Biological Cost of Single and Multiple Norfloxacin Resistance Mutations in *Escherichia coli* Implicated in Urinary Tract Infections

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Resistance to fluoroquinolones in urinary tract infection (UTIs) caused by *Escherichia coli* is associated with multiple mutations, typically those that alter DNA gyrase and DNA topoisomerase IV and those that regulate AcrAB-TolC-mediated efflux. We asked whether a fitness cost is associated with the accumulation of these multiple mutations. Mutants of the susceptible *E. coli* UTI isolate Nu14 were selected through three to five successive steps with norfloxacin. Each selection was performed with the MIC of the selected strain. After each selection the MIC was measured; and the regions of *gyrA*, *gyrB*, *parC*, and *parE*, previously associated with resistance mutations, and all of *marOR* and *acrR* were sequenced. The first selection step yielded mutations in *gyrA*, *gyrB*, and *marOR*. Subsequent selection steps yielded mutations in *gyrA*, *parE*, and *marOR* but not in *gyrB*, *parC*, or *acrR*. Resistance-associated mutations were identified in almost all isolates after selection steps 1 and 2 but in less than 50% of isolates after subsequent selection steps. Selected strains were competed in vitro, in urine, and in a mouse UTI infection model against the starting strain, Nu14. First-step mutations were not associated with significant fitness costs. However, the accumulation of three or more resistance-associated mutations was usually associated with a large reduction in biological fitness, both in vitro and in vivo. Interestingly, in some lineages a partial restoration of fitness was associated with the accumulation of additional mutations in late selection steps. We suggest that the relative biological costs of multiple mutations may influence the evolution of *E. coli* strains that develop resistance to fluoroquinolones.

Treatment of urinary tract infections (UTIs) is a major community indication for antibiotic usage (10, 14, 26) and affects 40 to 50% of women at some point during their lifetimes. *Escherichia coli* is the cause of more than 80% of community-acquired cases of uncomplicated UTIs (20). Fluoroquinolones are potent broad-spectrum antibacterial agents that are frequently used for the treatment of UTIs (14, 26, 27). Fluoroquinolones bind to DNA gyrase (topoisomerase II) and/or DNA topoisomerase IV and inhibit their activities in the control of DNA unwinding and supercoiling (25).

The management of UTIs is complicated by increasing rates of antimicrobial resistance. In the case of fluoroquinolone resistance among *E. coli* isolates, important mechanisms of resistance include mutations that reduce the affinity for the antibiotic targets, DNA gyrase and/or DNA topoisomerase IV (12, 13, 23, 31, 33, 34), and mutations that reduce fluoroquinolone accumulation in the cell, either by decreased uptake through outer membrane porins or by increased expression of drug efflux systems (1, 2, 29, 30, 32, 36). Target alterations due to mutations in the *gyrA*, *gyrB*, *parC*, and *parE* genes have been described, most of which are located in a small region of either *gyrA* or *parC*, known as the quinolone resistance-determining region.

Individual resistance mutations isolated experimentally confer only low-level resistance to fluoroquinolones. Experimental evidence from in vitro selection studies shows that higher-level fluoroquinolone resistance can be built up successively through the accumulation of several independently acquired mutations, including both target and efflux mutations. Similarly, evidence from studies with clinical isolates supports the conclusion that higher-level resistance is associated with multiple resistance mutations (23). This raises the question of whether the accumulation of multiple mutations is associated with a fitness cost for the resistant bacteria. For several other antibiotic resistance phenotypes, it has been shown that single mutations are frequently associated with fitness costs both in vitro and in vivo (5, 6, 28). Accordingly, we selected several lineages of *E. coli* for reduced susceptibility to successively higher levels of the fluoroquinolone norfloxacin and measured the associated changes in fitness. The exposure of bacteria to low levels of antibiotic has been postulated to be a significant factor in the development of clinical resistance (3, 4, 41). To mimic this situation we made each selection at the MIC of the selected strain.

MATERIALS AND METHODS

Bacterial strains. *E. coli* cystitis isolate Nu14 (15, 23, 24) was used for the in vitro selection of norfloxacin-resistant mutants. Nu14 (15, 24) is susceptible to norfloxacin (Etest MIC = 0.094 μg/ml) and to other antibiotics, including nalidixic acid, ciprofloxacin, tetracycline, chloramphenicol, amdinocillin, and trimethoprim (Table 1). *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213,

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TABLE 1. Genetic identification, MIC, and in vitro fitness of first-step mutants

Strain	Mutation[a]	MIC (μg/ml)[b]						Fitness[c]
		NOR	CIP	NAL	TET	CHL	MEC	
Nu14	Wild type	0.094	0.016	1.5	0.75	3	0.064	1.000
Nu98	gyrA D82G (1)[d]	0.38	0.047	16	0.75	3	0.064	ND
Nu118	gyrA S83A (1)	0.25	0.094	16	0.5	2	0.064	1.001
Nu41	gyrA D87Y (1)	0.5	0.19	96	0.75	4	0.047	0.995
Nu79	gyrA D87G (2)	1	0.125	96	0.75	3	0.064	0.999
Nu91	gyrA A119E (1)	0.5	0.125	64	0.75	3	0.047	ND
Nu87	gyrB R389S (1)	0.25	0.032	2	0.75	6	0.094	ND
Nu71	gyrB S464F (1)	0.50	0.094	6	1.5	3	0.064	1.023
Nu141	gyrB S464Y (1)	0.75	0.38	8	1.5	6	0.064	ND
Nu17	gyrB Δ9 nt[e] (2)	0.25	0.064	3	0.75	4	0.064	1.009
Nu96	marR + 1 nt[f] (1)	0.25	0.032	3	1.5	12	0.094	ND
Nu93	marR R77L (1)	0.5	0.064	3	2	12	0.125	0.987
Nu18	marR R94H (2)	0.38	0.064	3	2	24	0.125	0.997
Nu135	marOR Δ13 nt[g] (1)	0.19	0.047	3	1.5	16	0.094	1.003

