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# Effect of Subinhibitory Concentrations of Antibiotics on Intrachromosomal Homologous Recombination in *Escherichia coli*[▽]

Elena López and Jesús Blázquez*

*Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-CSIC, Campus UAM—Cantoblanco, 28049 Madrid, Spain*

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**Subinhibitory concentrations of some antibiotics, such as fluoroquinolones, have been reported to stimulate mutation and, consequently, bacterial adaptation to different stresses, including antibiotic pressure. In *Escherichia coli*, this stimulation is mediated by alternative DNA polymerases induced via the SOS response. Sublethal concentrations of the fluoroquinolone ciprofloxacin have been shown to stimulate recombination between divergent sequences in *E. coli*. However, the effect of ciprofloxacin on recombination between homologous sequences and its SOS dependence have not been studied. Moreover, the possible effects of other antibiotics on homologous recombination remain untested. The aim of this work was to study the effects of sublethal concentrations of ciprofloxacin and 10 additional antibiotics, including different molecular families with different molecular targets, on the rate of homologous recombination of DNA in *E. coli*. The antibiotics tested were ciprofloxacin, ampicillin, ceftazidime, imipenem, chloramphenicol, tetracycline, gentamicin, rifampin (rifampicin), trimethoprim, fosfomycin, and colistin. Our results indicate that only ciprofloxacin consistently stimulates the intrachromosomal recombinogenic capability of homologous sequences in *E. coli*. The ciprofloxacin-based stimulation occurs at concentrations and times that apparently do not dramatically compromise the viability of the whole population, and it is dependent on RecA and partially dependent on SOS induction. One of the main findings of this work is that, apart from quinolone antibiotics, none of the most used antibiotics, including trimethoprim (a known inducer of the SOS response), has a clear side effect on homologous recombination in *E. coli*. In addition to the already described effects of some antibiotics on mutagenicity, DNA transfer, and genetic transformability in naturally competent species, the effect of increasing intrachromosomal recombination of homologous DNA sequences can be uniquely ascribed to fluoroquinolones, at least for *E. coli*.**

In *Escherichia coli*, DNA-damaging agents, like ciprofloxacin (CIP), trigger the SOS response, which involves the induction of *recA* transcription. Contact with single-stranded DNA activates the coprotease activity of the RecA protein, promoting the self-cleavage of LexA, the SOS transcriptional repressor, and leading to the SOS response (24, 26). The autogenous control of *lexA* transcription supports a cellular response that is exquisitely proportional to the DNA damage level and prevents false triggering of the SOS response (8). RecA has multiple functions that affect different cellular processes, such as the rescue of stalled replication forks (12, 28) and coprotease action involved in the autocleavage of LexA and UmuD (17). Some antibiotics, such as fluoroquinolones, increase the frequency of mutants by inducing the SOS response in bacteria and the subsequent production of error-prone DNA polymerases (43). Thus, the phenomenon of SOS mutagenesis may also influence the appearance of antibiotic-resistant bacteria (10, 11). Another function of RecA is to promote homologous recombination between cDNA strands (20), which is crucial for the survival and evolution of bacterial cells. Intragenomic recombination helps to repair collapsed replication forks (30) and, as a consequence, may produce gene or operon rearrangements.

Recombination is a major driving force in bacterial evolution and survival (19, 20, 30, 40). Except for plasmids, which can replicate autonomously, and transposable elements, which do not require homologous recombination to be installed in the chromosome, the horizontally transferred DNA must be integrated into the bacterial chromosome by recombination. From an evolutionary point of view, the strategy of producing novel traits by recombinational shuffling may produce shortcuts to develop novel capacities, allowing microorganisms to accelerate the evolutionary process. This strategy is very efficient and may result in the development of novel abilities in a single or a few steps.

Some antibiotics, such as fluoroquinolones and beta-lactams, have been shown to increase the frequency of mutants by inducing the SOS response in bacteria (31, 36, 43). Recently, we demonstrated that sublethal concentrations of the cephalosporin ceftazidime (CAZ), an inhibitor of cell wall synthesis, elicit adaptive responses, including increases in mutation frequency, in *Pseudomonas aeruginosa* (5). Antibiotics may also act as promoters of the transference of pathogenicity island-encoded virulence factors (42) and the mobilization of integrating conjugative elements in *Vibrio cholerae* (3), suggesting that mobile genetic elements respond to stress by regulating their escape from damaged bacterial hosts. Some antibiotics

* Corresponding author. Mailing address: Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología-CSIC, Campus UAM—Cantoblanco, 28049 Madrid, Spain. Phone: 34 91 585 54 33. Fax: 34 91 585 45 06. E-mail: blazquez@cnb.csic.es.

