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Identification of a pKM101 Region Which Confers a Slow Growth Rate and Interferes with Susceptibility to Quinolone in *Escherichia coli* AB1157

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The effect of plasmid pKM101 on the survival of *Escherichia coli* AB1157, growing in minimal medium, in the presence of a 4-quinolone DNA gyrase inhibitor was investigated. The presence of this plasmid decreased susceptibility to the quinolone ciprofloxacin, whereas *mucAB* genes present in a multicopy plasmid did not. The same effect of pKM101 was detected in a *recA430* mutant, confirming that it was not really related to the SOS response. In contrast, when survival assays were performed under amino acid starvation conditions, pKM101 did not confer protection against ciprofloxacin. All of these results indicated that the synthesis of a product(s), different from MucAB, which was encoded by the plasmid pKM101 increased the rate of survival of the AB1157 strain in the presence of quinolone. To identify the gene(s) responsible for this phenotype, several plasmid derivatives carrying different portions of pKM101 were constructed. The 2.2-kb region containing *korB*, *traL*, *korA*, and *traM* genes was sufficient to decrease susceptibility to quinolone. This plasmidic fragment also made the AB1157 host strain grow more slowly (the Slo phenotype). Moreover, the suppression of the Slo phenotype by addition of adenine to the cultures abolished the decreased susceptibility to quinolone. These results are evidence that the protection against quinolone conferred by this region of pKM101 in strain AB1157 is a direct consequence of the slow growth rate.

pKM101 is a 35.4-kb self-transmissible broad-host-range IncN plasmid, which was derived from plasmid R46 (17) by a spontaneous deletion of 14 kb (2, 12). This plasmid has been extensively studied because it contains the *mucAB* genes (18), which are analogous to the *umuDC* genes whose products are involved in error-prone repair of DNA damage that can occur as a consequence of the SOS response. The ability of pKM101 to increase bacterial mutability has resulted in its introduction into *Salmonella typhimurium* strains used in the Ames test, enhancing the sensitivity of this system to detect chemicals as mutagens (16).

Besides *mucAB*, other genes of pKM101 that participate in plasmid replication, stable maintenance, and host range have been described. The conjugal transfer system of pKM101 consists of a mobilization gene cluster, providing functions required for DNA processing and mobilization, and a cluster of *tra* genes involved in the synthesis of the pilus and putative mating pore (23). Near this second cluster, the *kilA* and *kilB* genes were described to encode potentially lethal products whose lethality was prevented by the products of two other genes, *korA* and *korB* (24). In addition, the *kilA* gene, which is defined to a region between two Tn*5* insertions, was also thought to retard cell growth of certain *Escherichia coli* strains on defined minimal medium (24), and this phenomenon had previously been called the Slo phenotype (11, 22). More recently, this cluster of *tra* genes has been sequenced, and, on the basis of DNA sequence information, the *kilB* (renamed *traE*), *korA*, and *korB* genes have been identified, but no obvious sequence for the *kilA* gene has been found (19).

