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Characterization of Fluoroquinolone-Resistant Mutants of *Escherichia coli* Selected In Vitro

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Wild-type mutants highly resistant to fluoroquinolones were selected in vitro from a quinolone-susceptible *Escherichia coli* isolate by stepwise exposure to increasing concentrations of nalidixic acid and ciprofloxacin (CIP) either in liquid medium or on solid medium. Mutant R17 was selected by serial passage in liquid medium; the MIC of CIP for mutant R17 was 256 μg/ml. On solid medium, consecutive mutants MI, MII, MIII, MIVa, and MIVb were selected in four steps. The frequencies of mutations were between 10^{-9} and 10^{-11}, and the MICs of CIP ranged from 0.5 μg/ml (for mutant MI) to 256 μg/ml (for mutant MIVb). From the results of a dominance test with the *gyrB*$^+$ plasmid (pBP547), no *gyrB* mutations were detectable. In the first step, mutant MI, a mutation from a Ser to a Leu residue at position 83 (a Ser-83→Leu mutation), was detected in the quinolone resistance-determining region of the *gyrA* gene. In addition, the second-step mutation was associated with a reduced uptake of CIP and an altered outer membrane protein profile. The third mutation was identified as an Asp-87→Gly mutation in the quinolone resistance-determining region of the *gyrA* gene. Concomitantly, a slight increase in the doubling time was detected. For two different four-step mutants, mutants MIVa and MIVb, the MICs of only some quinolones, including CIP, increased. The accumulation of CIP in the mutants was comparable to that in their parent MIII. The doubling time of mutant MIVa was similar to that of mutant MIII, but differed by a factor of 3 from that of the very slow growing mutant MIVb. In contrast, a clinical isolate of *E. coli* (isolate 205096) described previously (P. Heisig, H. Schedletzky, and H. Falkenstein-Paul, Antimicrob. Agents Chemother. 37:696–701, 1993) which has the same double mutation in *gyrA*, had a doubling time comparable to that of the wild-type isolate.

Fluoroquinolones have excellent bactericidal activities against a broad range of bacteria, including *Escherichia coli* (5). These drugs act by inhibiting the bacterial type II topoisomerase DNA gyrase (60). Gyrase consists of two pairs of subunits A and B and introduces negative supercoils into covalently closed double-stranded DNA in an ATP-consuming reaction (for reviews, see references 16, 19, and 63). In the presence of inhibitory concentrations of quinolones, cleavable complexes of gyrase covalently bound to DNA can be isolated (35, 56). These complexes block DNA replication and are believed to be the inducing signal for a series of events which ultimately lead to cell death (32).

Resistance to quinolones involves at least one of two mechanisms: (i) The first involves mutations that alter the molecular target, DNA gyrase. All mutations in the structural gene *gyrA* coding for gyrase subunit A are located in a highly conserved region (the quinolone resistance-determining region [QRDR]) and cause an increase in the MICs of all quinolones (7, 11, 21, 26, 67, 68). Two mutations are known to exist in the *gyrB* gene coding for gyrase subunit B (65).

(ii) The second mechanism involves mutations that lead to impaired access of the drugs to gyrase, which causes alterations in the structural or regulatory elements of the outer membranes of gram-negative bacteria (for a review, see reference 53) or which affect energy-dependent efflux systems (10) in both gram-positive and gram-negative bacteria (37, 39). Additionally, in *E. coli*, the best characterized organism with respect to quinolone resistance mechanisms, mutations were detected in several unrelated loci like *crp*, *ctr*, *cya*, *hipQ*, *icd*, and *purB* (for a review, see reference 31) or an unknown locus coding for an analog of GroEL (22).

In *E. coli*, all of these one-step mutants isolated in vitro as well as most of the clinical isolates have significantly reduced susceptibilities to unfluorinated quinolones. These mutants, however, remain clinically susceptible to fluorinated compounds (11, 21, 29, 33, 34, 67, 68). One exception is a clinical isolate of *E. coli* that carries the novel *gyrA* mutation at the Asp residue at position 81 (Asp-81); this mutant shows clinically relevant levels of resistance to fluoroquinolones but hypersusceptibility to nalidixic acid (7). Since, in addition to the *gyrA* mutation, a reduction in quinolone accumulation was detected (43), it cannot be excluded that this unusual behavior is at least partly due to permeability changes that fluorinated and unfluorinated quinolones in a different manner.