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TABLE 1. Effects of different antibiotics on homologous recombination of strain ME12

| Drug | MIC (μg/ml) | Increase in recombination frequency (fold) ± SD[a] | | | |
|---|---|---|---|---|---|
| | | 1/4 MIC | 1/2 MIC | 1× MIC | 2× MIC |
| CIP | 0.16 | 2.00 ± 0.93 | 2.22 ± 1.19 | 5.80 ± 2.72[a] | 14 ± 7.72[a] |
| Ampicillin | 1 | 1.68 ± 0.78 | 1.29 ± 0.36 | 1.27 ± 0.36 | 1.28 ± 0.64 |
| CAZ | 0.32 | 1.76 ± 0.59 | 1.79 ± 0.52[a] | 1.62 ± 1.11 | 1.02 ± 0.31 |
| Imipenem | 0.05 | 1.43 ± 0.51 | 1.33 ± 0.71 | 0.78 ± 0.04 | 1.18 ± 0.35 |
| Chloramphenicol | 2 | 1.62 ± 0.79 | 1.29 ± 0.34 | 1.77 ± 1.03 | 0.89 ± 0.22 |
| Tetracycline | 0.5 | 0.96 ± 0.81 | 0.81 ± 0.05 | 0.97 ± 0.32 | 1.14 ± 0.58 |
| Gentamicin | 0.5 | 0.59 ± 0.39 | 0.51 ± 0.25 | 1.02 ± 0.42 | 0.46 ± 0.18 |
| Rifampin | 2 | 1.44 ± 1.20 | 1.30 ± 0.72 | 1.31 ± 1.28 | 1.67 ± 1.87 |
| Trimethoprim | 0.25 | 1.28 ± 0.42 | 1.38 ± 0.39 | 1.20 ± 0.34 | 1.49 ± 0.46 |
| Fosfomycin | 0.06 | 1.18 ± 0.36 | 1.10 ± 1.15 | 1.33 ± 0.82 | 1.34 ± 1.18 |
| Colistin | 2 | 1.83 ± 1.10 | 1.05 ± 1.06 | 1.33 ± 1.56 | 1.23 ± 0.80 |

[a] Statistically significant variation ($P < 0.05$).

also induce DNA transformation, via competence, in the naturally competent *Streptococcus pneumoniae* (38). CIP, a broad-spectrum antibiotic that blocks DNA replication by trapping DNA gyrase and DNA topoisomerase IV on DNA (15), has been recently described as a stimulator of recombination between divergent DNA sequences in *E. coli* (25). However, the possible effects of other antibiotics on the recombination frequency of bacteria remain to be studied.

In this work, we explore whether a representative group of antibiotics, including different molecular families with different molecular targets, may contribute to genetic variation by affecting homologous recombination in *E. coli*. In addition to the work previously developed with CIP (25), we have analyzed the effects of peri-MIC concentrations of CIP on homologous recombination and how RecA and the induction of the SOS response affect CIP-based stimulation of recombination.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The following *E. coli* K-12 strains were used: ME12 (MG1655 *lacZ*ΔT-*lacZ*ΔP-*yfp*), ME12 Δ*recA*::*kan*, and ME12 *lexA1* (ME12 *lexA1 malB*::Tn*9*) derivatives (16) and scavenger cells (MG1655 Δ*lacZ*::*cat*) (16). The construction of ME12 strains was previously described (16, 25). Both *recA* and *lexA1* phenotypes were verified by measuring UV sensitivity. Luria broth (LB) and minimal M9 medium with lactose as the sole carbon source were prepared according to the method of Miller (32). The MICs of antibiotics for ME12 and its mutant derivatives were determined according to NCCLS recommendations (33), except that the bacterial inocula were identical to those used in all subsequent recombination experiments. Chloramphenicol was used for selection at 20 μg/ml. Antibiotics tested for stimulation of recombination were used at different concentrations around their MICs. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were used at concentrations of 40 μg/ml and 1 mM, respectively.