[a] Six genes or regions (gyrA, gyrB, parC, parE, marOR, and acrR) were sequenced as described in Materials and Methods, resulting in each case in the identification of the one mutation listed. Amino acid substitution mutations, e.g., D82G, are written in the form original amino acid (D), amino acid position (82), mutant amino acid (G).

[b] The MIC is the modal value for at least five measurements made by Etest. MICs for trimethoprim were also measured but were not altered in any mutants (Nu14 trimethoprim MIC, 0.5). NOR, norfloxacin; CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; CHL, chloramphenicol; MEC, amdinocillin.

[c] Fitness is expressed as $1 + S$, the selection coefficient per generation determined in growth competition assays in urine (see Materials and Methods). ND, not determined. The mean standard error of the fitness values is 0.014. Values can be read as proportional to the growth rate of Nu14.

[d] Values in parentheses are the number of independent isolates (from different cultures) with each mutation.

[e] The mutation is a deletion of 9 nucleotides (nt; nucleotides 1427 to 1435) within the gyrB-coding sequence, where nucleotide 1 is the first nucleotide of the initiation codon. The deletion removes the underlined nucleotides from the sequence TGT GGT ATC GGT; and as a result, three amino acids, G477, I478, and G479, are predicted to be lost from the GyrB protein.

[f] Insertion of G into a run of 3 G residues at nucleotides 203 to 205, which results in a predicted reading frame shift and an altered protein sequence from amino acid 70 (A70R) in MarR.

[g] The mutation is a deletion of 13 nucleotides from position −3 to +10, where position 1 is the first nucleotide (underlined) of the marR-coding sequence (GTG). The deleted nucleotides are numbered 1617140 to 1617152 in the E. coli K-12 MG1655 genome sequence (note that the initiation codon of marR [GTG] is incorrectly identified in the genome annotation of MG1655 [GenBank accession number NC_000913] as ATG [codon 21]).

Enterococcus faecalis ATCC 29212, and Pseudomonas aeruginosa ATCC 27853 were used as quality control strains in antibiotic resistance testing.

Media and growth conditions. The liquid medium used for bacterial growth was Luria broth (LB) or sterile filtered urine. Midstream urine, collected from several healthy individuals at different time points during a day, was pooled, centrifuged at $4,000 \times g$, filtered through a 0.2-μm-pore-size filter (Sarstedt, Nümbrecht, Germany), stored at 4°C, and used within 2 weeks. Only urine with a pH of >5 was used in these experiments. The solid medium used was either Luria broth supplemented with 1.5% agar (LA; Oxoid Ltd., Basingstoke, England) or Mueller-Hinton medium (Sigma Aldrich, Stockholm, Sweden). Strains were grown at 37°C, and liquid cultures were incubated without shaking.

Determination of MICs. The MICs of norfloxacin, ciprofloxacin, nalidixic acid, chloramphenicol, tetracycline, amdinocillin, and trimethoprim were determined for each mutant by Etest, according to the instructions of the manufacturer (AB BIODISK, Solna, Sweden). Etests were performed on Mueller-Hinton agar plates, and the plates were incubated for 16 to 18 h at 37°C. In order to check the performance of the Etest with respect to the correct handling of the system, the recommended National Committee for Clinical Laboratory Standards (NCCLS) quality control reference strains for tests with the antibiotics concerned were tested on all occasions (see bacterial strains above). The variations in the Etest results for the American Type Culture Collection (ATCC) strains were measured on at least 10 independent occasions for the antibiotics tested and were always within ±1 concentration step.

Stepwise selection of norfloxacin-resistant mutants. Several independent cultures of the strain to be selected (initially Nu14) were grown overnight in LB. From each culture, approximately 10^8 CFU was spread on LA plates containing, for the first-step selection, norfloxacin at 0.1 μg/ml (the approximate MIC of Nu14). In this initial selection, colonies appeared at a frequency of 5×10^{-8} cells. All subsequent selections were made on norfloxacin at the approximate MIC of the strain being selected. The plates were incubated overnight at 37°C. Colonies were counted, picked, and streaked for the selection of single colonies on the selective medium. After each selection step, mutants were collected (10 from each independent culture) and frozen at −80°C in LB containing 7% dimethyl sulfoxide (Sigma Aldrich). The resistance patterns among the mutants were initially screened by streaking them on LB plates containing different levels of norfloxacin, nalidixic acid, and chloramphenicol. On the basis of the differ-

ences in antibiotic susceptibilities and their independence in origin from one another, a few mutants were chosen after each selection step for further analysis and future selections. Successive stepwise selections were continued for three to five steps.