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4-Quinolones (4-Qs) are synthetic antibacterial agents analogous to nalidixic acid which specifically inhibit the DNA gyrase. The interest in these compounds is due not only to their clinical applications as antimicrobial agents and potential antitumor drugs, but also to their action on this essential bacterial enzyme. Despite great efforts to understand their mode of action and the bacterial mechanisms of resistance to these compounds, today these subjects are still controversial (for reviews, see references 9, 10, and 15). Bacterial resistance to 4-Qs has usually been associated with mutations in chromosomal genes that produce alterations in DNA gyrase or changes in cellular permeability. Until now, no plasmids encoding resistance to 4-Qs have been found, and this has been mainly attributed to the dominance of the susceptible gyr^+ wild-type genes over the resistant *gyr* mutants. We had previously worked on the mutagenicity of 4-Qs in *S. typhimurium* (4, 25). When similar studies were carried out in *E. coli*, we realized that the presence of the pKM101 plasmid decreased the susceptibility of strain AB1157 growing in minimal medium to the quinolone ciprofloxacin and that this effect was apparently not related to the SOS response. In this work, we report the identification of the pKM101 region responsible for this phenomenon. Also, we present evidence that the slow growth rate of AB1157 promoted by this fragment of pKM101 confers protection against quinolones.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strain used for survival assays and growth kinetics was *E. coli* AB1157 (K-12 F^- *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*) (obtained from B. Bachmann). Strain IC41, which is a *recA430* derivative of AB1157, was provided by M. Blanco. DH5α (*recA1 hsdR17 gyrA96* F^- *lacZ*ΔM15) (Clontech Laboratories) was used as the host for plasmid constructions. The IncN plasmid pKM101 (obtained from G. C. Walker) was introduced into strains AB1157 and IC41 by conjugation. Plasmid pICV80 is a pBR322 derivative carrying the *mucAB* genes (1). Plasmids pK184 (provided by M. G. Jobling) and pBSK(+) (Stratagene) were used as vectors for cloning.

Plasmid constructions. Several plasmid derivatives containing different fragments of pKM101 were constructed, on the basis of physical and genetic maps of pKM101 reported earlier (19, 23). Standard procedures for DNA manipulation and transformation were performed essentially as described elsewhere (21). The pKM101 plasmid cleaved with *Sal*I generated two fragments: a 25.6-kb segment containing the origin of replication and ampicillin resistance, yielding pUA399 upon religation, and a 9.8-kb segment that was subcloned into pK184, obtaining pUA325. Plasmid pUA325 was digested with *Kpn*I, and two fragments were generated; the 7.9-kb segment was recircularized, obtaining pUA400, and the 4.3-kb segment was subcloned into pK184, obtaining pUA400-u. To construct the pUA323 plasmid, the 3.7-kb $HpaI_1$-$HpaI_2$ fragment from pKM101 was subcloned into *Hin*cII-cleaved pK184. The 4.7-kb $SalI_1$-*Bst*EII and the 4.2-kb *Acc*I-$KpnI_1$ fragments from pUA400 were blunt ended with T4 DNA polymerase and independently ligated into an *Sma*I-digested pK184 vector, obtaining plasmids pUA434 and pUA435, respectively. To construct plasmid pUA448, first the 3.3-kb *Acc*I-*Bst*EII fragment from pUA400 was blunt ended and ligated into pBSK digested with *Sma*I, obtaining pUA432. Afterwards, the 3.3-kb *Bam*HI-*Pst*I segment from pUA432 was subcloned into pK184, obtaining pUA448. The pUA483 plasmid was obtained by religating the 6.7-kb blunt-ended $KpnI_1$-*Nde*I fragment from pUA400. To construct pUA518, the 2.2-kb *Asn*I-*Nde*I segment from pUA448 was filled in and subcloned into *Sma*I-cleaved pK184.

Survival assays. Cultures were grown overnight at 37°C in AB salt solution (3) supplemented with glucose (0.2% [wt/vol]), Casamino Acids (0.4% [wt/vol]), thiamine (1 μg/ml), and the corresponding antibiotics at appropriate concentrations. For survival assays under growing conditions, cultures were diluted 1/100 in fresh medium and incubated at 37°C at 250 rpm until exponential phase was reached (optical density at 450 nm of 0.2 to 0.3). Two-milliliter samples were incubated with different concentrations of ciprofloxacin (purchased from Bayer, S.A.) for 2 h at 37°C at 250 rpm. Afterwards, the cells were diluted in 0.9% NaCl at room temperature and plated on AB agar plates supplemented with glucose (0.2% [wt/vol]), thiamine (1 μg/ml), and the amino acids arginine, leucine, threonine, proline, and histidine (50 μg/ml). Colonies were scored after a 48- to 72-h incubation at 37°C. When required, adenine was added to AB medium and agar plates at a final concentration of 60 μg/ml.

