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Isolation and Characterization of an *Escherichia coli* Strain Exhibiting Partial Tolerance to Quinolones

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Quinolone antimicrobial agents rapidly kill bacteria by largely unknown mechanisms. To study this phenomenon, a strain of *Escherichia coli* inhibited but inefficiently killed by (i.e., partially tolerant to) norfloxacin was isolated and characterized. *E. coli* KL16 (norfloxacin MIC, 0.10 μg/ml; MBC, 0.20 μg/ml) was mutagenized with nitrosoguanidine and cyclically exposed to 3 μg of norfloxacin per ml. After five cycles, a bacterial strain (DS1) which was killed 1,000-fold less than KL16 during 3 h of drug exposure was isolated. The MIC and MBC of norfloxacin for DS1 were 0.20 and 1.5 μg/ml, respectively. Over a range of norfloxacin concentrations, DS1 was killed 2 to 4 orders of magnitude less than KL16. DS1 grew more slowly than KL16 but after normalization for growth rate was killed four times less rapidly than KL16 at drug concentrations 10-fold higher than respective MICs. DS1 and KL16 cells filamented similarly upon exposure to norfloxacin. DS1 exhibited tolerance to other DNA gyrase A subunit antagonists (ofloxacin and ciprofloxacin) and DNA gyrase B subunit antagonists (novobiocin and coumermycin) but not to the aminoglycoside gentamicin, suggesting involvement of DNA gyrase. DS1 also appeared to be minimally tolerant to the β-lactam cefoxitin. DS1 exhibited increased susceptibility to the mutagen methyl methanesulfonate, implying a defect in DNA repair. This report describes the first use of quinolone enrichment for isolation of a bacterial strain partially tolerant to quinolones. The study of defects in such tolerant strains offers an approach to an increased understanding of the mechanisms of bacterial killing by quinolones.

The use of quinolone antimicrobial agents such as norfloxacin, ofloxacin, ciprofloxacin, and others for therapy of bacterial infections has increased markedly worldwide in recent years (21, 33). A primary bacterial target of quinolones and the earlier-developed agents nalidixic acid and oxolinic acid is the enzyme DNA gyrase (11, 12, 14, 31, 34). DNA gyrase contains two A subunits, encoded by the *gyrA* gene in *Escherichia coli* and antagonized by the quinolones, and two B subunits, encoded by the *gyrB* gene and antagonized by novobiocin and coumermycin, two antibiotics structurally unrelated to the quinolones (11, 12, 14, 34). Activities of DNA gyrase, which include supertwisting of DNA and reversible interlocking of DNA circles like links in a chain, are required for bacterial DNA replication and aspects of transcription and DNA repair (11, 14). Quinolones antagonize these activities and, in the presence of detergent and proteinase K, produce gyrase-mediated cleavage of DNA (11, 14).

Among the favorable characteristics of quinolones is rapid killing of many bacterial species (9, 10, 18, 27). The mechanism of quinolone killing apart from the involvement of DNA gyrase (3, 6, 29) remains largely unknown. To gain further insight into quinolone killing, we isolated and characterized a strain of *E. coli* inhibited but inefficiently killed by (i.e., tolerant to) quinolones.

Tolerance to a bactericidal agent is defined as a selective, disproportionate increase in the drug concentration which produces bacterial killing relative to that which inhibits growth (17). Resistance, in contrast, represents a coordinate, proportional increase in the drug concentration producing killing and growth inhibition.

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MATERIALS AND METHODS

Bacterial strains. Strains used were *E. coli* KL16 (Hfr *thi-1 relA spoT1*) (1), *E. coli* DS1 (partially quinolone tolerant, derived from KL16 as described below), and *E. coli* KL16-99 (KL16 *recA*) (1).