PCR amplification and DNA sequencing. The oligonucleotide primers used for PCR amplification and DNA sequencing, together with details of the specific regions sequenced in each gene, have been described previously (23). The PCR primers were designed to amplify the quinolone resistance-determining regions (7, 11, 12, 21, 35, 38–40) of gyrA, gyrB, parC, and parE and the whole of the marR and acrR genes. Conditions for PCR amplification and DNA sequencing were as described previously (23).

OST. Tolerance to the organic solvents hexane and cyclohexane is indicative of above-normal efflux by the AcrAB-TolC efflux pump (37). Organic solvent tolerance (OST) was measured in the selected mutants, as described previously (23).

Measurement of fitness in vitro. To determine the biological cost associated with different resistance mutations in vitro, each norfloxacin-resistant mutant was competed against Nu14 in urine. The experiment is a growth competition at 37°C in sterile urine, in which the outcome is evaluated as the change in the ratios of the competing strains as a function of the number of generations. Each competition was performed in triplicate by using independent starting cultures of each of the competing strains. In cases in which one of the outcomes differed significantly from the others, the experiment was performed again. The results are thus an average of at least three independent experiments in each case. Each competition was initiated from overnight cultures grown in urine, diluted in 0.9% NaCl, and mixed at a cell density of ~5,000 CFU/ml in 2 ml urine. The tubes were incubated without shaking overnight (24 h) to complete one cycle of competition (approximately 18 cell doublings). After each competition cycle the mixed culture was serially diluted and ~300 CFU was plated onto LB and LB plus 3 μg/ml nalidixic acid. This level of nalidixic acid was determined empirically to inhibit the growth of Nu14 but to permit the growth of each of the mutants with reduced norfloxacin susceptibility. After overnight incubation at 37°C, the ratio of the number of sensitive Nu14 cells to the number of cells of the resistant mutant was calculated. Successive cycles of growth competition were made by transferring on each day ~5,000 CFU from a competition tube to 2 ml urine and performing another overnight incubation. Up to six successive competition cycles

TABLE 2. MICs of strains selected stepwise for reduced susceptibility to norfloxacin

Lineage and strain (step no.)[b]	MIC (µg/ml)[a]					
	NOR	CIP	NAL	TET	CHL	MEC
Parent (Nu14)	0.064	0.016	1.5	0.75	3	0.064
Lineage 1						
Nu118 (1)	0.25	0.094	16	0.5	2	0.064
PL668 (2)	1	0.25	16	0.75	8	0.094
PL3637 (3)	2	0.38	24	0.25	1.5	0.032
PL3622 (3)	3	0.5	64	0.75	4	0.125
PL2090 (3)	1.5	0.38	256	1	6	0.25
PL2055 (3)	1.5	0.38	256	1	6	0.25
PL787 (2)	0.5	0.125	32	1	8	0.064
PL3665 (3)	1	0.25	32	1.5	8	0.094
PL3687 (3)	1	0.19	32	1.5	8	0.094
Lineage 2						
Nu41 (1)	0.5	0.19	96	0.75	4	0.047
PL213 (2)	1.5	0.5	>256	0.5	4	0.064
PL1660 (3)	6	2	>256	0.75	8	0.125
PL1965 (4)	16	3	>256	1	24	0.19
PL3511 (5)	64	8	>256	0.75	48	0.38
PL3515 (5)	32	3	>256	0.75	16	0.125
PL1710 (3)	8	4	>256	0.75	3	0.064
Lineage 3						
Nu71 (1)	0.5	0.094	6	0.75	4	0.047
PL136 (2)	1.5	0.38	8	1	12	0.094
PL3543 (3)	2	0.38	12	1	12	0.5
PL3547 (3)	3	0.5	8	1	12	0.094
Lineage 4						
Nu17 (1)	0.25	0.064	3	0.75	4	0.064
PL490 (2)	0.75	0.094	3	0.38	2	0.094
PL1270 (3)	1	0.25	64	0.38	3	0.064
PL3563 (4)	4	0.75	>256	1	24	0.125
Lineage 5						
Nu18 (1)	0.38	0.064	3	2	24	0.125
PL845 (2)	2	0.38	>256	3	24	0.125
PL1773 (3)	3	0.75	>256	1.5	12	0.094
PL1801 (3)	4	1	>256	2	64	4
PL1900 (3)	3	0.5	>256	2	24	0.094
PL3425 (4)	4	0.75	>256	2	32	0.094
PL3460 (4)	16	1.5	>256	2	16	0.125
PL858 (2)	0.75	0.125	6	2	32	0.5
PL1385 (3)	4	1.5	>256	2	24	0.5
PL2113 (4)	12	3	>256	2	16	0.38
PL2127 (4)	16	4	>256	1.5	12	0.38
Lineage 6						
Nu135 (1)	0.19	0.047	3	1.5	8	0.094
PL923 (2)	1	0.25	>256	1	8	0.094
PL1570 (3)	1.5	0.38	>256	1	6	0.064
PL1645 (3)	3	0.5	>256	0.75	1.5	1

[a] The MIC for trimethoprim was also measured for all selected strains and showed no significant variation as a function of selection from the value in the starting strain Nu14 (trimethoprim MIC, 0.5). See footnote *b* of Table 1 for definitions of the abbreviations.

[b] The selection step at which a particular mutant was isolated is provided in parentheses.

were made in each experiment. The in vitro selection coefficient (*S*) per generation of each mutant was calculated by linear regression as the slope [(number of mutant cells/number of wild-type cells)/(number of generations/number of cycles)] × ln 2. Relative fitness (with respect to that of the starting strain Nu14) was defined as 1 + *S*.