For survival assays under amino acid starvation conditions, fully grown cultures were diluted 1/100 in fresh medium and incubated at 37°C at 250 rpm until the optical density at 450 nm was approximately 0.8 to 1.0. Then, the cells were washed twice and resuspended in AB salt solution, and the solution was incubated for 1 h with gentle shaking to exhaust amino acid pools. Then, 2-ml samples were treated with ciprofloxacin and plated on AB agar plates as described above. The experiments were carried out at least three times.

Growth kinetics. Cultures grown at 37°C in AB salt solution supplemented with glucose (0.2% [wt/vol]), Casamino Acids (0.4% [wt/vol]), thiamine (1 μg/ml), and the corresponding antibiotics were diluted 1/100 in fresh medium and incubated at 37°C at 250 rpm. After the optical density at 450 nm was approximately 0.2 to 0.3, the A_{450} of the culture was monitored at half-hour intervals until stationary phase was reached. When required, adenine was added to AB medium at a final concentration of 60 μg/ml. The experiments were carried out at least twice.

RESULTS AND DISCUSSION

pKM101-mediated protection against quinolone in *E. coli* AB1157 is independent of *mucAB* genes. Under conditions that allow cell division and protein synthesis, rates of survival in minimal medium after ciprofloxacin treatment for the plasmid-free strain AB1157 and for AB1157 harboring pKM101 were compared. The presence of this plasmid increased the rate of survival in the presence of quinolone treatment (Fig. 1). Since pKM101 carries the *mucAB* operon, which of involved in the error-prone repair of DNA damage, the survival of an isogenic strain carrying *mucAB* in the multicopy plasmid pICV80 was also tested. However, the presence of *mucAB* genes did not increase the survival of AB1157 in the presence of this compound; instead, the lethality detected at high concentrations of quinolone was slightly higher (Fig. 1).

To corroborate that the effect of pKM101 was not related to the SOS response, the same assay was carried out with strain IC41 (*recA430*). This strain encodes a RecA protein active in recombination but deficient in protease activity; therefore, the RecA430 protein cannot cleave the LexA repressor, thus preventing induction of the SOS response. The results showed that pKM101 conferred a minor quinolone susceptibility to the *recA430* mutant, with levels of survival similar to those of the wild-type strain (Fig. 2). On the other hand, the lower rate of survival of strain IC41 compared with that of strain AB1157

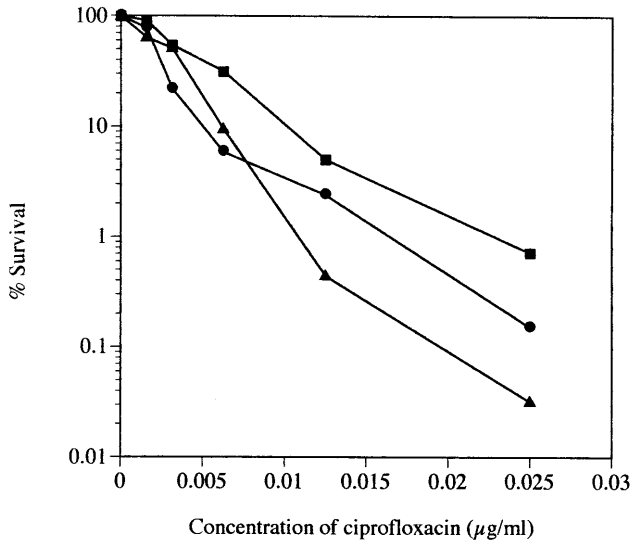

FIG. 1. Effect of plasmids carrying *mucAB* genes on the survival of strain AB1157 in the presence of ciprofloxacin under growing conditions. F, no plasmid; ■, pKM101; , pICV80.

agrees with previous results showing the role of the SOS system in the repair of quinolone damage (9).

