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Mutants of *Escherichia coli* K-12 Exhibiting Reduced Killing by Both Quinolone and β-Lactam Antimicrobial Agents

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Norfloxacin, ofloxacin, and other new quinolones, which are antagonists of the enzyme DNA gyrase, rapidly kill bacteria by largely unknown mechanisms. Earlier, we isolated, after mutagenesis, *Escherichia coli* DS1, which exhibited reduced killing by quinolones. We evaluated the killing of DS1 and several other strains by quinolones and β-lactams. In time-killing studies with norfloxacin, DS1 was killed 1 to 2 $\log_{10}$ units compared to 4 to 5 $\log_{10}$ units for the wild-type parent strain KL16, thus revealing that DS1 is a high-persistence (*hip*) mutant. DS1 exhibited a similar high-persistence pattern for the β-lactam ampicillin and reduced killing by drugs that differed in their affinities for penicillin-binding proteins, including cefoxitin, cefsulodin, imipenem, mecillinam, and piperacillin. Conjugation and P1 transduction studies identified a novel mutant locus (termed *hipQ*) in the 2-min region of the DS1 chromosome necessary for reduced killing by norfloxacin and ampicillin. *E. coli* KL500, which was isolated for reduced killing by norfloxacin without mutagenesis, exhibited reduced killing by ampicillin. *E. coli* HM23, a *hipA* (34 min) mutant that was isolated earlier for reduced killing by ampicillin, also exhibited high persistence to norfloxacin. DS1 differed from HM23, however, in the map location of its *hip* mutation, lack of cold sensitivity, and reduced killing by coumermycin. Results of these studies with strains DS1, KL500, and HM23 demonstrate overlap in the pathways of killing of *E. coli* by quinolones and β-lactams and identify *hipQ*, a new mutant locus that is involved in a high-persistence pattern of reduced killing by norfloxacin and ampicillin.

The quinolones are oral antimicrobial agents that are efficacious for the treatment of bacterial infections (44, 60). Quinolones include norfloxacin, ofloxacin, ciprofloxacin, and the less potent analogs nalidixic acid and oxolinic acid that were developed earlier. Quinolones are unusual in that they have as their target the essential enzyme DNA gyrase (bacterial topoisomerase II) (16, 17, 20, 28, 47, 58). This enzyme contains two A subunits, which are encoded by the *gyrA* gene (48 min on the *Escherichia coli* genetic map) and are targets of quinolones, and two B subunits, which are encoded by the *gyrB* gene (83 min) and are targets of novobiocin and coumermycin, two antibiotics that are structurally unrelated to quinolones. DNA gyrase introduces negative supertwists into DNA and reversibly interlocks (catenates) DNA circles; these activities are antagonized by quinolones. Quinolones also induce DNA gyrase to cleave DNA in the presence of detergent and proteinase K (57).

An important property of quinolones is their ability effectively to kill many bacterial species (11, 12, 47, 60). Exposure of *E. coli* to a quinolone results in an initial rapid decrease in cell viability followed by a slowing or cessation of killing (6, 8, 11, 12, 18, 21, 47, 62). The refractory subpopulation is composed of persisters (3, 24, 37) which are neither resistant mutants nor mutants that are less effectively killed on growth and reexposure to drug (62). Additional characteristics of killing by quinolones include a paradoxical reduction in killing at high drug concentrations (9, 12, 47, 59); reduced killing in the presence of chloramphenicol, rifampin, or other treatments that inhibit protein synthesis (suggesting a requirement for synthesis of a protein[s] for killing [8, 9, 11, 12, 47, 48, 51, 59, 65]); differences in the extent of killing of various bacterial species by the individual quinolones (31, 42, 47, 48); and a requirement for oxygen (48).

The exact mechanisms by which quinolones kill bacteria are not well understood (61). To study the killing phenomenon, we isolated DS1, a mutant strain of *E. coli* KL16 which exhibits reduced killing by quinolones and antagonists of the DNA gyrase B subunit but not by the aminoglycoside gentamicin (62). DS1 unexpectedly exhibited reduced killing by the β-lactam cefoxitin (62). We present here results of studies of killing of DS1 by quinolones and β-lactams and the initial genetic characterization of DS1. During our studies, *hipA* (high-persistence) mutants, which exhibit reduced killing by the β-lactam ampicillin (37, 38), were reported to be less effectively killed by nalidixic acid (46). We also present results of studies of killing of a *hipA* mutant by DNA gyrase antagonists.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study, their sources, and their relevant characteristics are given in Table 1.