Measurement of fitness in a mouse UTI model. All strains were checked for type 1 fimbriae by a mannose-sensitive hemagglutination assay (9) prior to in vivo testing. Single-strain infections showed that the individual strains could cause infections. The relative fitness of the wild-type and selected strains was measured in an ascending UTI model in mice by a published protocol (16, 22). Briefly,

wild-type Nu14 and selected norfloxacin-resistant bacteria were cultured overnight on 5% horse blood agar plates (no. 677; SSI Diagnostica, Hillerød, Denmark). One colony from each culture was suspended in 0.5 M phosphate-buffered saline (pH 7.4; no. 3892; SSI Diagnostica) to a cell density of 10^9 to 10^{10} CFU/ml determined with a colorimeter (Sherwood colorimeter 254). Nu14 was mixed with each of the norfloxacin-resistant bacteria in a 1:1 ratio. Infections were made in outbred female albino Ssc-CF1 mice (weight, 30 ± 2 g; Hvidesten, Allerød, Denmark). Anesthetized mice (five mice per competition) were inoculated transurethrally with 0.05 ml of the bacterial suspension by the use of plastic catheters, with 3×10^7 to 5×10^8 CFU/mouse placed into the bladder. Mice

TABLE 3. Mutations selected and associated fitness in *gyrA* lineages

Lineage and strain	Mutation at the following selection step:					Fitness[a]
	1	2	3	4	5	
Lineage 1						
Nu118	*gyrA* S83A					1.001
PL668		*marR* + 14 nt[b]				0.996
PL3637			*nim-1*[c]			0.518
PL3622			*nim-2*			0.512
PL2090			*nim-3*			0.823
PL2055			*nim-4*			0.845
PL787		*marR* + 1 nt[d]				1.000
PL3665			*nim-5*			1.017
PL3687			*nim-6*			1.022
Lineage 2						
Nu41	*gyrA* D87Y					0.995
PL213		*parE* S458P				0.834
PL1660			*marR* + 1 nt[e]			0.688
PL1965				*gyrA* D72G		0.563
PL3511					*nim-7*	0.770
PL3515					*nim-8*	0.568
PL1710			*gyrA* S83L			0.940

[a] Fitness is expressed as 1 + *S*, the selection coefficient per generation determined in growth competition assays in urine (see Materials and Methods). The mean standard error of the fitness values is 0.014. Values can be read as proportional to the growth rate of Nu14.
[b] A 14-nucleotide (nt) duplication (nucleotides 228 to 241) in the *marR*-coding sequence predicted to cause a reading frame shift.
[c] *nim*, nonidentified mutation.
[d] An additional A residue in a run of five A residues (nucleotides [nt] 181 to 185) in the *marR*-coding region predicted to cause a reading frame shift.
[e] An additional G residue in a run of five G residues (nucleotides [nt] 204 to 208) in the *marR*-coding region predicted to cause a reading frame shift from codon 70 onwards.

were killed by cervical dislocation 1, 3, or 7 days after infection. The bladder was removed and homogenized in 0.5 ml sterile 0.5 M phosphate-buffered saline. The bladder suspension was diluted in 0.9% saline (0.9% NaCl; no. 8901; Diagnostica) and plated on LA and on LA plus 3 μg/ml nalidixic acid to distinguish Nu14 from the norfloxacin-resistant mutant. A competition index was calculated as the ratio of the number of mutant cells/number of wild-type cells on day 3. Animal experiments were carried out at Statens Serum Institut, Copenhagen, Denmark, in compliance with institutional and national regulations.

RESULTS

Selection and characterization of first-step mutants. Norfloxacin-resistant colonies were selected from six independent cultures of Nu14. A total of 102 colonies were purified on the selection media and tested for norfloxacin MICs by Etest. On the basis of the increased MICs, 33 mutants were chosen for DNA sequencing of the genes and regions associated with mutations to fluoroquinolone resistance. A single mutation was identified in each of these 33 mutants (Table 1). In all, 13 different mutations were identified: 5 in *gyrA*, 4 in *gyrB*, and 4 in *marOR*. None were identified in *parC*, *parE*, or *acrR*. Each mutant type was subjected to tests for the MICs of several different antibiotics (Table 1). The norfloxacin MICs of the mutants were increased 2- to 10-fold (mean, 4-fold) over that of Nu14, and all mutants showed similar relative increases in

TABLE 4. Mutations selected and associated fitness in *gyrB* lineages

Lineage and strain	Mutation at the following selection step:				Fitness[a]
	1	2	3	4	
Lineage 3					
Nu71	*gyrB* S464F				1.023
PL136		*marR* Δ1 nt[b]			1.030
PL3543			*nim-9*		0.488
PL3547			*nim-10*		0.832
Lineage 4					
Nu17	*gyrB* Δ9 nt				1.009
PL490		*nim-11*[c]			0.868
PL1270			*gyrA* D87G		0.846
PL3563				*marR* Δ113 nt[d]	0.841

[a] Fitness is expressed as 1 + *S*, the selection coefficient per generation determined in growth competition assays in urine (see Materials and Methods). The mean standard error of the fitness values is 0.014. Values can be read as proportional to the growth rate of Nu14.
[b] Deletion of an A residue in a run of two A residues (codons 133 and 134) in *marR* predicted to cause a reading frame shift. nt, nucleotide.
[c] PL490 has an OST phenotype, suggesting that the *nim-11* mutation causes increased drug efflux, although its chloramphenicol MIC was not increased. *nim*, nonidentified mutation.
[d] Deletion of 113 nucleotides (nt; nucleotides −34 to +79 inclusive) spanning the start of *marR*.

