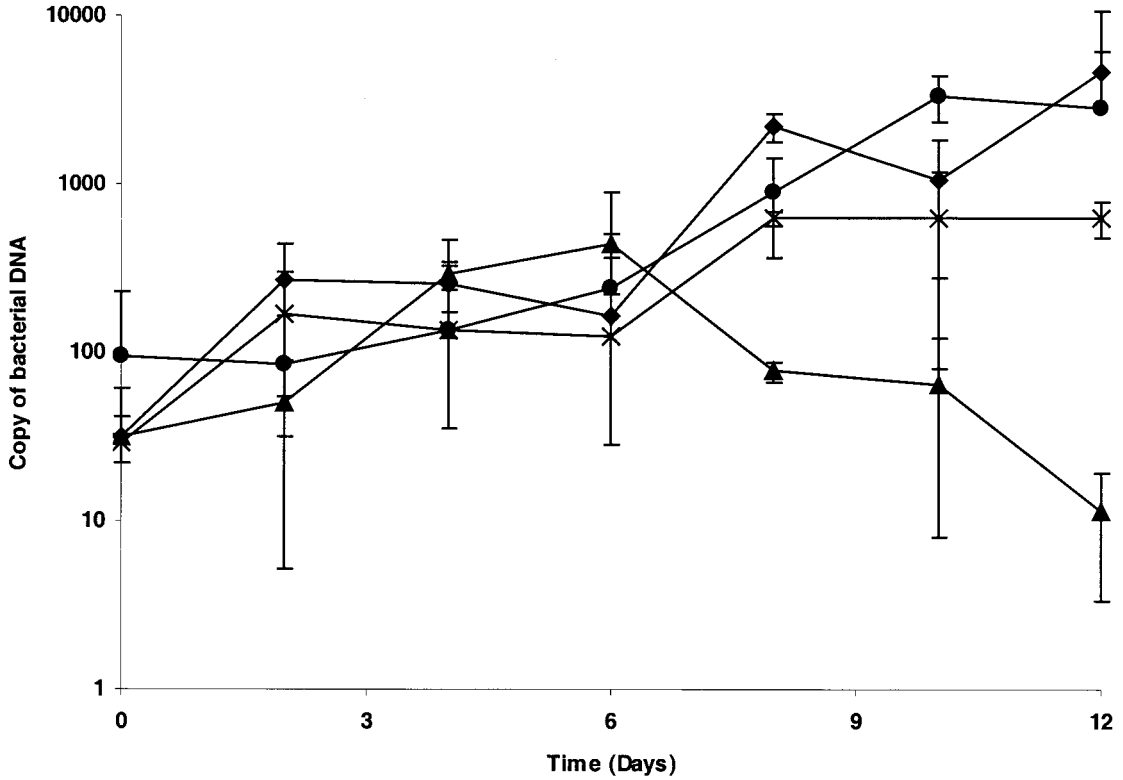

FIG. 1. Growth kinetics and susceptibilities to fluoroquinolones of *T. whipplei* Endo2. Bacterial loads were determined by enumeration of the bacterial DNA copies by quantitative PCR. Comparable results were found for the *T. whipplei* Twist and Slow2 strains. ◆, growth control; ●, ciprofloxacin at 1 μg/ml; ∗, ciprofloxacin at 4 μg/ml; ▲, ciprofloxacin at 8 μg/ml.