Antimicrobial agents. Stock solutions of drugs were as follows: norfloxacin (a gift of Merck Institute for Therapeutic Research, Rahway, N.J.) and ofloxacin (a gift of Ortho Pharmaceutical, Raritan, N.J.), 20 mg/ml in 0.1 N NaOH; ciprofloxacin (a gift of Miles Pharmaceuticals, West Haven, Conn.), 10 mg/ml in 0.1 N NaOH; novobiocin (Sigma Chemical Co., St. Louis, Mo.), 20 mg/ml in water; coumermycin A1 (Hoffmann-La Roche Inc., Nutley, N.J.), 10 mg/ml in dimethyl sulfoxide; and cefoxitin (Merck Sharp & Dohme, West Point, Pa.), 1 mg/ml in water. Gentamicin (Schering Corp., Kenilworth, N.J.) was purchased in solution (40 mg/ml). Antimicrobial agents were diluted in L (Luria) broth (19).

MICs, MBCs, time-kill curve studies, and drug concentration-kill studies. Except where noted, studies were performed with L broth (19) or agar at 37°C with inocula of exponential-phase cells. Viability was assayed by dilution and plating on agar. MICs and MBCs were determined by broth dilution with inocula of 2×10^4 cells in 1 ml of broth. MBCs were defined as the lowest drug concentrations which caused a >1,000-fold decrease in initial viable count after 18 to 24 h of drug exposure. For drug concentration-kill studies, cells at 2×10^8 CFU/ml were diluted 1:10 into 10 or 20 ml of broth containing a drug at the indicated concentrations and incubated with agitation, and viable counts were determined by plating on agar. For time-kill curve studies, cells at 2×10^8 CFU/ml were diluted 1:1,000 into 20 ml of broth and incubated with agitation, and viable counts were determined hourly by plating on agar; exposure to norfloxacin was initiated at 3 h by diluting cells 1:10 into broth containing a drug. For filter paper time-kill curve studies, a method for

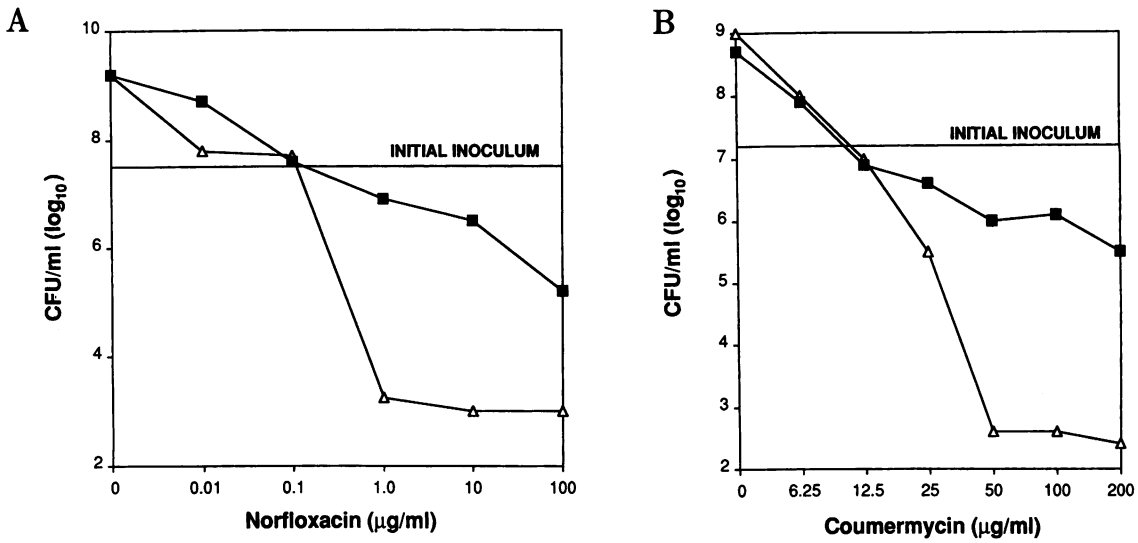

FIG. 1. Killing of *E. coli* DS1 (■) and *E. coli* KL16 (△) by exposure to different concentrations of norfloxacin (A) or coumermycin (B) for 3 h at 37°C. MICs of norfloxacin are 0.31 μg/ml for DS1 and 0.15 μg/ml for KL16. The MIC of coumermycin is 12.5 μg/ml for both strains.

measuring MICs and MBCs similar to that of Fernandes and co-workers (13) was used. Bacteria (2×10^6 CFU) were spread on nitrocellulose filters (0.45-μm pore size; Millipore Corp., Bedford, Mass.) resting on drug-free agar and incubated for 1 h. Filters were then moved to drug-containing agar, removed at the indicated times to drug-free agar for 1 h to allow diffusion of the drug from filters, and placed on drug-free agar and incubated for 18 to 24 h to permit growth of colonies.