TABLE 5. Mutations selected and associated fitness in *marR* lineages

Lineage and strain	Mutation at the following selection step:				Fitness[a]
	1	2	3	4	
Lineage 5					
Nu18	*marR* R94H				0.997
PL845		*gyrA* D87G			0.981
PL1900			*marR* + 1 nt[b]		0.503
PL3425				*nim-12*	0.954
PL3460				*nim-13*	0.965
PL858		*nim-14*			0.869
PL1385			*gyrA* S83L		0.848
PL2113				*nim-15*	0.767
PL2127				*nim-16*	0.835
PL905		*gyrA* D87G			0.964
PL1773			*nim-17*[d]		0.584
PL1801			*nim-18*		0.649
PL1860			*nim-19*		0.869
Lineage 6					
Nu135	*marR* Δ13 nt[c]				1.003
PL923		*gyrA* D87Y			0.994
PL1570			*nim-21*		0.971
PL1645			*nim-22*		0.449

[a] Fitness is expressed as $1 + S$, the selection coefficient per generation determined in growth competition assays in urine (see Materials and Methods). The mean standard error of the fitness values is 0.014. Values can be read as growth rate proportional to the growth rate of Nu14.
[b] An additional G in a run of five G residues (nucleotides [nt] 204 to 208) within the *marR*-coding sequence predicted to cause a reading frame shift.
[c] Deletion of 13 nucleotides (nt; nucleotides −3 to +10 inclusive) spanning the start of the *marR* coding sequence.
[d] *nim*, nonidentified mutation.

the MIC for ciprofloxacin. The MICs for nalidixic acid were also increased, in particular, for *gyrA* mutants. A correlation between single mutations in *gyrA* and high-level resistance to nalidixic acid has been noted previously for clinical UTI isolates (23). The MIC for chloramphenicol was increased in each of the four *marOR* mutants, as expected for efflux mutants (Table 1). OST was also increased in the four *marOR* mutants but not in any of the *gyrA* or *gyrB* mutants, supporting the suggestion that the four *marOR* mutations increase multidrug efflux. The MICs for amdinocillin, trimethoprim, and tetracycline were unaltered in the mutants. The relative fitness of eight of the mutants was measured in vitro (in urine) in growth competition assays against Nu14. None of the mutants showed

TABLE 6. Relative fitness in vitro and in vivo for lineage 2

Lineage and strain	CI[a] in the bladder (in vivo) (SE[b])	Fitness[c] in urine (in vitro)
Lineage 2-1		
Nu41	0.86 (0.22)	0.995
PL213	0.06 (0.02)	0.834
PL1660	0.002 (0.002)	0.688
PL1965	0.04 (0.02)	0.563
Lineage 2-2		
Nu41	0.86 (0.22)	0.995
PL213	0.06 (0.02)	0.834
PL1710	0.74 (0.2)	0.940

[a] CI, competition index, calculated as the ratio of the number of mutant cells/number of wild-type Nu14 cells in mouse bladders 3 days after infection with equal numbers of each competing strain. Competition indices are the mean of the ratios for several bladders (Nu41, $n = 12$; PL213, $n = 6$; PL1660, $n = 4$; PL1965, $n = 7$; PL1710 $n = 5$).
[b] SE, standard error of the mean for the competitions in mice.
[c] Fitness in vitro is $1 + S$ (selection coefficient per generation) calculated from competitions between Nu14 and the mutant strain in urine, as described in Materials and Methods. The mean standard error of the fitness values is 0.014.

any significant deviation in fitness from Nu14 (Table 1). Six mutants, two *gyrA*, two *gyrB*, and two *marR* mutants, were chosen as starting points for subsequent selections and are referred to as lineages 1 to 6, respectively.