differentiated from ParC by constructing a phylogenetic tree with known GyrA and ParC sequences by using MEGA (Molecular Evolutionary Genetics Analysis) software (version 2.1) and the neighbor-joining method (amino acid proportion distance, with bootstrap testing). The GyrA sequences used were those of *E. coli* K-12 (16130166), *Haemophilus influenzae* (16271976), *Pseudomonas aeruginosa* (15595198), *Rickettsia conorii* (15892196), *Rickettsia prowazekii* (3860770), *Neisseria gonorrhoeae* (2120873), *Neisseria meningitidis* (15794492), *Bartonella bacilliformis* (18920720), *Agrobacterium tumefaciens* (17933925), *Mycobacterium leprae* (15826871), *Mycobacterium tuberculosis* (13879047), *Mycobacterium smegmatis* (1346234), *Streptomyces coelicolor* (1093585), *Treponema pallidum* (15639000), *Enterococcus faecalis* (15982568), *Streptococcus pneumoniae* (15903142), *Listeria monocytogenes* (16802048), *S. aureus* (21203170), *Clostridium perfringens* (18143657), *Bacillus subtilis* (16077075), *Corynebacterium urealyticum* (21518737), *M. pneumoniae* (13507743), *Ureaplasma urealyticum* (13357558), *Chlamydia trachomatis* (13627049), and *Chlamydia pneumoniae* (12644500). The ParC sequences used were those of *H. influenzae* (16271976), *E. coli* K-12 (16130915), *P. aeruginosa* (4176378), *R. prowazekii* (3860637), *R. conorii* (15891923), *N. gonorrhoeae* (2120882), *N. meningitidis* (15794693), *A. tumefaciens* (17739557), *S. pneumoniae* (20530728), *L. monocytogenes* (21328243), *E. faecalis* (15982571), *S. aureus* (21204410), *C. perfringens* (18311050), *B. subtilis* (3914271), *C. urealyticum* (21518734), *M. pneumoniae* (13507739), and *U. urealyticum* (13357558).

Determination of *gyrA* and *parC* QRDRs in *T. whipplei* Twist. The *T. whipplei* Twist *gyrA* and *parC* QRDRs were defined by best alignment with known homologous QRDR sequences from other species, including *E. coli* K-12 (16127994), *M. tuberculosis* (13879047), *M. leprae* (1041443), *Mycobacterium avium* (1041431), *Mycobacterium intracellulare* (2815375), *M. smegmatis* (1346234), and *Mycobacterium fortuitum* (2815371).

Determination of *gyrA* and *parC* QRDR sequences in an additional 11 samples and comparison of their protein counterparts with the BLAST program. *T. whipplei* DNA was extracted as described above from MRC5 cells infected with strain Slow2 or Endo2 and from eight duodenal biopsy specimens and one cardiac valve specimen obtained from patients with Whipple's disease (Table 1). Tissue samples were preincubated in 200 μl of digestion buffer at 55°C for 3 h. *T. whipplei* DNA in biopsy samples was previously demonstrated by amplification and sequencing of the *T. whipplei rpoB* and *its* genes (5).

The extracted DNA was used to amplify the *T. whipplei gyrA* QRDR with primers *gyr*AF1 and *gyr*AR1 (Table 2) or the *T. whipplei parC* QRDR with primers *par*CF1 and *par*CR1 (Table 2). The sequences of these primers were defined from the *T. whipplei* Twist *gyrA* and *parC* sequences. PCRs were performed with a Perkin-Elmer 9600 thermocycler under the following conditions: a first denaturation step at 95°C for 2 min; 40 three-step cycles of 94°C for 30 s, 48°C for 30 s for primers *gyrA*F1 and *gyr*AR1 or 55°C for 30 s for primers *par*CF1 and *par*CR1, and 68°C for 1 min; and a final 6-min extension step at 68°C.

DNA sequencing was performed with an internal dRhodamine terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase (FS; Perkin-Elmer Applied Biosystems, Warrington, United Kingdom), according to the instructions of the manufacturer. The 3′ and 5′ ends of the amplified fragments obtained in the different PCR assays were sequenced after precipitation and purification with 70% ethanol and 0.5 mmol $MgCl_2$. The primers used were *gyr*AF2 and *gyr*AR2 for the *gyrA* QRDR sequences and *par*CF2 and *par*CR2 for the *parC* QRDR sequences (Table 2). The cycle sequencing reaction mixtures comprised 4 μl of ready reaction mixture, 1 μl (i.e., 10 pmol) of forward primer for direct DNA strand sequencing or 1 μl (i.e., 10 pmol) of reverse primer for cDNA strand sequencing, and 4 μl (i.e., 200 ng) of template DNA. The mixture was brought to 10 μl with deionized water. Amplification was performed by use of 30 cycles of 95°C for 20 s, 50°C for 10 s, and 60°C for 2 min. Electrophoresis was performed with an ABI PRISM 310 genetic analyzer (Perkin-Elmer).