**Recombination experiments.** Recombination frequencies were studied as described previously (16, 25). Briefly, two nonfunctional *lacZ* alleles were cloned in close proximity (569 bp) in the *E. coli* chromosome. The first *lacZ* gene contains a C-terminal deletion (*lacZ′* Δ*C*), whereas the second contains an N-terminal deletion (*′lacZ* Δ*N*). The two alleles share overlapping DNA regions of 1.3 kb that are 100% identical at the sequence level. The functional *lacZ* gene can be reconstituted by a single recombination event between the two gene fragments cited. Recombination events can be visualized as colonies able to grow on minimal M9 medium with lactose as the sole carbon source. For the $Lac^+$ recombinant measurement, aliquots of 2 ml of exponentially growing cells ($10^8$ cells/ml) were incubated with different concentrations of antibiotics for 4 h at 37°C with shaking (250 rpm). One milliliter of these cultures was centrifuged for 10 min at 6,000 rpm, and the pellet was resuspended in 2 ml of fresh LB medium and incubated overnight at 37°C with shaking. This step was necessary to resolve the filaments formed after CIP, CAZ, and trimethoprim treatment. To prepare the recombinant test plates, scavenger cells were grown to saturation in LB medium. The resulting culture was diluted, and a new culture was started in fresh LB medium. When this culture reached saturation, about $10^8$ scavenger cells were washed three times with 10 mM $MgSO_4$ (to clear any contaminating sources of sugar) and were inoculated in 4 ml of soft M9 minimal medium agar containing X-Gal and IPTG. The cells were spread on M9 minimal medium agar plates containing X-Gal, IPTG, and lactose as the sole carbon source. After 1 hour at room temperature, appropriate dilutions of the antibiotic-treated cultures, washed with 10 mM $MgSO_4$, were inoculated in 4 ml of soft M9 minimal medium agar containing X-Gal and IPTG and spread over the scavenger cells. The plates were incubated for 48 h at 37°C. Viable cells were determined by plating appropriate dilutions on LB agar plates. Recombination events were measured as the production of $Lac^+$ colonies. The frequency of recombination was calculated as the number of $Lac^+$ colonies per viable cell. At least three independent experiments were performed for each antibiotic concentration. For the experiments with *recA* and *lexA1* mutants, between six and nine independent experiments were performed.

**Statistical analysis.** Statistical evaluation was done by using the Mann-Whitney U test when two groups were compared or Kruskal-Wallis analysis for more than two groups. Differences were considered significant for $P$ values of $<0.05$.

**Effect of antibiotics on the growth of *E. coli*.** For the viability measurements after antibiotic treatments, appropriate dilutions from the above-described 4-hour antibiotic-treated cells were plated on LB plates, and the colonies were scored after 24 h.

## RESULTS

**Effects of different concentrations of 11 antibiotics on homologous recombination.** In a previous work, we demonstrated that low concentrations of CIP stimulate recombination of divergent DNA sequences in *E. coli* (25). In this work, we extended the study to 10 other antibiotics. Table 1 shows the effects of different concentrations of the tested antibiotics on the homologous-recombination frequency. The table also indicates the MIC of each antibiotic under our testing conditions. CIP clearly stimulates homologous recombination in *E. coli* at different concentrations. Treatment with CAZ stimulated homologous recombination very slightly (close to twofold), though significantly. None of the other nine antibiotics showed any effect on homologous recombination.

**Effects of RecA and SOS response on the CIP-mediated stimulation of recombination.** Only CIP produced a clear stimulation of homologous recombination (Table 1). Thus, we studied the molecular basis of this stimulation. RecA is, as expected, absolutely necessary for the stimulation of homologous recombination (Table 2). The same requirement was previously demonstrated for recombination of divergent sequences in *E. coli* (25). On the other hand, in a mutant unable

TABLE 2. Effects of lack of RecA (ME12 *recA*) and the inability to induce the SOS response (ME12 *lexA1*) on the stimulation of homologous recombination

| Strain | Recombination frequency ± SD (no CIP) | Increase in recombination frequency (fold) with CIP ± SD[a] | | |
|---|---|---|---|---|
| | | 1/4 MIC | 1/2 MIC | MIC |
| ME12 | $6.9 \times 10^{-3} \pm 3.8 \times 10^{-3}$ | 2.00 ± 0.93 | 2.22 ± 1.19 | 5.80 ± 2.72[b] |
| ME12 *recA* | $3.5 \times 10^{-3} \pm 1.4 \times 10^{-3}$ | 0.82 ± 0.38 | 1.05 ± 0.79 | 1.09 ± 0.68 |
| ME12 *lexA1* | $2.0 \times 10^{-3} \pm 0.6 \times 10^{-3}$ | 1.84 ± 0.71 | 2.66 ± 1.29[b] | 3.12 ± 1.42[b] |

[a] The values are the increases produced over the baseline of the corresponding strain.
[b] Statistically significant value ($P < 0.05$).

to develop the SOS response (*lexA1*), CIP still stimulated homologous recombination, although to a lesser extent than in the wild-type strain. This result indicates that CIP-dependent stimulation of homologous recombination is partially dependent on SOS induction. This suggests that the CIP-mediated stimulation does not require SOS induction, yet the induction further increases the level of stimulation (probably via the increased levels of RecA).