International studies show a generally low incidence of clinical resistance to fluoroquinolones (i.e., MICs, >1 μg/ml) in isolates of *E. coli* (14, 40); however, clinically resistant *E. coli* isolates are emerging in some areas of the world (1, 38). Two such isolates (ciprofloxacin [CIP] MICs, 64 and 32 μg/ml, respectively) were examined to determine the underlying mechanism(s) of resistance. One isolate had a double mutation in *gyrA* alone (26), while the other one had a combination of mutations in *gyrA* and an unidentified locus resulting in a reduced amount of the outer membrane protein (OMP) F (3).

Selection of such mutants in vitro was successful only in a few cases and required repeated exposure of the cells to increasing quinolone concentrations (18, 32, 62). One mutant (CIP MIC, 20 μg/ml) was selected with CIP (32); another was selected with norfloxacin (NOR; NOR MIC, 16 μg/ml) (62). In both cases, at least six intermediate isolates were isolated to yield mutants with high-level resistance. Information on the nature of the individual mutation steps was limited. Another study reported the isolation of two-step mutants from different members of the family *Enterobacteriaceae* in vitro. For only one mutant of *E. coli* was there shown to be a clinically relevant

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increase in the MIC of CIP, from 0.015 to 4 μg/ml (51). These findings are in apparent contrast to data suggesting that only two mutations in the *gyrA* gene of clinical strain 205096 account for high-level resistance to CIP (64 μg/ml) (26). However, since it is rather difficult to be certain how many mutation steps occurred in vivo, we performed the present study in order (i) to select mutants of *E. coli* with high-level resistance toward fluoroquinolones, comparable to that of strain 205096, (ii) to determine the number of mutation steps and the respective mutation frequencies, and (iii) to examine the underlying alterations associated with individual mutation steps.

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MATERIALS AND METHODS

Antibiotics. Antibiotics were kindly supplied by the indicated manufacturers: ampicillin (AMP), CIP, and Bay Y 3118 (BAY), Bayer AG, Wuppertal, Germany; cefoxitin (COX) and NOR, Merck Sharp & Dohme, Munich, Germany; enoxacin (ENO), Parke-Davis, Berlin, Germany; fleroxacin (FLX), Hoffmann-LaRoche, Basel, Switzerland; nalidixic acid (NAL), Sterling-Winthorp, Guildford, United Kingdom; ofloxacin (ofx), Hoechst AG, Frankfurt, Germany; pefloxacin (PFX) and sparfloxacin (SPA), Rhône-Poulenc-Rorer, Cologne, Germany; and temafloxacin (TFX), Abbott, Laboratories, North Chicago, Ill. Chloramphenicol (CLM) was purchased from Boehringer, Mannheim, Germany, and kanamycin (KAN) was purchased from Life Technologies, Eggenstein, Germany.

Biochemicals, chemicals, and media. Unless otherwise stated, biochemicals were from Boehringer or New England Biolabs, Schwalbach, Germany. Chemicals were from Merck, Darmstadt, Germany, or Sigma, Munich, Germany. Media were purchased from Merck; Mueller-Hinton broth, however, was purchased from Difco, Detroit, Mich.

***E. coli* K-12 strains.** The *E. coli* K-12 strains used in the study were JM83 (66), DH5α (Life Technologies), and JF701 (*ompC* mutant) (8). The clinical isolate *E. coli* 205096 has been described previously (26, 26a). It was isolated from the urine of a hospitalized patient and was obtained from C. Krasemann. An *E. coli* wild-type (WT) strain was isolated from a stool specimen from a healthy volunteer who had never received quinolone therapy.

Plasmids. Plasmids pBP514 (*gyrA*$^+$) and pBP547 (*gyrB*$^+$) have been described recently (26). Plasmid pSPORT1 was purchased from Life Technologies.

Isolation and manipulations of DNA. Isolation and manipulations of DNA were done by standard procedures described by Sambrook et al. (54) and Ausubel et al. (4).

Cloning and DNA sequencing of a PCR product carrying the QRDR of the *gyrA* gene. A 740-bp fragment carrying the 5′ end of the *gyrA* gene (nucleotides −249 to 491) was amplified by PCR. PCRs were performed in 10 mM Tris-HCl (pH 8.3)–1.5 mM $MgCl_2$–50 mM KCl–0.01% gelatin containing 100 μmol (each) of the deoxynucleotide triphosphates (Boehringer), 2.5 U of *Taq* DNA polymerase (Life Technologies), 20 pmol each of primers PCR5-4 and PCR3-1 (26), and 500 ng (each) of chromosomal DNAs of the WT strain and mutants MI, MII, MIII, MIVa, MIVb, and R17 in a total volume of 100 μl per reaction mixture. Thirty cycles with the following temperature profile were run for each reaction: 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. Each amplification reaction yielded a single DNA fragment of the identical size (740 bp), and this fragment was purified by using Qiaquick spin columns (Diagen, Hilden, Germany). Via a *Bam*HI site and an *Aat*II site introduced with primers PCR5-4 and PCR3-1, respectively, the PCR fragments were cloned separately into the *Bam*HI and *Aat*II sites of plasmid pSPORT1. After transformation of competent DH5α cells, recombinant clones were identified by blue-white screening on AMP-containing agar plates. Two single colonies from each transformation were taken for plasmid DNA isolation. Each plasmid was sequenced by the chain termination technique (55) by using Sequenase 2.0 (United States Biochemicals, Bad Hamburg, Germany) and either primer A or primer B (26). Primers were synthesized on a PCR-mate 391 synthesizer (Applied Biosystems, Weiterstadt, Germany).