The DNA and amino acid sequences of the QRDRs obtained for the 12 *T. whipplei* strains studied were aligned and compared by using the CLUSTAL multialignment package (11).

RESULTS

***T. whipplei* growth kinetics.** Growth of *T. whipplei* isolates was exponential, with a 2.7 ± 1.9 log increase in bacterial concentration over 12 days of incubation for strain Twist, a

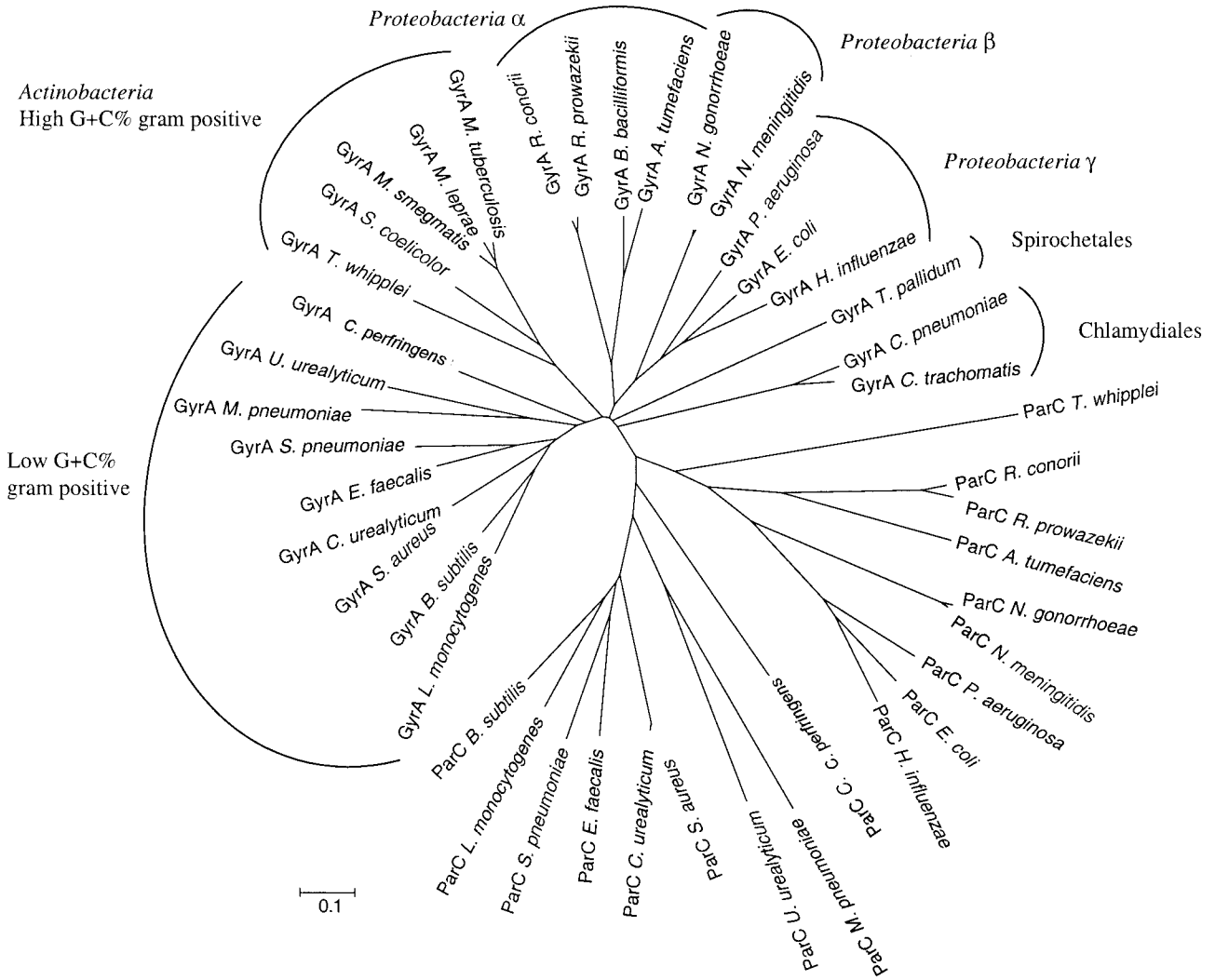

FIG. 2. Phylogenetic tree of GyrA and ParC sequences from *T. whipplei* and various representative species of the domain *Bacteria*. Protein sequences were aligned by using the ClustalW program from the Pôle BioInformatique Lyonnais (Lyon, France). The tree was constructed with MEGA software (version 2.1) by the neighbor-joining methodology (amino acid p-distance, including site, complete deletion, and bootstrap testing).

2.25 ± 0.98 log increase for strain Endo2, and a 1.96 ± 1.16 log increase for strain Slow2. During this period, the doubling time of *T. whipplei* Twist was found to be between 32 and 36 h, whereas that of Endo2 was between 34 and 38 h and that of strain Slow2 was between 41 and 48 h. Bacterial growth then reached a plateau.

Fluoroquinolone susceptibility testing of *T. whipplei* Twist, Slow2, and Endo2 strains. As determined by quantitative PCR assay, increases in the number of DNA copies of *T. whipplei* Twist, Slow2, and Endo2 were completely abolished with ciprofloxacin at 4 