Isolation of *E. coli* DS1. *E. coli* KL16 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (ICN/K & K Laboratories Inc., Plainview, N.Y.) (19). Cells in exponential growth phase were washed twice in 0.1 M citrate buffer (pH 5.5) and exposed to 50 μg of nitrosoguanidine per ml in citrate buffer for 25 min without aeration, resulting in 98% killing. Cells were washed with 0.1 M phosphate buffer (pH 7), suspended in L broth, incubated overnight at 37°C, diluted 25-fold into L broth, and aerated to allow onset of logarithmic growth. At a density of about 2×10^8 CFU/ml, viable count was determined, and cells were diluted 10-fold into L broth containing 3 μg of norfloxacin per ml and incubated with aeration at 37°C for 3 h, at which time the viable count was determined and the culture (0.1 ml) was spread undiluted on agar. The following day, cells from confluent growth from the agar plate which received undiluted culture were grown in L broth, and exposure to norfloxacin was repeated. After five such exposures to norfloxacin, an isolate (DS1) was colony purified twice and used for subsequent studies.

Susceptibility to methyl methanesulfonate (MMS). Susceptibility of strains to MMS (Aldrich Chemical Co., Milwaukee, Wis.) was determined by efficiency of plating on tryptone-yeast extract agar (26) containing 520 μg of MMS per ml (0.04%) (15).

RESULTS

Norfloxacin killing curve of *E. coli* KL16. Exposure of *E. coli* KL16 (norfloxacin MIC, 0.10 μg/ml; MBC, 0.20 μg/ml) in exponential growth phase to norfloxacin (1, 3, or 30 μg/ml) in broth resulted in a rapid 3- to 4-log_{10} decrease in viable count over 1 to 2 h followed by a marked slowing in the rate of killing, as has been observed by others (9, 10, 18, 27). Technical problems can cause broth studies of antibiotic killing to be inaccurate (17). For example, cells may attach to glass above the meniscus of broth, survive because of lack of drug exposure, and subsequently be reintroduced into the broth with mixing, forming colonies when the titer is determined. Therefore, time-kill curve studies were performed with cells spread on nitrocellulose filters on agar containing 3 μg of norfloxacin per ml, with subsequent removal of filters to drug-free agar to allow growth of survivors. A time-kill curve similar to that obtained with broth was obtained (data not shown), confirming the accuracy of the broth studies. Therefore, subsequent killing studies were done with broth.

Evaluation of cells surviving the initial rapid killing revealed that progeny were neither more resistant to norfloxacin nor more likely to survive repeated exposure to norfloxacin (data not shown). Thus, these less rapidly killed cells appear to represent a subpopulation that is phenotypically and not genotypically tolerant to quinolones.

The amount of killing of cells exposed for 3 h to increasing concentrations of drug was determined (drug concentration-kill study; Fig. 1A, △). Maximum killing occurred at concentrations ≥10-fold higher than the MIC (≥1 μg/ml).

Enrichment of a partially quinolone-tolerant strain. An attempt was made to enrich for spontaneously quinolone-tolerant mutants. *E. coli* KL16 was cycled 15 times by exposure of exponential-phase cells to 3 μg of norfloxacin per ml for 3 h followed by regrowth in drug-free medium, but no decrease in killing was observed. Cells were therefore mutagenized with nitrosoguanidine and similarly cyclically exposed to norfloxacin. By five exposures to norfloxacin, the amount of killing decreased from 3 to 4 log_{10} to <1 log_{10}. An isolate (DS1) exhibiting this property was colony purified twice and further studied.