Susceptibility testing. Susceptibility testing was done by following the guidelines of the National Committee for Clinical Laboratory Standards (44) and by using unsupplemented Mueller-Hinton broth. The susceptibilities of the cells carrying either plasmid pBP514 or plasmid pBP547 were determined as the MICs for single cells essentially as described previously (26a).

Identification of *E. coli* strains. The WT *E. coli* strain and the subsequently isolated mutants were identified biochemically by using the API 20E identification system.

Accumulation of CIP. The accumulation of CIP was measured as described previously (24). Briefly, the cells were grown in standard broth no. I (NI broth) (Merck) at 30°C to the mid-log phase (A_{570} = 0.7). CIP was added to a final concentration of 100 μg/ml, and the cells were incubated at 30°C with aeration. At different times (30, 90, 180, 600, and 1,200 s), eight samples of 1 ml each were withdrawn and placed in prechilled tubes containing 0.5 ml of silicon oil (final density, 1.0012 g/ml at 20°C; one-volume AK100, two-volume AR200; Wacker Chemie, Munich, Germany), and the tubes were centrifuged immediately at 13,000 × *g* for 120 s at 4°C. The supernatant was discarded and the pellets were dried overnight in the dark. The dried pellets were resuspended in 1.0 ml of 0.1 M glycine hydrochloride (pH 3.0), and the mixture was incubated for >90 min at room temperature. The samples were centrifuged again, and the supernatant was obtained for determination of the intracellular CIP concentration by a fluorimetric assay described by Chapman and Georgopapadakou (9); the result was compared with a standard curve obtained with 0.02 to 0.2 μg of CIP per ml in 0.1 M glycine hydrochloride (pH 3.0). Emission at 445 nm was measured with excitation at 276 nm by using a Perkin-Elmer 1000 fluorescence spectrophotometer. The experiments were done in duplicate, and the data are expressed as the mean of the data obtained from all assays (standard deviation, ≤10%). Protein levels were measured by the method of Stickland (59) by using bovine serum albumin as the standard. Assuming a protein content of 0.165 g of protein per g of cells (wet weight) and a cellular density of 1.0 g/ml (45), the results were calculated and expressed as nanograms of CIP per ml.

OMPs. OMPs were isolated by the method of Piddock et al. (52) and were separated on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel. The protein content was determined as described previously (41).

Selection of quinolone-resistant mutants. Selections of quinolone-resistant mutants were performed in liquid culture and on agar plates. For the selection in liquid culture, 1 ml of an overnight culture of the WT isolate was diluted into 24 ml of NI bouillon containing NAL (in the first selection step) or CIP (in the following selection steps) at four different concentrations (ranging between 1× and 16× the MICs), and the solutions were incubated under aeration at 37°C overnight (one passage). Cultures which showed visible growth were diluted 1:25 into fresh NI bouillon containing the same selec-

TABLE 1. Stepwise selection of quinolone resistant mutants

Step	No. of cells plated	Selecting agent (concn [μg/ml])	No. of colonies	Mutation frequency[a]	Clone	MIC (μg/ml)			
						NAL	CIP	CLM	COX
					WT	4	0.015	16	8
I	2.0×10^{11}	NAL (128)	190	9.5×10^{-10}	I	512	0.5	16	8
II	4.2×10^{11}	CIP (4)	625	1.5×10^{-9}	II	ND[b]	2	32	16
III	5.4×10^{10}	CIP (16)	33	6.2×10^{-10}	III	ND	64	64	16
IV	1.2×10^{11}	CIP (256)	249	2.1×10^{-9}	IVa	ND	128	64	16
					IVb	ND	256	128	16

[a] The mutation frequencies were calculated by dividing the number of CFU per milliliter selected on agar plates containing the respective selecting agent in the concentration indicated by the number of CFU per milliliter plated in total.
[b] ND, not determined.

tion concentration for three additional passages. Simultaneously, single colonies isolated after each passage were taken for the determination of the susceptibilities to CIP, CLM, COX, and NAL.