Survival assays with ciprofloxacin were also performed under amino acid starvation conditions that did not allow either cell division or protein synthesis. In this case, the pKM101 plasmid did not decrease the susceptibility of strain AB1157 to ciprofloxacin treatment (Fig. 3). On the contrary, killing of AB1157 was greater in the presence of pKM101 than in its absence. The cause of this phenomenon remains unknown.

The results obtained with ciprofloxacin and its progenitor, nalidixic acid (data not shown), indicated that the synthesis of a product(s) different from MucAB that was encoded by plasmid pKM101 modified the susceptibility to quinolone of strain AB1157 growing in minimal medium.

Moreover, these data suggest that the interference of genes

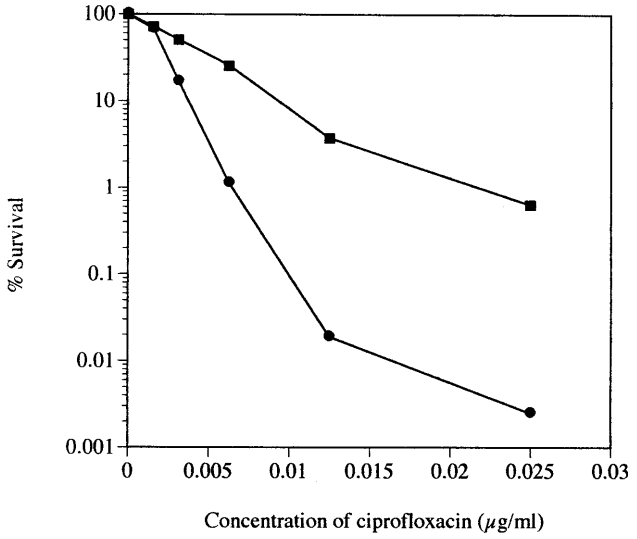

FIG. 2. Effect of pKM101 on the survival of the *recA430* strain AB1157 (IC41) in the presence of ciprofloxacin under growing conditions. F, no plasmid; ■, pKM101.

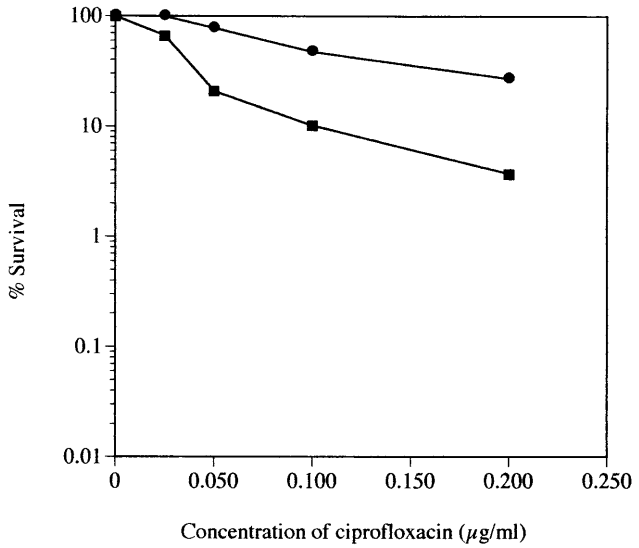

FIG. 3. Effect of pKM101 on the survival of strain AB1157 in the presence of ciprofloxacin under amino acid starvation conditions. F, no plasmid; ■, pKM101.

other than *mucAB* with the susceptibility of bacteria to certain chemicals such as 4-Qs could also modify the mutagenic response. According to this, a region of pKM101 carrying the *nuc* gene increases the sensitivity of *E. coli* to the antibiotic phleomycin E and affects its mutagenic response to this compound (8). Both findings reinforce the importance of replacement of pKM101 by plasmids containing only the *mucAB* operon in the *S. typhimurium* tester strains used in the Ames test for increasing the sensitivity of this assay.