Characterization of DS1. For DS1, the MIC and MBC of norfloxacin were 0.20 and 1.5 μg/ml, respectively, consistent with partial tolerance to this agent. Tolerance of DS1 lessened with passage on drug-free medium but was restored to

TABLE 1. Comparison of growth rates (generation times) and initial killing rates (halving times during exposure to norfloxacin) for *E. coli* DS1 and KL16

E. coli strain	Generation time (min)	Initial killing rate[a] (min)	Growth rate/ killing rate
DS1	30	36	0.8
	60	90	0.6
	90	108	0.8
KL16	18	≤6	≥3.0
	24	≤6	≥4.0
	24	≤6	≥4.0
	37[b]	18	2.1
	39[b]	12	3.3
	42[b]	12	3.5

[a] Norfloxacin concentrations were 10-fold higher than the MIC. The initial half time of killing was determined by viability counts taken at intervals after addition of norfloxacin.

[b] Experiments were performed at 30°C rather than at 37°C to slow the growth rate of KL16.

initial levels in survivors of cells cycled once or twice by exposure to 3 μg of norfloxacin per ml for 3 h.

The maximal amount of killing of DS1 exposed to various concentrations of norfloxacin for 3 h was significantly less than that of KL16 (Fig. 1A).

DS1 grew variably slower than KL16. Therefore, killing rates were normalized to growth rates (30) in individual experiments for both DS1 and KL16 exposed to norfloxacin concentrations 10-fold higher than their respective MICs (Table 1, Fig. 2). DS1 was found to be killed about fourfold more slowly than KL16, independent of growth rate.

Partial tolerance of DS1 to norfloxacin was present at 30°C (MIC, 0.31 μg/ml; MBC, >5.0 μg/ml) and 42°C (MIC, 0.31 μg/ml; MBC, 2.5 μg/ml) as well as at 37°C.

The ability of norfloxacin to induce filamentation in DS1 and KL16 was compared. At concentrations 10- to 20-fold higher than the MIC, no differences in filamentation between the two strains were observed by measuring lengths of Gram-stained cells by light microscopy (data not shown).

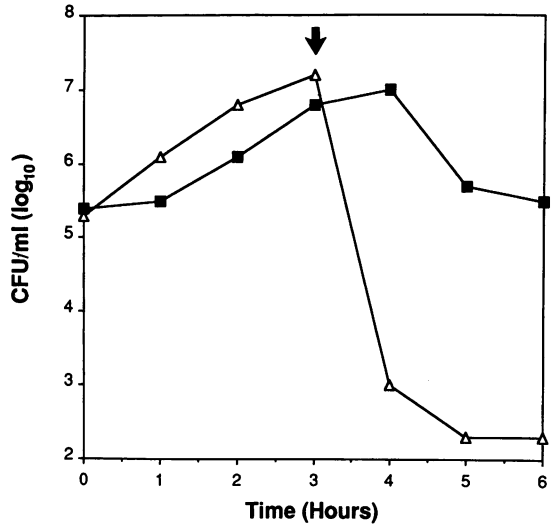

FIG. 2. Time-kill curves of *E. coli* DS1 (■) and *E. coli* KL16 (△) at 37°C. At 3 h, cells were diluted 1:10 into broth containing norfloxacin (arrow) at concentrations 10 times higher than the MIC for each strain (3 μg/ml for DS1 and 1.5 μg/ml for KL16). Cell concentrations thus decreased 10-fold with addition of the drug; CFU per milliliter in the presence of drug, however, are displayed at 10-fold-higher concentrations to simplify the graph.