For the selection on agar plates, about 10^{11} cells were plated on China blue-lactose agar containing NAL (in the first selection step) or CIP (in the following selection steps) at four different concentrations (between 2× and 64× the MICs). Single colonies from each selection step were purified on agar plates containing the appropriate quinolone concentration. Purified single colonies were taken for the determination of the susceptibilities to CIP, CLM, COX, and NAL. For the subsequent selection step, four individual clones were chosen.

Doubling times. Cells from a stationary-phase culture were diluted 1:100 in NI broth and were grown under aeration at 37°C. Every 15 min a sample of 0.5 ml was withdrawn and was serially diluted 10-fold. Aliquots of 50 μl each of the last four dilutions were plated onto NI agar plates. After incubation at 37°C for 24 to 48 h, the viable cell count was determined. Doubling times were determined graphically from the linear part of a semilogarithmic plot of the viable cell count against time. Experiments were performed in duplicate, and the doubling times are given as the means of both determinations. The differences between two experiments were <10%.

RESULTS

Stepwise selection in vitro of fluoroquinolone-resistant mutants of *E. coli*. The mutation frequencies, the selecting agent and its concentration, and the susceptibilities to different drugs for a set of four mutants that were obtained in steps I to IV (MI, MII, MIII, and MIVa or MIVb, respectively) and that were subsequently selected on agar plates are summarized in Table 1. The mutation frequencies of the different steps ranged between 5×10^{-10} and 2×10^{-9}. Mutation step I resulted in an increase in the MICs of all quinolones by 8- to 32-fold, and mutation step III resulted in an increase in the MICs of all quinolones except PFX and ENO by 16- to 64-fold (the MICs of PFX and ENO increased by ≥64-fold). Mutation steps II and IV had less dramatic effects, with MIC increases of fourfold or less (Table 2). One exception involved mutant MIVa, for which the MICs were equal to (FLX, NFX, SPA, TFX) or even less than (BAY, OFX, PFX) those for the preceding mutant, mutant MIII. The MIC of CLM for mutants MII, MIII, and MIVb increased by 1 dilution step each, while the MIC of COX was for MII and the following mutants elevated by only 1 dilution step.

Mutant R17, which was selected after serial passage in liquid culture, is similar to mutant MIVb with respect to the pattern of quinolone MICs except for the MICs of SPA and TFX, while the MICs of all quinolones for clinical isolate 205096 differed significantly from those for all mutants selected in vitro (Table 2). For 205096, the MICs of CLM and COX were comparable to those of the unrelated WT strain (data not shown).

Identification of gyrase mutations in the different mutants. Dominance tests were performed by introducing either plasmid pBP514 (*gyrA*$^+$) or plasmid pBP547 (*gyrB*$^+$) into the cells. As shown in Table 3, the transfer of pBP547 did not significantly alter the susceptibilities of any clone to CIP or NAL. Transfer of pBP514 did not alter the quinolone susceptibilities of the WT strain but had a marked effect on all mutants (Table 3). Mutant MI carrying pBP514 was as susceptible as the WT strain to CIP and NAL (complete dominance), while the MICs of CIP and NAL for mutants MII to MIVb and mutant R17 were increased by 4- to 16-fold compared with those for the WT strain (partial dominance). However, transfer of pBP514 to mutant MIII decreased the MICs of CIP and NAL to the level of those for the preceding mutant MII (complete dominance).

TABLE 2. Quinolone susceptibilities of *E. coli* WT, its mutants, and clinical isolate 205096

Strain	MIC (μg/ml)								
	CIP	BAY	ENO	FLX	NOR	OFX	PFX	SPA	TFX
WT	0.015	0.015	0.25	0.25	0.125	0.125	0.125	0.06	0.06
I	0.5 (32)[a]	0.125 (8)	2 (8)	2 (8)	1 (8)	1 (8)	2 (16)	0.5 (8)	1 (16)
II	2 (4)	0.5 (4)	8 (4)	8 (4)	4 (4)	4 (4)	8 (4)	2 (4)	4 (4)
III	64 (32)	16 (32)	512 (64)	128 (16)	256 (64)	128 (32)	2,048 (≥256)	64 (32)	128 (32)
IVa	128 (2)	8 (−2)	128 (−4)	128 (0)	512 (1)	64 (−2)	1,024 (−2)	64 (0)	128 (0)
IVb	256 (4)	16 (0)	512 (0)	1,024 (8)	1,024 (2)	256 (2)	2,048 (0)	256 (4)	1,024 (8)
R17	256	8	512	1,024	1,024	256	2,048	128	256
205096	64	2	512	128	512	32	512	8	16

[a] The numbers in parentheses indicate the fold changes in the MIC for a strain and its immediate parent.