MICs, mutations, and fitness in lineages 1 to 6. The genealogies of lineages 1 to 6, together with the corresponding MICs for norfloxacin and other antibiotics, are shown in Table 2. Each of the selected strains listed in Table 2 was subject to DNA sequence analysis of genes and regions associated with reduced susceptibility to fluoroquinolones and to competition experiments against the standard wild-type, Nu14 (Tables 3 to 5). Tables 3 to 5 include details for each of the mutations identified. All strains in which a *mar* mutation was identified had an increased MIC for chloramphenicol and were also positive for OST, supporting the suggestion that they have increased drug efflux. There are several interesting correlations between decreased susceptibility to norfloxacin and changes in susceptibilities to unrelated antibiotics (see Discussion). Fitness in vitro is presented as the growth rate per generation relative to that of Nu14. Each of the six first-step mutants had fitness values very close to the value for parental strain Nu14 (Table 1). Among the nine second-step mutants, six were fit within ±3% of the value for Nu14, close to the standard error of these measurements, while three were less fit, with generation times 13 to 16% slower than those of Nu14. Of 18 third-step mutants, only 3 retained fitness close to that of Nu14 (±2 to 3%), while the remaining 15 mutants had severe or very severe fitness losses, with generation times increased by between 6% and 55% (mean increase in generation time, 31%). The six fourth-step mutants tested all had reduced fitness, with generation times increased by between 4% and 44%. Two fifth-step mutants tested had generation times that increased by 23% and 43%, respectively, relative to that of Nu14.
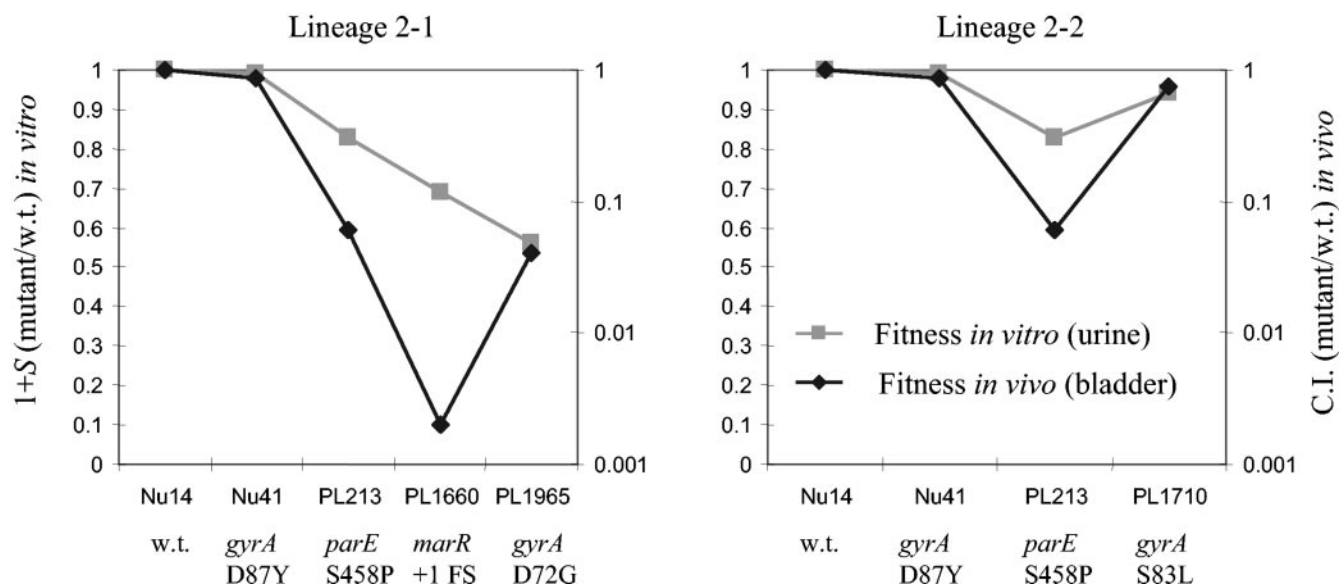
FIG. 1. Fitness costs (and compensation), in vitro (squares) and in vivo (diamonds), associated with the accumulation of mutations causing a loss of susceptibility to norfloxacin. Each successive point on the *x* axis represents a successive selection step, with Nu14 being the parental strain starting point. The new mutation associated with each successive selection step is given on the *x* axis below the strain name. C.I., competition index; w.t., wild type.

Relative fitness of lineage 2 strains in vitro and in vivo. In lineage 2 we identified specific resistance-associated mutations for each of selection steps 1 to 4 and therefore chose lineage 2 for the measurement of relative fitness both in vitro and in vivo. In lineage 2-1 (Table 6; Fig. 1), an increasing loss of fitness in vivo was associated with the first three selection steps, but this was partially reversed by the fourth selection step. In contrast, in vitro the same strains showed a progressive decline in fitness with each of the four selection steps. In lineage 2-2, the pattern both in vivo and in vitro was a small loss of fitness associated with the first selection step, a further loss with the second step, and finally, a partial restoration of fitness associated with the third selection step (Table 6; Fig. 1).

DISCUSSION

Increases in norfloxacin MICs and breakpoints. After three successive selection steps no strain (0 of 16 strains) had crossed the NCCLS-defined breakpoint for resistance to norfloxacin (≥ 16 µg/ml). Resistant strains were selected only after a fourth selection step (three of six strains) and a fifth selection step (two of two strains). This suggests that there is a reservoir of potential genetic alterations capable of causing small reductions in susceptibility to norfloxacin before the current breakpoint is reached.

Cross-resistance to unrelated antibiotics. We found evidence for cross-resistance to other antibiotic classes among the norfloxacin-selected mutants. In lineage 1 third-step mutants PL2090 and PL2055, in which the norfloxacin MIC increased slightly from 1 to 1.5 µg/ml, have an increase in the amdinocillin MIC from 0.094 to 0.25 µg/ml (Table 2). The mutations responsible were not identified. In lineage 2, the second-, third-, fourth-, and fifth-step mutants (mutants PL213, PL1660, PL1965, and PL3511, respectively) showed parallel increases in

the MICs for norfloxacin (1.5, 6, 16, and 64 µg/ml, respectively), chloramphenicol (4, 8, 24, and 48 µg/ml, respectively), and amdinocillin (0.064, 0.125, 0.19, and 0.38 µg/ml, respectively) (Table 2). The mutations associated with these selection steps are in *parE, marR, gyrA,* and *nim-7*, respectively (Table 3). Correlations between decreased susceptibility to norfloxacin and amdinocillin were also found among mutants (mutants PL845 → PL1801, Nu18 → PL858, and PL923 → PL1645) in lineages 5 and 6 (Table 2). The mutations causing these parallel changes in MICs were not identified. These correlations show that selection for decreased susceptibility to a fluoroquinolone may coselect for decreased susceptibility to an unrelated class of antibiotic. One possible explanation is that mutations that affect cell wall biosynthesis and that decrease susceptibility to amdinocillin may reduce the transport of quinolones into the cell.