TABLE 2. Tolerance of *E. coli* DS1 and KL16 to different antimicrobial agents

Drug	*E. coli* strain	MIC/MBC (μg/ml)	Drug concentration-kill study result
Norfloxacin	DS1	0.20/1.5	Partial tolerance
	KL16	0.10/0.20	
Ofloxacin	DS1	0.16/0.63	Partial tolerance
	KL16	0.16/0.16	
Ciprofloxacin	DS1	0.08/0.64	Not done
	KL16	0.04/0.08	
Novobiocin	DS1	125/2,000	Partial tolerance
	KL16	125/125	
Coumermycin	DS1	12.5/25	Partial tolerance
	KL16	12.5/25	
Gentamicin	DS1	2.0/4.0	No tolerance
	KL16	4.0/8.0	
Cefoxitin	DS1	12.5/12.5	Minimal tolerance
	KL16	6.3/6.3	

Tolerance of DS1 to other antimicrobial agents. DS1 was evaluated for tolerance to additional DNA gyrase A subunit antagonists (ofloxacin and ciprofloxacin), DNA gyrase B subunit antagonists (novobiocin and coumermycin), the aminoglycoside gentamicin, and the β-lactam agent cefoxitin by MIC and MBC studies and drug concentration-kill studies (Table 2). Partial tolerance was found to ofloxacin, ciprofloxacin, novobiocin, and coumermycin but not to gentamicin. Coumermycin tolerance was demonstrated in drug concentration-kill studies (Fig. 1B) but not in MIC and MBC studies, emphasizing that lack of tolerance as determined by MIC and MBC determinations may be inaccurate for coumermycin, as is known for β-lactam drugs (17). For cefoxitin, drug concentration-kill studies and time-kill curve studies suggested minimal tolerance (data not shown).

Killing of DS1 and KL16 by ofloxacin or norfloxacin. Ofloxacin is known to kill KL16 more effectively than norfloxacin (27). Therefore, killing of DS1 and KL16 by ofloxacin and norfloxacin was compared. Ofloxacin was confirmed to kill KL16 to a greater extent than DS1 in drug concentration-kill studies (data not shown). DS1 was also found to be killed to a greater extent by ofloxacin than norfloxacin at equivalent concentrations (Fig. 3).

Susceptibility to MMS. Susceptibility to the DNA-damaging agent MMS was determined for KL16, DS1, and KL16-99 (KL16 *recA*). DS1 was more susceptible to MMS than KL16 and less susceptible than KL16-99 (which served as a positive control) (Table 3). These results suggest that DS1 may be deficient in DNA repair. Whether increased susceptibility of DS1 to MMS is related to tolerance is unclear.

DISCUSSION

The mechanism of bacterial killing by nalidixic acid, oxolinic acid, and the newer quinolones is not well understood. Four categories of observations, however, give insights into the phenomenon.

First, killing appears to involve DNA gyrase, because quinolone resistance mutations in the *gyrA* gene of DNA gyrase result in increased drug concentrations necessary to kill bacteria (3, 6, 29). Also, a *gyrA* mutant strain of *E. coli* selected with nalidixic acid appears to be partially tolerant to quinolones (6, 29).

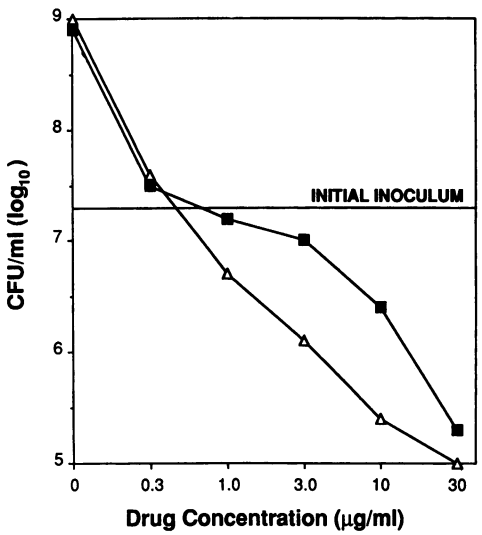

FIG. 3. Killing of *E. coli* DS1 by exposure to different concentrations of ofloxacin (△) or norfloxacin (■) for 3 h at 37°C. The MIC of both drugs for DS1 is 0.31 μg/ml.