Drugs. Stock solutions of the following drugs were used: ampicillin sodium (Sigma Chemical Co., St. Louis, Mo.), 10 mg/ml in water; cefoxitin (Merck Sharp & Dohme, West Point, Pa.), 1 mg/ml in water; cefsulodin sulfate (Sigma), 10 mg/ml in water; coumermycin A1 (a gift from Hoffmann-La Roche Inc., Nutley, N.J.), 10 mg/ml in dimethyl sulfoxide; diaminopimelic acid (Sigma), 3 mg/ml in water; imipenem monohydrate (Merck & Co., Inc., Rahway, N.J.), 1 mg/ml in water; mecillinam (Hoffmann-La Roche), 10 mg/ml in water; nalidixic acid (Calbiochem-Behring, La Jolla, Calif.), 10 mg/ml in 0.1 N NaOH; nitrofurantoin (Sigma), 10 mg/ml in dimethyl sulfoxide; norfloxacin (a gift from Merck & Co.)

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TABLE 1. *E. coli* K-12 strains used in this study

Strain	Relevant characteristic	Source or reference
KL16	Hfr *thi-1 relA spoT1*	B. Bachmann (1)
DS1	KL16, reduced killing by quinolones	Mutagenesis of KL16 and cycled exposure to norfloxacin (62)
DS1-2	Reduced killing by quinolones	Spontaneous partial revertant of DS1
KL497	AT984, *dapE purC*::Tn*10*	H. Moyed
KL498	KL16, *dapE purC*::Tn*10*	P1 transduction from KL497 into KL16 (tetracycline selection)
KL499	KL498, reduced killing by quinolones	Cycled exposure of KL498 to norfloxacin
KL500	KL499, Dap^+, reduced killing by quinolones	Spontaneous Dap^+ derivative of KL499
SG13107	*his pyrD leu*::Tn*10 rpsL*	S. Gottesman (23)
χ697	F^- *leuB6 purE42 trpE38 his-208 argG77 ilvA681 met-160 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 azi-6 rpsL104 tonA23 tsx-67 supE44*	B. Bachmann
HM21	AT984 *dapE zde-264*::Tn*10*	H. Moyed (37)
HM23	AT984 *dapE zde-264*::Tn*10 hipA9*	H. Moyed (37)
DL-2	F^- *thi zei-723*::Tn*10 gyrA glpR araD139 ΔlacU169 rpsL relA fldB ptsF25*	E. Lin (29)
GM100	KL16 *zei-723*::Tn*10 gyrA*$^+$	P1 transduction from DL-2 into KL16 (tetracycline selection, norfloxacin susceptible)

and ofloxacin (a gift from Ortho Pharmaceuticals, Raritan, N.J.), 1 mg/ml in 0.02 N NaOH; piperacillin sodium (Lederle Laboratories, Pearl River, N.Y.), 1 mg/ml in water; rifampin (Calbiochem-Behring), 5 mg/ml in dimethyl sulfoxide; and tetracycline hydrochloride containing 2.5 g of ascorbic acid per g of tetracycline (Lederle Laboratories), 50 mg/ml in water. Antimicrobial agents were diluted in L (Luria) broth (36).

Measurement of drug susceptibility and killing. Cells were in the exponential phase of growth when they were exposed to drugs, and viability was measured by determination of CFU. Studies were performed with L or LB broth or on L or TYE agar (36) at 37°C. Media were supplemented with 75 μg of diaminopimelic acid per ml for diaminopimelic acid-requiring strains. Determinations of MICs and MBCs were performed as described previously (62), except for studies with mutants HM21 and HM23, for which initial inocula were 10^6 CFU (rather than 10^4 CFU) in 1 ml of broth. Time-killing studies and drug concentration-killing studies were done as described previously (62), except for drug concentration-killing studies with rifampin, for which bacteria were exposed to drug for 4 and 7 h as well as 3 h. All experiments were done two or more times.

A rapid assay for scoring large numbers of colonies for reduced killing by norfloxacin was done, as follows. Cells grown overnight in L broth as a standing culture were diluted (10-fold for χ697 or 100-fold for DS1) in 0.9% NaCl. Ten microliters of diluted culture was transferred with a Steers replicator to agar containing norfloxacin at concentrations near the MIC. After reincubation for 18 h, cells were replica-plated with velvet to agar plates containing no drug and drugs at the same concentrations, and the plates were incubated for 18 to 24 h. Growth of ≥10 colonies on drug-free agar with no growth or growth of pinpoint colonies on drug-containing agar was scored as reduced killing.