Genetic locations of resistance mutations. Among 16 independent first-step mutants, we identified one mutation in *gyrA* ($n = 6$), *gyrB* ($n = 5$), and *marOR* ($n = 5$) in each strain (Table 1). Among second-step mutants we identified new mutations in *gyrA* ($n = 3$), *marR* ($n = 3$), and *parE* ($n = 1$) in seven of nine strains. Among third-step mutants we identified mutations in *gyrA* ($n = 3$) and *marR* ($n = 2$) in only 5 of 18 strains. Mutations were also identified in two of six fourth-step mutants, one each in *gyrA* and *marOR*. No mutation was identified in either of the two fifth-step mutants tested. In these last two mutants the entire coding sequences of *gyrA, gyrB, parC,* and *parE* were sequenced, ruling out the possibility that a novel mutation within these coding sequences was responsible for their high norfloxacin MICs. Thus, mutations in genes and regions known to be associated with fluoroquinolone resistance were identified in virtually all (23 of 25) first- and second-step mutants but in less than one-third (7 of 26) of third-, fourth-, and fifth-step mutants. The paucity of mutations identified in the later selec-
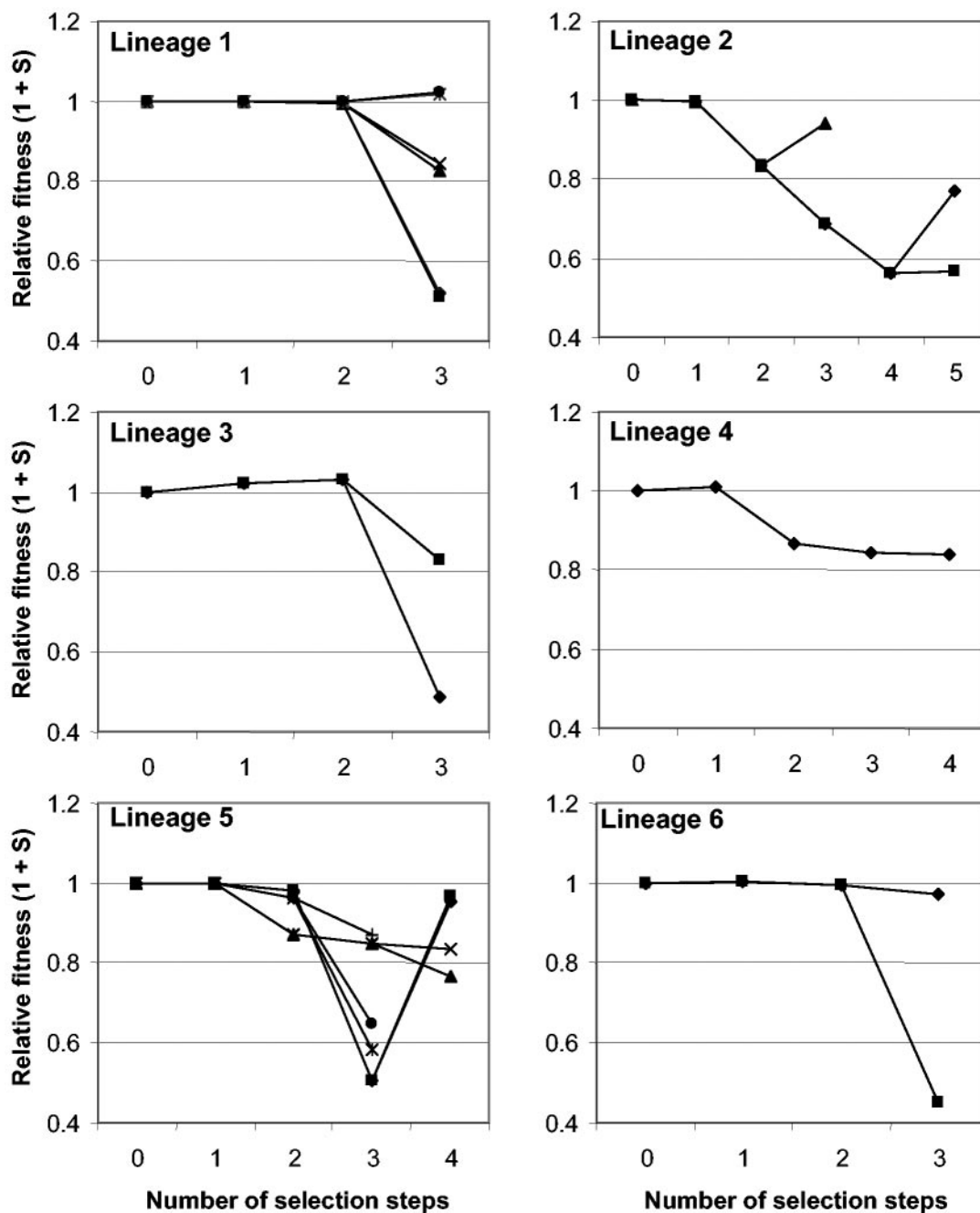
FIG. 2. Graphic representation of the relative fitness in vitro of each of the mutants selected and tested as a function of the number of successive selection steps. Different symbols (squares, diamonds, triangles, etc.) represent different sublineages (Tables 3, 4, and 5).

tion steps suggests that the reservoir of potential mutations capable of reducing susceptibility to fluoroquinolones may be significantly larger than is currently appreciated. Nu14 is refractive to transduction with bacteriophage P1, and so we have not attempted to genetically map these unknown mutations.