or 8 μg/ml (Fig. 1) but not with ciprofloxacin at 1 or 2 μg/ml. Thus, the MIC of ciprofloxacin for *T. whipplei* was evaluated to be 4 μg/ml. Levofloxacin was more effective, with MICs ranging from 0.25 to 0.5 μg/ml. The antibiotic dilution was stable for 30 days and had the same activity against control strains.

***T. whipplei* Twist *gyrA* and *parC*.** The putative *T. whipplei* *gyrA* sequence is a 2,460-bp ORF (AE014184). The start codon, TTG, was determined with the ORF finder (28). The ORF extends to a stop codon, TAG, at nucleotide 2,460. The ITS G+C content is 49.3%. Translation of the ORF corresponds to a putative 819-amino-acid (aa) protein, with a calculated molecular mass of 90,668 kDa, comparable to that of *S. coelicolor* GyrA (i.e., 818 aa with a calculated molecular mass of 88.586 kDa) (3).

The putative *T. whipplei parC* ORF is 2,475 bp (GenBank accession number AE014184). The start codon, TTG, was determined with the ORF finder, as described above (28). The ORF extends to a stop codon, TAG, at nucleotide 2475. The ITS G+C content is 43.92%. Translation of the ORF corresponds to an 824-aa protein, with a calculated molecular mass of 91.190 kDa.

For the *T. whipplei* GyrA sequence, we found identities with the *M. pneumoniae* GyrA and ParC sequences of 40 and 29%, respectively, and identity with the *M. tuberculosis* GyrA sequence of 54%. For the *T. whipplei* ParC sequence, we found