Identification of the region of pKM101 that decreases the susceptibility to quinolone. Several plasmid derivatives containing different fragments of pKM101 were obtained and introduced into AB1157. The regions of pKM101 DNA retained in each of these plasmid constructions as well as their ability to confer ciprofloxacin resistance are indicated in Fig. 4. All derivatives were stable, with the exception of pUA400-u, which was highly unstable, since it underwent spontaneous curing and changes in its restriction patterns.

The pUA399 plasmid, which contains *mucAB*, did not promote the phenotype described above, whereas the pUA325 plasmid, lacking these genes, did. These results confirmed our previous supposition that a gene(s) different from *mucAB* was responsible for protection against quinolones. Survival assays with the other plasmid derivatives lead us to conclude that the 2.2-kb *Asn*I-*Nde*I fragment, encoding the *korB*, *traL*, *korA*, and *traM* genes, is sufficient to decrease the susceptibility of strain AB1157 to quinolone. Further attempts to delineate more accurately the minimal region that confers this phenotype were not successful, because the constructions were nonviable. For example, subcloning of the *Acc*I-*Hpa*I_1 and *Acc*I-*Hin*cII fragments was not possible in the absence of the *korA* gene in *trans* (data not shown).

Identification of the region of pKM101 responsible for the Slo phenotype. In the course of this study, we noted that the host strain AB1157 grew more slowly when carrying some of these plasmid constructions, and we investigated whether this could be related to the decreased susceptibility to ciprofloxacin.

The Slo phenotype was first described as the ability of pKM101 (or its parental plasmid R46) to make *E. coli* AB1157 form small colonies on defined minimal agar plates (11, 22). The fact that adenine and hypoxanthine and their nucleosides suppressed slow growth suggested that these plasmids interfered in the de novo purine synthesis pathway (22); however, at present, the basis of such interference in the AB1157 lineage has not been clarified. Earlier genetic experiments indicated that the *kilA* locus was responsible for the Slo phenotype (24); however, a specific gene has not been identified thus far (19).

Studies of the growth kinetics of AB1157 containing the different plasmid derivatives in AB minimal medium were performed, and the doubling time (τ) was determined from the slope of the linear region of curves of optical density versus time. The presence of pKM101 increased the τ of AB1157 from 42 to 65 min, and all of the other plasmids that conferred protection against quinolone also decreased the growth rate of this strain (2.2- to 3.4-fold) (Fig. 4). Similar results were obtained when τ was determined from the slope of the linear region of curves of viable cells versus time (data not shown). All of these data indicated that the 2.2-kb *Asn*I-*Nde*I fragment of pKM101 containing *korB*, *traL*, *korA*, and *traM* genes also provides the Slo phenotype and suggested that the slow growth rate could be related to the increase in cell survival. Moreover, our results indicate that the pKM101 fragment which carries the *korB*, *traL*, and *korA* genes is sufficient to promote the Slo phenotype because plasmid pUA323, carrying the *traM*, *traA*, and *traB* genes, is Slo^-. This agrees with previous results showing that the Slo phenotype was associated to the region between the *korB* and *korA* genes (24). We could not clearly demonstrate the minimal region of pKM101 responsible for the Slo phenotype because of nonviability of plasmid constructions without the *korB* and *korA* genes in *trans*. This is in agreement with Pohlman and coworkers (19), who have suggested that at least the product of the *traL* gene might be conditionally lethal in *E. coli*.

Suppression of slow growth also abolishes protection against quinolone by pKM101. The data presented above suggested that the Slo phenotype provided by this fragment of plasmid pKM101 could modify the susceptibility to quinolone. To test it, first we determined that the addition of adenine at 60 μg/ml to AB minimal medium was necessary and sufficient to suppress slow growth by the pUA518 plasmid, which contains the 2.2-kb *Asn*I-*Nde*I fragment that confers an increase in the rate of survival in the presence of quinolone (Fig. 5). Thus, the presence of adenine reduced the doubling time of AB1157 carrying pUA518 from 116 to 39 min, which was similar to that of strain containing the pK184 vector either in the absence (τ, 44 min) or in the presence (τ, 42 min) of the nucleotide. Afterwards, we studied the effect of the addition of adenine (60 μg/ml) on the survival of strain AB1157 in the presence of ciprofloxacin. The rate of survival of AB1157 carrying pUA518 growing in the presence of adenine was much lower than that in its absence and was quite similar to that of strain AB1157 carrying pK184 regardless of the presence of the nucleotide (Fig. 6). These data provide evidence that the slow growth rate promoted by this 2.2-kb region of pKM101 in AB1157 results in a minor susceptibility to quinolone in this strain.