Genetic spectrum of norfloxacin resistance mutations. Approximately one-third of the first-step mutations arose in *gyrB*, a class which almost never appears in fluoroquinolone-resistant clinical isolates. A possible explanation for their absence in clinical isolates is that they have a high fitness cost, although

this was not apparent, at least in vitro, among the mutants selected here (Table 1). A second possibility is that *gyrB* mutations are selected only at very low antibiotic concentrations, like those used here, and that in the clinical situation, selection at a high antibiotic concentration is more usual, precluding their emergence. In support of the latter possibility, no *gyrB* mutations were selected after the first selection step. A second feature of the mutation spectrum is the absence of mutations in *parC*. Among the fluoroquinolone-resistant *E. coli* UTI isolates, *parC* mutations were identified in 25 of 30 resistant

isolates (23). One possibility is that the frequency of *parC* mutations may increase when selection is for higher-level resistance and that the selections in this study do not meet this criterion. As defined by the NCCLS breakpoint, only five of the mutants selected here would be defined as resistant to norfloxacin. An alternative possibility is that *parC* mutations arise more frequently in strains that already carry a double mutation in *gyrA*. Thus, among 25 fluoroquinolone-resistant UTI isolates with *parC* mutations, all but 2 also carried double mutations at positions 83 and 87 in *gyrA* (23). Only one of the strains isolated in this study had acquired double mutations in *gyrA* at positions 83 and 87 (PL1710, lineage 2-2; Table 3). A third feature of the mutation spectrum is that mutations in *marR* arose frequently (in all six lineages), whereas no mutations arose in *acrR*, although such mutations are associated with fluoroquinolone resistance in clinical isolates (23, 35, 36). Finally, most mutations selected in the third and subsequent steps were not identified, suggesting the existence of other common classes of mutations that reduce *E. coli* susceptibility to norfloxacin.

Fitness of mutant strains. Our starting strain for lineage selection, Nu14, belongs to the B2 phylogenetic group of *E. coli*, a group associated with a high degree of virulence (8). Previous studies of clinical *E. coli* isolates have shown a negative correlation between fluoroquinolone resistance and bacterial virulence (17–19). Analysis of those data suggests that the negative correlation may be due to the preferential emergence of resistance in phylogenetically distinct strains with an intrinsically low level of virulence, and there is no evidence for any loss of virulence associated with the acquisition of resistance (17, 18). Furthermore, it should be noted that virulence and fitness are not directly comparable parameters.

To determine whether the accumulation of resistance-associated mutations is associated with a loss of fitness, each of the mutants was tested in a growth competition assay in urine against the parental Nu14 strain. In addition, strains from lineage 2 were also tested against Nu14 in a mouse UTI model. In general, both in vitro and in vivo, there was little or no difference in fitness between Nu14 and any of the first-step mutants (Tables 1 and 6). In contrast, 3 of 9 second-step mutants, 15 of 18 third-step mutants, and 6 of 8 fourth- and fifth-step mutants suffered large decreases in growth rates relative to that of Nu14 (Tables 3 to 5). In many cases the loss of fitness was extreme, with the generation times being doubled. One can therefore conclude that the accumulation of mutations that decrease susceptibility to norfloxacin, in particular, three or more mutations, is strongly associated with a severe loss of bacterial fitness, defined as a decrease in the growth rate. However, a closer examination of the data reveals a more interesting and complicated picture (Fig. 2). Thus, in lineages 2-1 and 2-2, the loss of fitness, both in vitro and in vivo (Fig. 1), associated with the initial accumulation of mutations is partially reversed by later mutations (Tables 3 and 6). A similar pattern of a loss of fitness followed by a partial restoration of fitness was also seen in lineage 5 (Fig. 2). This suggests the possibility that particular combinations of resistance-associated mutations can effect a partial compensation for the fitness losses associated with individual mutations.

The reversal of fitness costs associated with the accumulation of mutations is interesting in terms of the clinical devel-

opment of fluoroquinolone resistance. Thus, if the multiple mutations required for resistance impose a severe fitness cost, resistant bacteria will be under selection pressure to avoid, minimize, or compensate for these costs. A naïve expectation is that as resistance-associated mutations accumulate, there could be a parallel accumulation of fitness-compensating mutations either within or outside of the genes associated with resistance. In the natural setting, these fitness-compensatory mutations might be acquired in the time intervals between antibiotic selections, as has been shown to occur in selections made in the laboratory in the absence of antibiotic selection (5, 28). A general conclusion from these published experiments is that fitness compensation is not necessarily associated with any loss of antibiotic resistance. Our experiments differed from those described above, in that we made each successive selection in the presence of the antibiotic. Thus, we may have selected for a subset of the potential fitness-compensatory mutations, i.e., those that are also associated with increased antibiotic resistance. Whether the apparent coselection of fitness-compensating and resistance mutations observed in this study is relevant to the clinical evolution of fluoroquinolone resistance will require further genetic analysis.

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