Conjugation and P1 transduction. Conjugation was carried out by mixing DS1 and χ697 in the exponential phase of growth at a donor to recipient ratio of 1:10 on a filter (pore size, 0.45 μm; diameter, 13 mm; Millipore Corp., Bedford, Mass.) which was placed on an L-agar plate and incubated for 2.5 h. Cells were washed from the filter with 0.9% NaCl and plated onto selective media. Transduction with P1 *vir* (a gift from E. Brinkman) was done as described previously (36).

RESULTS

Killing studies with DS1 and KL500. In time-killing studies, exposure of strain DS1 and its parent strain KL16 to norfloxacin at concentrations 10-fold above their MICs resulted in similar initial rapid killing of both strains. The magnitude of killing differed substantially, however, being 1 to 2 $\log_{10}$ units for DS1 and 4 to 5 $\log_{10}$ units for KL16 (Fig. 1A). When originally isolated, DS1 in general grew more slowly than KL16 did after single-colony purification (62). After additional passage, DS1 grew at a rate similar to that of KL16 (Fig. 1A), eliminating slower growth as an explanation for the reduced killing of DS1. These time-killing studies indicate that DS1 is a high-persistence (*hip*) mutant, as defined by Moyed and Bertrand (37) in their studies of the killing of *E. coli* by ampicillin.

DS1 exhibited reduced killing by nitrofurantoin (Table 2). The killing effects of drugs tested previously (62) are also given in Table 2. In contrast, DS1 was as effectively killed by rifampin as KL16 was, a finding similar to that for gentamicin (62).

Because DS1 exhibited reduced killing by cefoxitin in drug concentration-killing studies (62), killing by additional β-lactams was evaluated. DS1 was less effectively killed by ampicillin in MIC and MBC studies (Table 2), in time-killing studies in which a high-persistence pattern was present (Fig. 1B), and in drug concentration-killing studies (Fig. 2A).

Ampicillin and cefoxitin bind to multiple penicillin-binding proteins (PBPs) (35, 50). Killing of DS1 and KL16 by β-lactams with different affinities for PBPs was evaluated (Table 2). DS1 was less effectively killed by cefsulodin (which binds preferentially to PBP 1a [53]), mecillinam (which binds preferentially to PBP 2 [49]), and piperacillin (which binds preferentially to PBP 3 [4]) in drug concentration-killing studies which included testing of drugs at concentrations near the MIC to favor selective binding to PBPs. DS1 also exhibited reduced killing by imipenem, a carbapenem which binds to multiple PBPs, including PBP 7 (53).

Reduced killing of DS1 by quinolones and β-lactams suggests that there is an overlap in the bactericidal actions of these two classes of drugs. Because DS1 was isolated after mutagenesis with nitrosoguanidine, however, reduced killing by quinolones and β-lactams might represent the effects of unrelated mutations. DS1-2, a spontaneous partial revertant

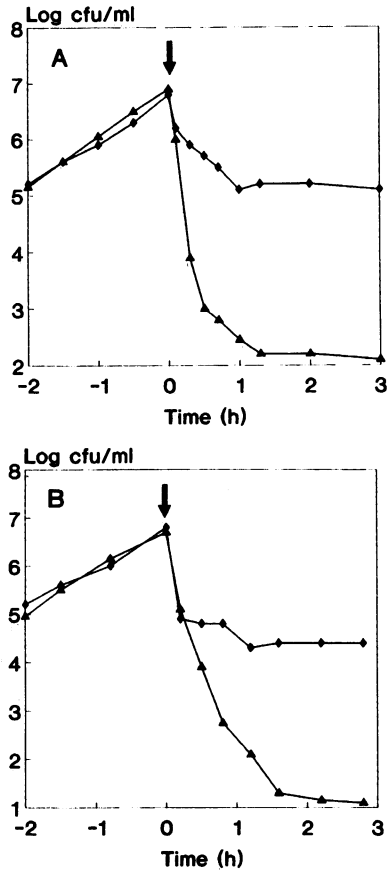

FIG. 1. Time-killing study of *E. coli* DS1 (◆) and KL16 (▲) exposed to norfloxacin (A) at 10-fold the MICs (MICs, 0.2 and 0.1 μg/ml, respectively) and ampicillin (B) at 10-fold the MIC (MIC, 3.1 μg/ml for both strains). Arrows indicate the time of drug addition.