In agreement with this finding, the results of other groups bear out the importance of the stage of the cell cycle and the growth rate in the bactericidal activity of these compounds. 4-Qs less effectively kill nondividing *E. coli* (20, 26, 27), *Enterococcus faecalis* (13), and staphylococci (14) than exponentially growing cells. Reduced killing of *Pseudomonas aeruginosa* in the stationary phase of growth by 4-Qs has also been reported elsewhere (6). Likewise, the bactericidal activity of ciprofloxacin is reduced as growth rates decrease in chemostat cultures (7). Furthermore, a specific correlation between the generation time of *E. coli* growing at different temperatures

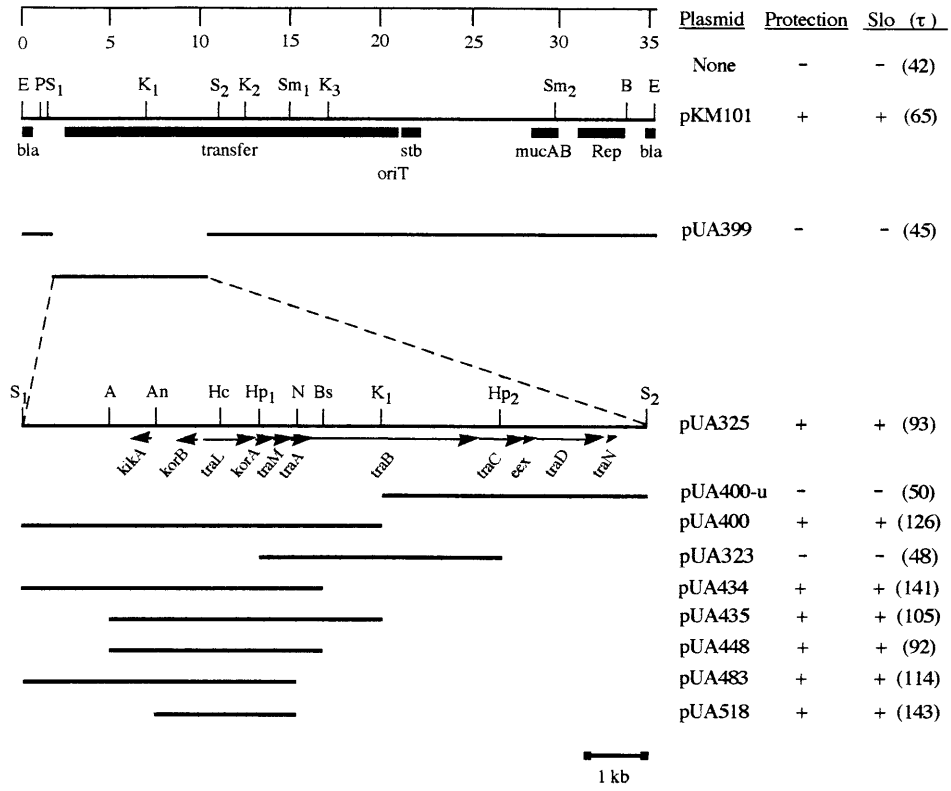

FIG. 4. Plasmid derivatives containing different fragments of pKM101 tested for their abilities to decrease susceptibility to quinolone and to promote the phenotype Slo in strain AB1157. The numbers in parentheses (τ) are the doubling times expressed in minutes. + and −, ability and inability, respectively, to confer protection against quinolone or result in a Slo phenotype; single horizontal lines, DNA from pKM101 remaining on each plasmid construction. Physical and genetic maps of pKM101 are based on those reported earlier (19, 23). The top line indicates kilobase coordinates on pKM101. *bla*, β-lactamase; Rep, origin of replication; *stb*, gene required for stability in recombination-proficient hosts. The transfer region includes a cluster of *tra* genes involved in the synthesis of pilus, a mobilization gene cluster, the origin of transfer (*oriT*) lying at one end of this cluster, the *kikA* gene (probably sufficient to kill a conjugal recipient strain of *Klebsiella