TABLE 3. Results of the dominance test with plasmids pBP514 (*gyrA*$^+$) and pBP547 (*gyrB*$^+$)

Strain	Gene introduced	MIC (μg/ml) for a single cell[a]	
		CIP	NAL
WT		0.015	4
WT	*gyrA*$^+$	0.008	2
WT	*gyrB*$^+$	0.008	4
I		0.5	512
I	*gyrA*$^+$	0.015	2
I	*gyrB*$^+$	1	512
II		2	2,048
II	*gyrA*$^+$	0.06	16
II	*gyrB*$^+$	1	2,048
III		64	2,048
III	*gyrA*$^+$	0.125	32
III	*gyrB*$^+$	64	2,048
IVa		128	2,048
IVa	*gyrA*$^+$	0.125	16
IVa	*gyrB*$^+$	128	2,048
IVb		256	2,048
IVb	*gyrA*$^+$	0.25	32
IVb	*gyrB*$^+$	256	2,048
R17		256	2,048
R17	*gyrA*$^+$	0.125	32
R17	*gyrB*$^+$	256	2,048
205096		64	1,024
205096	*gyrA*$^+$	0.015	2
205096	*gyrB*$^+$	64	1,024

[a] Susceptibilities were determined as the MICs of CIP and NAL for single cells (26a).

The DNA sequences of the *gyrA* genes determined for the WT strain and all mutants extended from nucleotides 75 to 430, including the QRDR (nucleotides 199 to 319), which was sequenced from both strands. The DNA sequences of the cloned PCR fragments from two individual transformants of each cloning experiment were identical. A comparison of the DNA sequences of the different mutants with that of the WT strain revealed a Ser-83→Leu mutation in mutants MI and MII and a double mutation, Ser-83→Leu in combination with Asp-87→Gly, in mutants MIII, MIVa, MIVb, and R17 (Fig. 1). Compared with the DNA sequence of *E. coli* K-12, all mutants and the WT strain contained two silent mutations at nucleotides 255 (C→T) and 267 (T→C) in the region between residues 80 and 90 (Fig. 1).

OMP profiles. To investigate if partial dominance is associated with non-*gyrA* mutations like alterations in the OMP profile, the OMPs of the WT strain and all mutants were isolated and separated electrophoretically. The results shown in Fig. 2 indicate that a protein that comigrates with OMP F is present in the WT and mutant MI but is absent from mutants MII to MIVb and R17. The OMP profile of clinical strain 205096 differed from that of the WT strain and all other mutants by the lack of bands that comigrated with OMP F and OMP A and the appearance of a new band that migrated between OMP F and OMP A; the new band had an apparent molecular size of about 36 kDa (data not shown).

Accumulation of CIP. The results of CIP accumulation

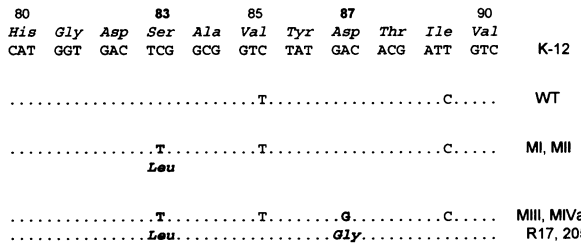

FIG. 1. DNA sequence of the QRDR of the *gyrA* genes from *E. coli* K-12, WT, its mutants (MI to MIVb and R17), and clinical isolate 205096. Nucleotides 238 to 270 of the *gyrA* gene coding for amino acids 80 to 90 from *E. coli* K-12 (K-12) are shown (61). Of the corresponding region in the *gyrA* genes from the WT strain, its mutants (MI, MII, MIII, MIVa, MIVb, R17), and clinical isolate 205096 (26), only those nucleotides which differ from those in the sequence of *E. coli* K-12 are shown. Of these mutations, those resulting in an amino acid change are indicated as boldface letters. The amino acid exchanged is given below the base.

experiments are shown in Fig. 3. CIP accumulation followed a biphasic kinetic; after a rapid initial phase, concentrations close to the steady-state level were reached within 5 min. The highest steady-state level after 20 min was obtained for the WT strain, while the level for mutant MI was slightly reduced (85% that for the WT strain). For all other mutants, the corresponding levels ranged between 35% (MII) and 24% (R17) of that for the WT. The level for clinical isolate 205096 (36% that for the WT) was similar to that for mutant MII.