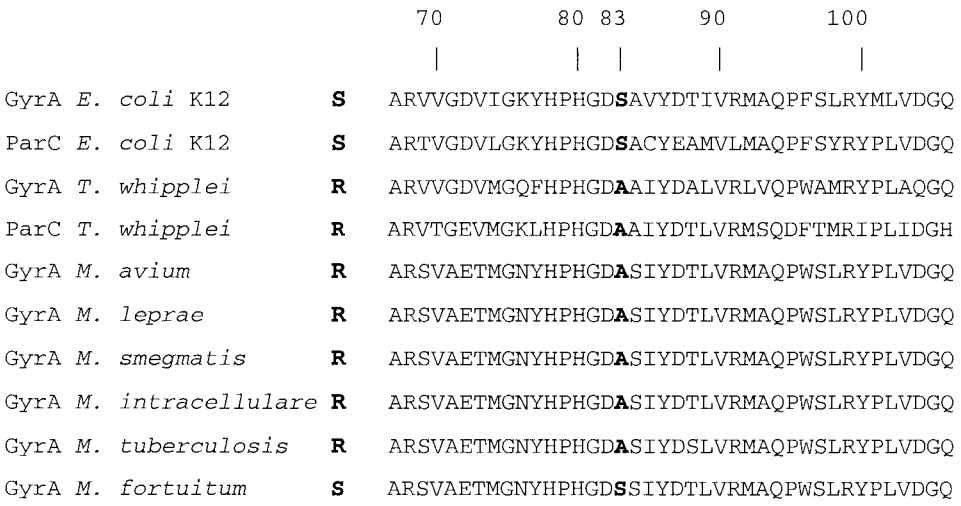

FIG. 3. *T. whipplei* GyrA and ParC QRDR alignment with homologous sequences from *E. coli* K-12 (16127994), *M. tuberculosis* (13879047), *M. leprae* (1041443), *M. avium* (1041431), *M. intracellulare* (2815375), *M. smegmatis* (1346234), and *M. fortuitum* (2815371).

identities with the *M. pneumoniae* GyrA and ParC sequences of 33 and 38%, respectively, and identity with the *M. tuberculosis* GyrA sequence of 34%.

Also, a phylogenetic tree constructed with known GyrA and ParC protein sequences clearly distinguished the two types of protein sequences and allowed differentiation of putative GyrA and ParC sequences in *T. whipplei* (Fig. 2).

Determination of *gyrA* and *parC* QRDRs in *T. whipplei* Twist. The *T. whipplei* GyrA and ParC QRDRs are presented in Fig. 3. The *T. whipplei* GyrA QRDR extends from the alanine at position 65 to the glutamine at position 104. Its ParC QRDR extends from the alanine at position 80 to the histidine at position 119. An alanine residue was found at position 81 of the *T. whipplei* GyrA QRDR, corresponding to the serine at position 83 in the *E. coli* GyrA sequence. An alanine residue was also found at position 96 of the *T. whipplei* ParC QRDR, corresponding to the serine at position 80 in the *E. coli* ParC sequence (19).

Determination of *gyrA* and *parC* QRDRs in an additional 11 *T. whipplei* strains. The primers whose sequences were defined from the *T. whipplei* Twist *gyrA* and *parC* sequences allowed us to amplify and sequence the *gyrA* and *parC* QRDRs from two other *T. whipplei* strains cultured (i.e., strains Endo2 and Slow2), as well as from the *T. whipplei* DNA contained in uncultured digestive or cardiac valve biopsy specimens from nine additional patients with Whipple's disease.

For the *gyrA* QRDR, a 460-bp DNA fragment was amplified from the 11 additional specimens tested. When these DNA fragments were aligned with the *T. whipplei* Twist *gyrA* QRDR, nucleotide sequence variations were found only at positions 120 (A versus G) and 354 (T versus C), which corresponded to silent mutations. An alanine residue at position 81was found in the sequences of all strains. For the *parC* QRDR, 400-bp DNA fragments were amplified and aligned with the *T. whipplei* Twist *parC* QRDR, as described above. Variations in nucleotide sequences were found at positions 432 (T versus G), 489 (C versus T), and 531 (C versus T), which corresponded to silent mutations. An alanine residue was found at position 96 in the sequences of all strains tested.

DISCUSSION

T. whipplei, the agent of Whipple's disease, is extremely fastidious. Its recent in vitro propagation in our laboratory provided us the opportunity to test its antibiotic susceptibility for the first time. As phenotypic methods were not applicable, we determined the in vitro growth kinetics of *T. whipplei* using quantitative PCR technology, allowing enumeration of specific target *its* DNA copies. We found a doubling time of 32 to 36 h for the *T. whipplei* Twist strain, much lower than that previously reported (i.e., 18 days) for the same strain at the time of primary isolation (18) (Fig. 1). This suggests in vitro selection of bacteria most adapted to the MRC5 cell culture system after multiple passages. This doubling time, however, remains higher than that of the slowly growing bacterium *M. tuberculosis* (14.3 h minimum to 24 h) (15).