oxytoca*), and the *eex* gene (for the conjugal recipient entry exclusion of other IncN plasmids). Arrows, directions of transcription of genes. Only relevant restriction enzymes sites are indicated. Abbreviations: A, *Acc*I; An, *Asn*I; B, *Bam*HI; Bs, *Bst*EII; E, *Eco*RI; Hc, *Hin*cII; Hp, *Hpa*I; K, *Kpn*I; N, *Nde*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.

and the rate of killing after treatment with norfloxacin has been found (5). Our study confirms these observations, providing a clear picture of how slow growth, which is produced in this case by a plasmidic product(s), results in higher rates of survival in the presence of 4-Qs and how the suppression of the Slo phenotype also abolishes this effect on drug susceptibility.

Plasmid-mediated resistance to 4-Qs has not yet been demonstrated up to date. However, in this work, we report that the

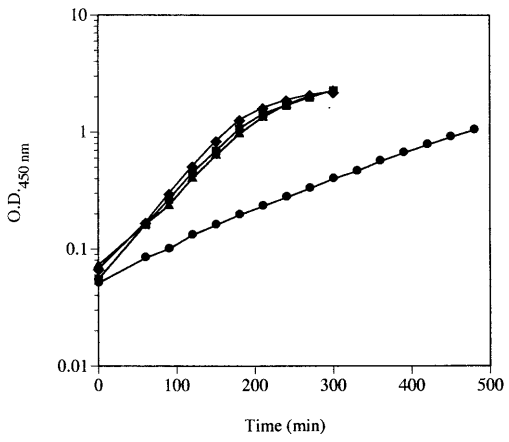

FIG. 5. Growth kinetics of strain AB1157 carrying the pK184 vector (and ■) and pUA518 plasmid (F and }) in AB minimal medium without (and F) and with (■ and }) adenine at 60 μg/ml. OD_{450}nm, optical density at 450 nm.

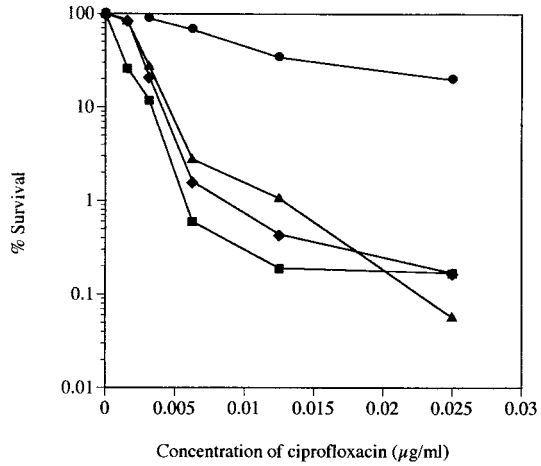

FIG. 6. Survival of strain AB1157 carrying the pK184 vector (and ■) and pUA518 (F and }) in AB minimal medium without and with adenine at 60 μg/ml.

interference of a naturally occurring plasmid in cellular metabolism decreases bacterial susceptibility to treatment with these DNA gyrase inhibitors. Experiments are in progress to further elucidate the molecular basis of the Slo phenotype as well as to determine the relationship between slow growth rate and quinolone susceptibility.

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