Interestingly, the level of homologous recombination in the absence of antibiotic challenge was only reduced to one-half in the *recA* mutant (Table 2). This suggests that half of the events of reconstitution of the *lacZ* gene were probably caused by polymerase slippage. However, the CIP-based stimulation of recombination was due to true recombinational events, as it was abolished in the *recA* mutant.

As stated above, treatment with CAZ stimulated homologous recombination very slightly (close to twofold), though significantly. This stimulation seems to be dependent on both RecA and SOS induction, as the stimulation was lost in both *recA* and *lexA1* mutants, although the small increase in homologous recombination did not allow appropriate measurements (data not shown).

**Effects of sublethal concentrations of antibiotics on *E. coli* growth.** Figure 1 shows the effects produced on the growth of *E. coli* cells by 4 hours of treatment with different concentrations of all the antibiotics used. Treatment with ampicillin, CAZ, imipenem, colistin, gentamicin, and rifampin (rifampicin) produced very small effects on bacterial growth, even at concentrations two times the MIC. Treatment with tetracycline, chloramphenicol, CIP, and trimethoprim also produced small effects at concentrations below the MIC, with full reduction of growth at the MIC or higher. Finally, fosfomycin produced the most dramatic effect, with a drastic reduction of growth at one-fourth the MIC and a clear killing effect at concentrations of one-half the MIC and higher. At the MIC and 2× MIC, fosfomycin killed more than 99.999% of the population.

Because the number of viable bacteria in the inoculum might have affected the observed frequency of recombination, we performed experiments with different inoculum sizes of un-

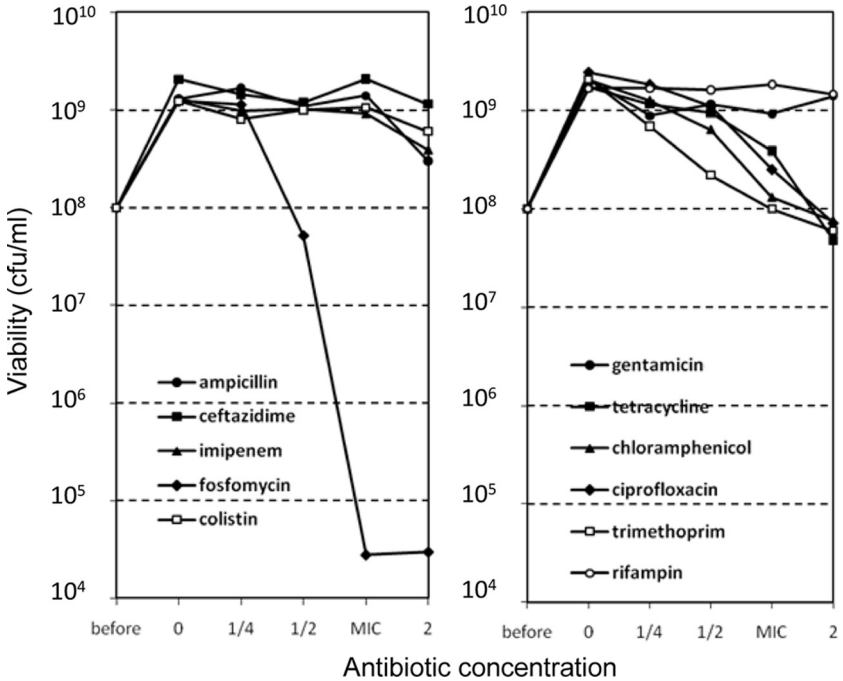


FIG. 1. Effects of peri-MIC concentrations of 11 antibiotics on the viability of strain ME12 after 4 h of treatment. Viability is expressed as the number of CFU per ml. Viability before the antibiotic treatment (i.e., time zero) is indicated as "before." The concentrations of antibiotics are expressed relative to the MIC of each antibiotic (Table 1).

treated ME12 cells, ranging from $10^9$ to $10^5$ cells. No differences were observed in the recombination frequencies (not shown).

In conclusion, the viability of the cultures treated with different antibiotics is not the cause of the stimulation or lack of stimulation of homologous recombination.