Growth characteristics of the WT and the different mutants. During the selection procedure, differences in the time required to detect visible colonies were apparent between the WT strain and the late mutants MIII, MIVa, and MIVb and mutant R17. For a quantification of these differences, the doubling times were calculated from growth curves. The doubling times of the WT, MI, MII, MIII, MIVa, MIVb, R17, and 205096 were 25, 26, 27, 35, 40, 127, 37, and 21 min, respectively. Mutants MI and MII showed doubling times comparable to that of the WT; the doubling times of mutants MIII, MIVa, and R17 were slightly longer (by about 40 to 60% that of the WT). Mutant MIVb showed a significantly longer doubling time (500% that of the WT). In contrast, the doubling time of clinical isolate 205096 was even shorter (by 20% that of the WT).

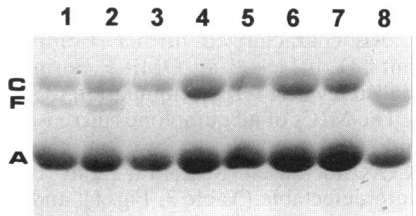

FIG. 2. OMP profiles of WT, its mutants (MI to MIVb, R17), and JF701 (an *ompC* mutant). OMPs were isolated from the WT (lane 1), its mutants MI (lane 2), MII (lane 3), MIII (lane 4), MIVa (lane 5), MIVb (lane 6), R17 (lane 7), and strain JF701 (ompC; lane 8). About 15 μg of protein was loaded onto each lane and protein was separated on an 12% SDS–polyacrylamide gel. The bands were stained with Coomassie brilliant blue R250. The positions of proteins OMP F (F), OMP C (C), and OMP A (A) are indicated.

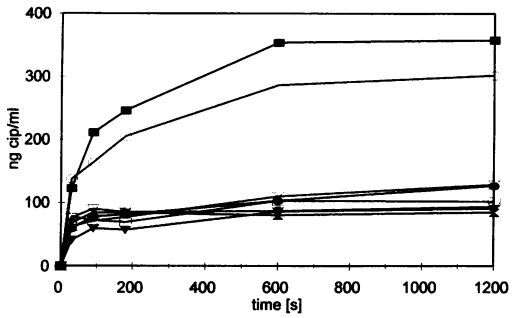

FIG. 3. Accumulation of CIP. CIP accumulation was determined as described in the text and is given as concentration in nanograms of CIP per milliliter. ■, WT; ○, MI; ●, MII; □, MIII; ▼, MIVa; ▽, MIVb; ⧗, R17; ⧖, 205096.

DISCUSSION

By exposing a randomly chosen isolate of *E. coli* (WT) to increasing concentrations of quinolones, mutants highly resistant to fluoroquinolones (CIP MIC, up to 256 μg/ml) could be selected in vitro in liquid culture (mutant R17) and on solid medium (mutants MI to MIVb) (Table 1). By use of the latter procedure, mutation frequencies of 10^{-9} to 10^{-11} for individual steps were determined.

The first of these consecutive mutants (MI) resembled *gyrA* mutants in two features: (i) a simultaneous increase in the MICs of all quinolones (parallel resistance; Table 2) (67) and (ii) complete dominance after introduction of the *gyrA*$^+$ gene (Table 3) (23). Loss of the plasmid carrying the *gyrA*$^+$ gene during susceptibility testing is one limitation of the dominance test (26a, 57). Therefore, the quinolone susceptibility was determined as the MIC for a single cell (26a). DNA sequencing revealed a C→T mutation at nucleotide 248 in the QRDR of the *gyrA* gene resulting in a Ser-83→Leu exchange (mutant MI). The accumulation of CIP (Fig. 3) and the doubling time of MI resembled those for the WT, with slight differences.

Alterations of Ser-83 result in the most effective increase in the quinolone MIC and are the most frequent cause of quinolone resistance in mutants of *E. coli* selected in vitro or isolated from patients receiving quinolone therapy (11, 48, 57, 67, 68). In *Staphylococcus aureus* and *Campylobacter jejuni*, quinolone resistance is also associated with alterations of the corresponding residues Ser-84→Leu and Thr-86→Ile, respectively (58, 64). These findings together point to the central role of the Ser-83 residue (or its homologs) in the development of quinolone resistance.