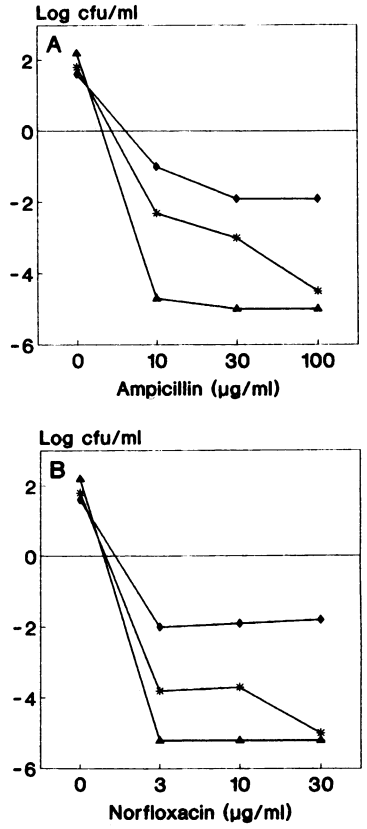

FIG. 2. Drug concentration-killing studies of DS1 (◆), DS1-2 (*), and KL16 (▲) exposed for 3 h to ampicillin (A) and norfloxacin (B). Cell titers are normalized to $\log_{10} = 0$ for initial inocula.

of DS1, was noted to be killed by norfloxacin at a level intermediate between that for DS1 and KL16 (Fig. 2B). DS1-2 was also killed to intermediate levels by exposure to different concentrations of ampicillin (Fig. 2A), suggesting that a mutation(s) affecting killing by quinolones also affected killing by β-lactams. *E. coli* KL500 was isolated without mutagenesis by cycled exposure of KL498 (KL16 *dapE*) to norfloxacin and selection for loss of growth requirement for diaminopimelic acid. MICs and MBCs of norfloxacin were 0.2 and 3.2 μg/ml, respectively, for KL500 and 0.1 and 0.2 μg/ml, respectively, for KL16; and MICs and MBCs of ampicillin were 6.3 and 50 μg/ml, respectively, for KL500 and 3.1 and 6.3 μg/ml, respectively, for KL16, indicating

TABLE 2. Reduced killing by drugs of *E. coli* DS1 compared with KL16 in MIC, MBC, and drug concentration-killing studies

Drug	MIC/MBC (μg/ml)		Log_{10} reduced killing of DS1 compared with that of KL16	Range of concn tested (factor above MIC)
	DS1	KL16		
Norfloxacin[a]	0.20/1.5	0.10/0.20	2.0–4.0	5–50
Ofloxacin[a]	0.16/0.63	0.16/0.16	1.5–3.0	6–62
Novobiocin[a]	125/2000	125/125	0.5–1.0	2–8
Coumermycin[a]	12.5/25[b]	12.5/25	1.0–3.5	2–16
Nitrofurantoin	31/63	31/31	1.0–3.5	2–10
Ampicillin	3.1/12.5	3.1/6.3	2.9–4.0	10–50
Cefoxitin[a]	12.5/12.5[b]	6.3/6.3	1.0–2.5	4–32
Cefsulodin	63/ND[c]	31/ND	2.6–3.0	2–4
Imipenem	0.4/ND	0.2/ND	2.8–3.5	5–25
Mecillinam	0.16/ND	0.16/ND	1.0–3.0	6–600
Piperacillin	3.2/ND	1.6/ND	2.0–2.4	2–4
Gentamicin[a]	2.0/4.0	4.0/8.0	No difference	2–4
Rifampin	1.6/ND	1.6/ND	No difference	2–60

[a] MICs and MBCs were published previously (62).

[b] Reduced killing of DS1 was demonstrated by drug concentration-killing but not MIC and MBC studies, indicating the reduced sensitivity of the MIC and MBC method for detecting differences in killing by these agents (62).

[c] ND, Not done.

reduced killing of KL500 by both drugs. Reduced killing was confirmed by drug concentration-killing studies (data not shown). These results with KL500, a nonmutagenized strain, again suggest a relationship between the killing of *E. coli* by quinolones and β-lactams.