Molecular evaluation of fluoroquinolone MICs, defined as the minimum concentration of antibiotic that allowed complete inhibition of an increase in *its* copy numbers, revealed the relative resistance of three *T. whipplei* strains to ciprofloxacin, with the MICs being comparable to those previously reported for *M. tuberculosis* (26).

Comparison of the whole *T. whipplei* Twist genome with known *gyrA* sequences with the BLAST program allowed identification of two *gyrA*-type sequences, which were differentiated into *gyrA* and *parC* genes on the basis of the sequence homologies of their protein counterparts with known GyrA and ParC sequences. Both genes are considered paralogs and are found in many of the complete bacterial genomes that are available.

In both the *T. whipplei* GyrA and the *T. whipplei* ParC sequences, alanines were found at positions 81 and 96, respectively, corresponding to the serine at position 83 in *E. coli* GyrA (10) and the serine at position 80 in *E. coli* ParC (29), respectively. We sequenced the same QRDRs of both genes

from two other *T. whipplei* isolates as well as from nine uncultured strains whose DNA was directly extracted from tissue specimens from Whipple's disease patients.

GyrA-mediated resistance to fluoroquinolones has been well characterized in many bacteria. Many examples exist to demonstrate that species naturally bearing a serine residue at position 83 (*E. coli* numbering) are usually susceptible to fluoroquinolones (14), whereas the presence of an alanine at this critical position usually corresponds to natural or acquired resistance to these antibiotics (4, 13, 13, 14, 21, 31, 34). GyrA-mediated natural resistance to fluoroquinolones has been described in *Mycobacterium* species, which are phylogenetically closely related to *T. whipplei*. Poor susceptibilities to fluoroquinolones are found in *M. tuberculosis*, *M. avium*, *M. intracellulare*, *Mycobacterium marinum*, *Mycobacterium chelonae*, *Mycobacterium abcessus*, and *M. smegmatis*, species that bear an alanine residue at position 83 (*E. coli* numbering) in the GyrA QRDR (8, 9, 26). In contrast, a serine residue is found in *M. fortuitum*, *Mycobacterium peregrinum*, and *Mycobacterium aurum*, which are naturally more susceptible to these antibiotics (8, 9, 26). Other critical positions in the GyrA QRDR have been described as positions 84 and 87 in *E. coli* (31) and corresponding positions in other species (23, 31, 32). High-level resistance to fluoroquinolones has been described in *M. tuberculosis* due to amino acid substitution in the wild-type GyrA sequence at the critical positions 83, 84, and 87, with MICs being more than 100-fold those for wild-type strains when these mutations accumulate (16). ParC-mediated resistance has been described in gram-negative bacteria such as *E. coli* (17, 29). Critical positions in *E. coli* ParC correspond to positions 80 and 84, and increased fluoroquinolone resistance has been specifically associated with replacement of the serine at position 80 by an alanine. In gram-negative bacteria, these mutations are usually combined with amino acid sequence alterations in GyrA. In contrast, topoisomerase IV is considered the primary target of fluoroquinolones in the gram-positive species of the genera *Staphylococcus* and *Streptococcus* (22, 30). Surprisingly, levofloxacin was efficient, and this may suggest another resistance mechanism.

In conclusion, we have presented the first in vitro evaluation of the antibiotic susceptibilities of *T. whipplei*. Because of the fastidious nature of this bacterium, only a molecular method based upon quantitative PCR technology allowed determination of the MICs. Relative resistance to ciprofloxacin was found in three human strains of *T. whipplei*. Identification of *gyrA* and *parC* in the *T. whipplei* genome allowed us to correlate fluoroquinolone resistance with specific QRDR sequences. These sequences were then demonstrated to be the wild types by amplification and sequencing of homologous sequences in two additional isolates as well nine uncultured strains. We speculate that *T. whipplei* has a topoisomerase-mediated natural low-level resistance. The *T. whipplei* model can be considered a paradigm for an obligate molecular approach to the testing of antibiotic susceptibility in a fastidiously growing microorganism.

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