In the second selection step, only mutants for which CIP MICs were ≤4 μg/ml could be selected. One of these mutants, mutant MII, was characterized further. Compared with its parent, mutant MI, it lacked an OMP F porin (Fig. 2) and showed a reduced level of CIP accumulation (35% that of the WT) (Fig. 3). The MICs of all quinolones increased by fourfold (Table 2), and simultaneously, those of unrelated drugs like CLM and COX increased twofold (Table 1). No gyrase alterations were detectable (Table 3; Fig. 1), and the doubling times were similar for the WT strain and mutant MI. Phenotypically, the mutant obtained in this second mutation step resembles mutants with the *cfxB* or the *nfxC* mutation, which have been shown to be alleles of *marA* (33, 34). Like MII, mutants with these mutations have reduced levels of OMP F porin and a reduction in the accumulation of CIP (to about 35% of that of their parent). Recently, another two-step mutant of *E. coli* similar to mutant MII has been selected in vitro; it carries an unidentified gyrase mutation in combination with a permeation mutation that mediates resistance to multiple quinolones and unrelated drugs (51). Thus, data from the present study are in agreement with those of previous studies demonstrating that *E. coli* mutants showing clinically relevant levels of resistance to fluoroquinolones can be selected in vitro in only two mutation steps. However, because of the favorable pharmacokinetic properties of these drugs, their concentrations at the site of infection can exceed the levels in serum severalfold (6, 30). This might explain the generally low frequency of fluoroquinolone resistance in clinical isolates of *E. coli* seen in international studies (14, 40). The increasing incidence of fluoroquinolone resistance in some areas may be due to a high degree of selective pressure (1, 50).

Like mutant MI, the third-step mutant, mutant MIII, showed characteristics of mutants with a *gyrA* mutation (cross-resistance to all quinolones tested) (Table 2) and complete dominance after transfer of the *gyrA*$^+$ gene in comparison with mutant MII but partial dominance in comparison with the WT strain (Table 3). With respect to the latter finding, mutant MIII differed from mutants with the *nfxD* mutation described recently (57): the *nfxD* phenotype, i.e., four- to eightfold increase in the NOR MIC, was detectable only in *gyrA* mutants, but not in strains with the *gyrA*$^+$ background. Additionally, in the presence of a plasmid-encoded *gyrA*$^+$ gene, a *gyrA nfxD* mutant retains wild-type susceptibility, indicating complete dominance.

DNA sequencing revealed an A→G mutation at nucleotide 260 of the *gyrA* gene of mutant MIII, resulting in an Asp-87→Gly exchange (Fig. 1). This mutation, which has been detected recently in combination with a Ser-83→Leu mutation in clinical isolate 205096 (26), has not previously been selected as a single-step mutation. However, introduction of this mutation into the chromosomal *gyrA* gene of *E. coli* K-12 by gene replacement yielded a mutant for which MICs to quinolones were increased (25). Thus, the Gly-87 mutation resembles other *gyrA* mutations like Leu-83 and is likely to contribute to the resistance of MIII to CIP. Compared with MII, the level of CIP accumulation (Fig. 3) and the OMP profile of MIII were not altered (Fig. 2). The generation time was increased slightly (by about 40%), indicating an impaired viability of MIII under in vitro conditions. It is tempting to speculate that viability is affected by the combination of three mutations in MIII; however, isolate 205096, which, in comparison with mutant MIII, has the identical double mutation in *gyrA*, a comparable reduction in the level of CIP accumulation, and an altered OMP profile, has a doubling time comparable to that of the WT. Although no data indicating impaired viability because of *gyrA* mutations at Ser-83 or Asp-87 are available, some low-level quinolone-resistant mutants exhibiting alterations in supercoiling have been described previously (2). This implies that certain, not further characterized *gyrA* mutations might by themselves affect the growth rate because of an altered enzyme function.

Four-step mutants MIVa and MIVb were isolated from mutant MIII at a frequency of 10^{-9}. In contrast to their precursors, the MICs of only some quinolones (including CIP) for these mutants increased, while those of other quinolones decreased or were unchanged (Table 1). Incomplete cross-resistance was described for the *norC* mutation affecting the composition of the lipopolysaccharide (29). No additional mutation in the QRDR of *gyrA* was detectable (Fig. 1), which is in agreement with the results of the dominance test (Table 3). Parameters concerning cell wall-associated alterations in mutants MIVa and MIVb, i.e., the loss of OMP F porin (Fig. 2) and a reduction in the level of CIP accumulation (Fig. 3),

were comparable to those in MIII. In contrast, a dramatic increase in the growth rate of MIVb (500% that of the WT) was detectable, while the growth rate of mutant MIVa was similar to that of MIII. This increased growth rate could also be detected by measuring the time required to form visible colonies of MIVb on agar plates or to enhance the optical density of a liquid culture.