## DISCUSSION

It is now clear that the extended use of antibiotics over the past 6 decades has had a major impact on human pathogens, leading to the selection and spread of resistant bacteria. The exposure of bacteria to antibacterial agents results in the selection of preexisting resistant variants that ultimately become fixed in the population (23, 27, 34). However, some studies have suggested that bacteria are not simply passive subjects during the process of evolution by mutation and natural selection (4, 9, 39). For instance, it has been reported that fluoroquinolone antibiotics, by means of SOS induction, may stimulate mutations and the horizontal transfer of DNA sequences (3, 5, 10, 36, 37, 42, 43). Antibiotic stress induces transformability via competence in *S. pneumoniae* (38), and antibiotic pressure may also select for cells with an increased frequency of mutation (hypermutators) (29).

The evolution of antibiotic resistance is based on genetic variation and selection of the genotypes generated by this variation. In nature, the spontaneous generation of genetic variation in bacteria is based on the strategies of small local changes in the nucleotide sequence of the genome (mutation), intragenomic reshuffling of genomic sequences (intrachromosomal recombination), and the acquisition of DNA sequences from other organisms via horizontal gene transfer. Horizontal gene transfer is a major diversification mechanism in prokaryotes (19, 21, 22), and recombination is crucial to different traits, allowing bacteria to evade the host immune response, distributing genes that increase virulence, and providing increased resistance to antibiotics (14).

Recombination has had a major impact on bacterial evolution (18, 35, 40, 43). This impact may be even greater than that of mutation, as, for *E. coli* in nature, any single nucleotide change is about 50 times more likely to have occurred by recombination than by a de novo mutation (19).

In a previous work, we demonstrated that low concentrations of the fluoroquinolone antibiotic CIP stimulate recombination of homologous and divergent DNA sequences in *E. coli* (25). The stimulation of divergent sequences required the presence of the recombinase RecA. However, despite CIP being a good inducer of the SOS response, the stimulation of recombination was, curiously, independent of the induction of this response. The results presented here demonstrate that the CIP-based stimulation of homologous recombination occurs at concentrations and times that apparently do not dramatically compromise the viability of the whole population and that it is dependent on RecA and partially dependent on SOS induction.

The fact that other antibiotics stimulate adaptive responses, including mutation, competence, and DNA transference, led us to perform an exhaustive study of the effects of some antibiotics, representing several molecular families with different molecular targets, on the rate of homologous recombination of DNA.

Here, we have shown that none of the 10 additional antibiotics tested in addition to the fluoroquinolone CIP (an inhibitor of type II DNA topoisomerases) produced a stimulation of homologous recombination. Only the cephalosporin CAZ (an inhibitor of cell wall synthesis) produced a slight stimulation of recombination in *E. coli*. However, the effect of CAZ is so weak that it probably has no effect on the evolution of antibiotic resistance in *E. coli*.

Our results show that only a small window of antibiotic concentrations stimulates recombination. However, due to different factors, a huge diversity of spatial and temporal antibiotic concentration gradients may occur in the human body (2). Consequently, any recombination-stimulating concentration is probably not difficult to find. Moreover, although antibiotics are mainly used to combat pathogens, they also challenge commensals collaterally. Thus, there are vast numbers of bacteria challenged by antibiotics. Infections are usually caused by a relatively small number of cells ($10^8$ to $10^9$); however, about $10^{14}$ prokaryotic cells belonging to hundreds of different species compose our commensal flora (1), and these species have different intrinsic levels of antibiotic susceptibility (44). Thus, some treatments with fluoroquinolones may modify genetic recombination in treated bacteria, increasing the probability of acquiring and/or developing new antibiotic resistance. For instance, fluoroquinolone treatments may accelerate the evolution of new extended-spectrum β-lactamase variants by stimulating recombination between single mutants instead of accumulating successive mutations (13). This is a very likely scenario, as extended-spectrum β-lactamases are the result of combining a reduced number of mutations (6, 7), and the isolation of bacteria producing multiple β-lactamases simultaneously is not rare (41).

The main finding of this work is that, apart from quinolone antibiotics, none of the tested antibiotics (including the ones most used clinically) has a side effect on the recombinogenic capacity of *E. coli*. However, an effect of some antibiotics at concentrations different from those tested here cannot be ruled out. In addition to the already described effects of some antibiotics on mutagenicity, increased DNA transfer from treated donor bacteria, and increased genetic transformability in naturally competent species, the increased genetic recombination of DNA sequences can be uniquely ascribed to fluoroquinolones, at least for *E. coli*.

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