Second, bacterial killing by quinolones is blocked by chloramphenicol, rifampin, 2,4-dinitrophenol, or nutrient starvation (6, 9, 27, 29, 35), all of which inhibit protein synthesis, suggesting that synthesis of a protein may be required for quinolone killing. Such a requirement may explain the paradoxical effect of decreased killing of some bacterial strains by high concentrations of quinolone (7, 10, 27, 29, 32), because low drug concentrations inhibit DNA synthesis, whereas high concentrations inhibit RNA and protein synthesis as well (14, 29, 32). There are, in addition, differences in bactericidal activity among quinolones. Analog 17606 is only bacteriostatic (29), and ofloxacin and ciprofloxacin, in comparison with norfloxacin and nalidixic acid, produce greater killing of *E. coli* in the presence of chloramphenicol (27) or nutrient starvation (35).

Third, the extent of degradation of the *E. coli* chromosome in response to nalidixic acid (8, 9, 24) is associated with the extent of cell death (4), although some of these effects may reflect induction of lysogenic bacteriophages (2). Chloramphenicol, however, inhibits killing but not DNA degradation (C. S. Lewin and J. T. Smith, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 541, 1988), suggesting that DNA degradation is unrelated to drug killing. Quinolones are potent inducers of the *recA* SOS DNA repair-recombination system of *E. coli* (5, 16, 22, 23), and *recA* mutants are hypersusceptible to quinolones (18), indicating that the SOS system is needed to repair DNA damage. It is also possible, however, that quinolone-induced DNA damage occurring in a wild-type cell is completely repaired and that killing occurs by mechanisms unrelated to DNA damage. Consistent with this possibility is the finding that intact chromosomes have been isolated from cells exposed to lethal concentrations of oxolinic acid (28).

TABLE 3. Susceptibility of *E. coli* DS1, KL16, and KL16-99 to MMS

Strain	Relevant characteristic	Efficiency of plating[a]
KL16	Wild type	1.4
DS1	Quinolone tolerant	4×10^{-3}
KL16-99	*recA*	1×10^{-6}

[a] Efficiency of plating represents the ratio of growth (CFU per milliliter) of the indicated strain on tryptone-yeast extract agar with 520 μg of MMS per ml to growth on tryptone-yeast extract agar without MMS.

Fourth, quinolones antagonize the DNA replication fork, because DNA synthesis is rapidly inhibited at drug concentrations near the MIC (3, 11, 12, 16, 24). Nalidixic acid, however, inhibits DNA synthesis in the presence of chloramphenicol concentrations which block killing (9, 32), suggesting that inhibition of DNA synthesis alone is insufficient to explain killing by nalidixic acid.

Some of these observations are consistent with quinolone-induced DNA damage by DNA gyrase causing quinolone killing (4, 11, 22, 23, 27); lethal DNA damage, such as a nonrepairable double-strand break, might be introduced in the unreplicated portion of the bacterial chromosome by DNA gyrase itself or by a repair enzyme attempting to repair DNA gyrase-mediated DNA damage. As discussed above, data supporting this hypothesis, however, are thus far lacking.

E. coli hipA mutants, isolated because of decreased killing by the β-lactam ampicillin, have recently been found to be killed less effectively by nalidixic acid (20, 26). These and other properties of *hipA* mutants suggest the possibility of a common pathway for killing by β-lactams and quinolones involving cell division (26).

Because of gaps in our understanding of bacterial killing by quinolones, we undertook isolation of a quinolone-tolerant organism. After mutagenesis and cyclical exposure to norfloxacin, *E. coli* DS1 was identified, representing the first partially quinolone-tolerant bacterial strain isolated by this approach. The strain is partially tolerant to DNA gyrase A subunit antagonists (norfloxacin, ofloxacin, and ciprofloxacin) and DNA gyrase B subunit antagonists (novobiocin and coumermycin) but not to the aminoglycoside gentamicin, suggesting that tolerance may be caused by mutation in the *gyrA* or *gyrB* gene of DNA gyrase. DS1, however, is hypersusceptible to MMS and appears to be partially tolerant to cefoxitin, suggesting tolerance by other mechanisms, such as mutations in DNA repair genes (23), autolysis genes (17, 25), *hipA* (26), or cell division genes (26). Genetic and other studies of DS1 are under way to attempt to differentiate among these possibilities.

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