Mutant R17, which was selected in liquid culture by exerting the same selective pressure (increasing quinolone concentrations), was nearly identical to mutant MIII with respect to all of the characteristics examined in the present study (MIC, *gyrA* mutations in the QRDR, OMP profile, accumulation of CIP, doubling time). However, the selection technique that was applied did not allow the determination of the order of the individual mutation steps. The sequence of mutations (MI to MIV) might be influenced by the use of NAL instead of CIP in the first step; CIP is a hydrophilic quinolone which enters the cells of gram-negative bacteria mainly via the OMP F porin, while NAL, a hydrophobic quinolone, is believed to penetrate the lipid layer of the outer membrane directly (28). Therefore, CIP might favor the selection of a permeability mutant with reduced amounts of OMP F in the first step, while NAL could favor the selection of *gyrA* mutants. As pointed out earlier in this report, *gyrA* mutations, especially those around Ser-83, can be selected in vivo in one step and seem to play a major role for quinolone resistance in clinical isolates of *E. coli*. Therefore, starting the selection in vitro with a *gyrA* mutant probably reflects the situation encountered in vivo. Nevertheless, the possibility that these selection conditions place constraints on what other mutations may be compatible in subsequent selections cannot be excluded.

Included in the present examination was a clinical isolate of *E. coli*, 205096, with high-level resistance to fluoroquinolones (26a). A previous study revealed a double mutation in *gyrA* (Leu-83 and Gly-87) in this strain. Additionally, the CIP concentration necessary to inhibit by 90% the supercoiling activity of DNA gyrase reconstituted from a recombinant subunit A carrying this double mutation and a B subunit from a WT strain was more than 4,000-fold greater than that of gyrase from a WT strain (26). Several additional findings strongly suggested that this double mutation is responsible for the high level of fluoroquinolone resistance: (i) the difference in the 90% inhibitory concentrations correlated well with the difference in the MIC of CIP, (ii) the introduction of a $gyrA^+$ gene restored susceptibility identical to that of the WT strain (complete dominance; Table 3), (iii) introduction of the *gyrB* gene did not alter the quinolone susceptibility, (iv) the susceptibility to CLM, which was decreased in mutants like *marA* showing reduced amounts of OMP F porin, was not altered in 205096 compared with that in susceptible WT strains, and (v) CIP accumulation after 20 min was comparable but not identical to that in a randomly chosen quinolone-susceptible isolate of *E. coli*. However, in the present study, reexamination revealed a reduction in the CIP accumulation to 35% of that of the WT. Intrinsically varying levels of quinolone accumulation because of variations in the outer membrane composition or efflux systems with different activities together with variations of up to ±15% for individual determinations (42) may account for most of the observed differences in the CIP accumulation of the two quinolone-susceptible WT isolates. Since the susceptible parent of 205096 was not available, the importance of the reduced level of CIP accumulation compared with that of the WT and the unique OMP profile of 205096, which lacked an OMP F band, for quinolone resistance remains to be determined.

E. coli 205096 differs from all mutants selected in the present study by at least two parameters. The most likely candidate for a counterpart is mutant MIII. However, for MIII the MICs of some quinolones were enhanced (Table 2), and it had a different OMP profile (Fig. 2) and a prolonged doubling time. This indicates decreased viability under laboratory conditions. Since this effect was not found in mutant MII, it is tempting to speculate that the double mutation in *gyrA* affects not only the structural features of the cellular target for quinolone action but also the enzymatic function of gyrase. A functional gyrase is essential for maintaining in balance the degree of DNA supercoiling within the cell (for a review, see reference 17). Changes in the degree of supercoiling result in pleiotropic effects on the expression of several unrelated genes (20, 27, 47). Major changes may also be deleterious to the cell and, thus, may require compensatory mutations. Such mutations have been detected previously and include mutations in genes coding for topoisomerases type I, *topA* (46, 49), and type II, *gyrA* and *gyrB* (2, 12), or genes coding for histone-like DNA-binding proteins like H-NS (15, 36) and HU (15) or *tolC* (13). *E. coli* 205096 might have acquired such a compensatory mutation which would restore its viability. Consequently, *gyrA* double mutants should be not viable in the absence of a compensatory mutation. This is consistent with the fact that a *gyrA* double mutant has not yet been selected in a two-step procedure, despite the finding that each mutation in *gyrA* occurred at a frequency of about 10^{-9} to 10^{-10} (Table 1). The conditions required for the selection of fluoroquinolone-resistant mutants in vitro (i.e., increasing amounts of a fluoroquinolone and growth in aerated enriched broth) presumably differ from the conditions required in vivo (i.e., changing fluoroquinolone concentrations, low oxygen availability, and the presence of limited nutrients), resulting in different compensatory mutations adapted to the respective environment. Assuming that only one such compensatory mutation is required to isolate *gyrA* double mutants, an inoculum of at least 10^{27} cells is necessary.

Further analysis of the mutants described in this report is in progress to reveal the nature of such presumed compensatory mutations.

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