Genetic studies with DS1. DS1 is a derivative of KL16, an Hfr strain which transfers loci in its chromosome in descending order from an origin at 61 to 63 min (1). In conjugation experiments with strains DS1 and χ697, recombinants were selected for reversion to prototrophy and scored for reduced killing by norfloxacin by using a rapid assay method. None of 44 *his*$^+$ (54 min), 1 of 45 *trp*$^+$ (28 min), 2 of 45 *pur*$^+$ (12 min), 7 of 44 *leu*$^+$ (2 min), and 2 of 44 *ilv*$^+$ (85 min) recombinants exhibited reduced killing by norfloxacin. These results indicated that the mutation(s) that produced reduced killing in DS1 could be genetically transferred by conjugation and suggested that one or more of these mutations is more closely linked to *leu* than it is to the other markers tested.

To define more precisely the location of the locus involved in reduced killing, DS1 was transduced with P1 *vir* grown on strain SG13107 (*leu*::Tn*10*, 2 min) (23), selecting for tetracycline resistance. For 8 of 35 transductants (23%) evaluated by exposure to norfloxacin for 3 h, the rapid assay method, or both, the extent of killing by norfloxacin returned near or to wild-type levels. Similar transductions with donor strains with Tn*10* located near 34 min (strain HM21) or 48 min (strain GM100) revealed no change in reduced killing of DS1 in 12 recipients of each strain. These results indicate that DS1 contains at least one mutation about 0.7 min (64) from the *leu* locus (2 min) that is necessary for a high-persistence pattern of reduced killing by norfloxacin. We designated this mutant locus *hipQ*.

Two DS1 transductants effectively killed by norfloxacin were effectively killed by ampicillin in drug concentration-killing studies, while two transductants which continued to exhibit reduced killing by norfloxacin still exhibited reduced killing by ampicillin (data not shown). Thus, *hipQ* is necessary for reduced killing by both norfloxacin and ampicillin. These results confirm a relationship between the bactericidal activities of these two drugs.

Studies with *hipA* mutants. *E. coli* HM22 (*hipA7*, 34 min), which was isolated for reduced killing by ampicillin (37, 38), was recently reported to be less effectively killed by nalidixic acid in MIC and MBC studies (46). Reduced killing by ampicillin and nalidixic acid was confirmed for *E. coli* HM23 (*hipA9*, 34 min) compared with that for the isogenic wild-type strain *E. coli* HM21 in MIC and MBC studies (data not shown). HM23 exhibited reduced killing by norfloxacin in a high-persistence pattern in time-killing studies (Fig. 3) and by norfloxacin and ofloxacin but not by coumermycin in drug concentration-killing studies (data not shown).

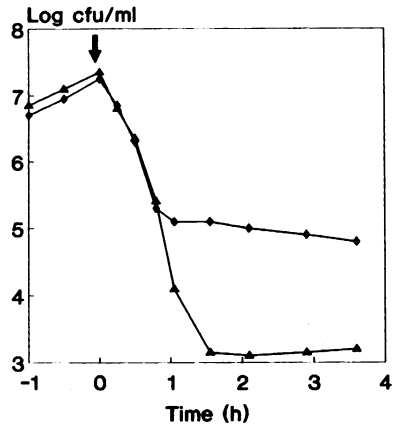

FIG. 3. Time-killing study of *E. coli* HM23 (*hipA9*) (◆) and HM21 (isogenic wild-type) (▲) exposed to norfloxacin at 20-fold the MICs (MIC, 0.4 μg/ml for both strains). The arrow indicates the time of drug addition.

DISCUSSION

Results of these studies with strains DS1, KL500, and HM23 demonstrate that there is an overlap in the pathways of killing of *E. coli* by quinolones and β-lactams and identify *hipQ*, a new mutant locus which is necessary for the high-persistence pattern of reduced killing by norfloxacin and ampicillin.

Five observations with DS1, KL500, and *hipA* mutants indicate that there is a relationship between killing of *E. coli* K-12 by quinolones and β-lactams. First, DS1 isolated for reduced killing by quinolones (62) exhibited reduced killing by β-lactams. Second, DS1-2, a spontaneous derivative of DS1 exhibiting an extent of killing by norfloxacin intermediate between that for DS1 and the wild-type strain KL16, exhibited an intermediate extent of killing by ampicillin. Third, transductants that regained effective killing by norfloxacin also regained effective killing by ampicillin. Fourth, KL500, a nonmutagenized derivative of KL16 isolated for reduced killing by norfloxacin, exhibited reduced killing by ampicillin. Fifth, *hipA* mutants isolated for reduced killing by ampicillin (37) exhibited reduced killing by nalidixic acid (46) and new quinolones. These findings indicate that there is an overlap in killing of *E. coli* by DNA gyrase A-subunit antagonists and cell wall-active agents. An alternative possibility is the presence of a process which, when altered, reduces the lethal effects of both classes of drugs.

Although DS1 (*hipQ*) and *hipA* mutants exhibited reduced killing by quinolones and β-lactams, they nevertheless differed in a number of ways. *hipA* mutants are genetically distinct from DS1 and, in contrast to DS1 (62), are cold sensitive (46) and are effectively killed by coumermycin. Thus, the mechanisms of reduced killing of *hipA* mutants and DS1 by quinolones and β-lactams are not identical.

DS1 exhibited reduced killing by drugs which bind to multiple PBPs (ampicillin, cefoxitin, and imipenem) and which bind preferentially to PBP 1a (cefsulodin), PBP 2 (mecillinam), and PBP 3 (piperacillin). Thus, reduced killing by drugs extended to drugs with varied affinities for PBPs.

Time-killing studies revealed DS1 to be a high-persistence (*hip*) mutant (37). Tolerance, which may be related to high persistence, has also been used to describe bacteria that are poorly killed by a bactericidal agent (26, 45, 62, 63), and thus, DS1 has also been called a quinolone-tolerant strain (62). High persistence and tolerance should not be mistaken for resistance. Strains exhibiting high persistence or tolerance to a drug often exhibit low-level or no resistance, as measured by MICs.

Insertion of wild-type DNA into the 2-min region of DS1 restored killing by quinolones. It has not yet been possible to transfer the reduced killing trait from a DS1 donor to a wild-type strain by P1 transduction (unpublished data), suggesting the presence of an additional necessary mutation(s). This putative additional mutation might have occurred during mutagenesis with nitrosoguanidine or cycled exposure to norfloxacin (62). It was possible, however, to transfer the Hip phenotype by conjugation, which allows transfer and recombination of larger segments of DNA.

The mechanisms of bacterial killing by quinolones are poorly understood (61). Killing involves DNA gyrase (5, 8, 51, 61); but whether its role is direct, such as by introduction of nonrepairable cuts in DNA, or indirect, such as the trigger of a cascade of events which ultimately lead to bacterial death, is unknown. Inhibition of DNA synthesis (5, 7–9, 22, 51, 59) and degradation of DNA (7, 10, 11, 16, 27, 41) have been proposed to contribute to killing (5, 6, 27), but their involvement has been questioned because nalidixic acid-induced inhibition of DNA synthesis (11, 59) and degradation of DNA (32) occur in the presence of concentrations of chloramphenicol that inhibit cell killing. Induction of the SOS DNA repair system of *E. coli* (7, 25, 39, 40) has also been suggested to contribute to killing (5, 12, 40), because maximal killing concentrations of quinolones are similar to concentrations that maximally induce the SOS system (39). Killing by nalidixic acid, however, is not reduced in *lexA3* mutants in which the SOS system is not induced by quinolones (30) (repair-deficient mutants are generally hypersusceptible to quinolones), favoring a role for the SOS response in the repair of damaged DNA rather than in killing (16, 30).

The relationship of reduced killing in DS1, KL500, and *hipA* mutants to other proved or postulated killing mechanisms and phenomena for quinolones (48, 61) and β-lactams (such as induction of autolysins [26, 43]) is unclear. It is noteworthy, however, that both quinolones (33) and β-lactams (19, 52) antagonize cell division, which suggests a common site or overlapping pathway of killing activity (33, 46). Exposure of *E. coli* to quinolones causes growth of bacterial cells as aseptate filaments (2, 8, 12, 15, 18, 21, 47, 54–56), at least in part by induction of the SOS (7, 25, 39, 40) and other (*sfiC*) (33) systems which interfere with the *sulB* (*sfiB*) gene product required for septum formation (13, 23). Interaction of β-lactams with PBPs, particularly PBP 3 (49, 52), also results in the formation of aseptate filaments, by interactions involving the products of filamentation temperature-sensitive genes such as *ftsI* (which encodes PBP 3), *ftsA*, *ftsQ*, and *ftsZ*, all of which are required for septum formation (13). Interestingly, *sulB* is an allele of *ftsZ* (34). *ftsI*, *ftsA*, *ftsQ*, and *ftsZ* (*sulB*) are among about 15 genes in the major morphogene cluster at 2 min on the *E. coli* chromosome that are involved in the synthesis of the septum and the lateral cell wall (2, 13, 14). The relationship of *hipQ* to these morphogenes is as yet unknown.

Study of *hip* mutants offers an approach to determine whether the bactericidal action of quinolones and β-lactams occurs by interference with cell division, induction of autolysins, or other processes.

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