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Ciprofloxacin-Mediated Mutagenesis Is Suppressed by Subinhibitory Concentrations of Amikacin in *Pseudomonas aeruginosa*

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ABSTRACT Resistance to antibiotics is a global health problem. Activation of the SOS response, and the subsequent elevation in mutagenesis, contributes to the appearance of resistance mutations. Among currently used drugs, quinolones are the most potent inducers of the SOS response. In the present study, we show that amikacin inhibits ciprofloxacin-mediated SOS induction and mutagenesis in *Pseudomonas aeruginosa*.

KEYWORDS *Pseudomonas aeruginosa*, SOS response, ciprofloxacin, *recA*

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Antibiotics may cause genetic changes involving different pathways, and one of them is the induction of error-prone polymerases mediated by SOS response (1, 2). Ciprofloxacin (CIP), one of the antimicrobials of choice for the treatment of *Pseudomonas aeruginosa*, induces the SOS response (3–5) by interfering with gyrase or topoisomerase activity (6, 7). The SOS regulon controls 15 genes, including *imuABC* and *dinB* (4, 5), which encode error-prone polymerases (8, 9). By inducing the SOS response, ciprofloxacin increases mutagenesis (e.g., see references 10 and 11), facilitating the appearance of drug resistance.

We investigated the effect of antibiotic combinations on the SOS response and mutagenesis induced by ciprofloxacin in *P. aeruginosa* PAO1. *recA* is among the SOS-regulated genes in this organism (4, 5). To analyze the induction of the SOS response, we constructed a chromosomal P*recA-lux* reporter. The regulatory region upstream from *recA* (−501 bp relative to the start codon) was cloned into the pUC18T-mini-Tn*7*T-*lux*-Gm plasmid (12) and transferred to *P. aeruginosa* for integration at *att*Tn*7*, resulting in strain *att*Tn*7*::P*recA-lux*. The effect of antibiotics on the expression of the reporter was evaluated in solid medium using disk-based qualitative assays. The P*recA-lux* strain was diluted to an optical density at 260 nm (OD_{600}) of 0.1. Fifty microliters of this dilution was seeded on Mueller-Hinton (MH) agar, and test antibiotic disks and CIP (5 μg) disks (Sensifar-Cefar, Brazil) were placed close to one another to observe the effect of the antibiotic interaction on *recA* expression. Luciferase activity was detected in the ChemiDoc MP system (Bio-Rad, USA).

Amikacin (AMI), imipenem, meropenem, polymyxin B, ceftazidime, cefepime, and aztreonam were tested, representing different drug classes. We found that amikacin is not an inducer of the SOS response but is in fact a strong inhibitor of *recA* induction by sub-MICs of ciprofloxacin (Fig. 1A). Amikacin is an aminoglycoside derived from kanamycin (13). Aminoglycosides bind, with high affinity, to the A-site on the 16S rRNA of the 30S ribosome (14) and can cause mRNA decoding errors, block mRNA and tRNA translocation, and inhibit ribosome recycling (15).

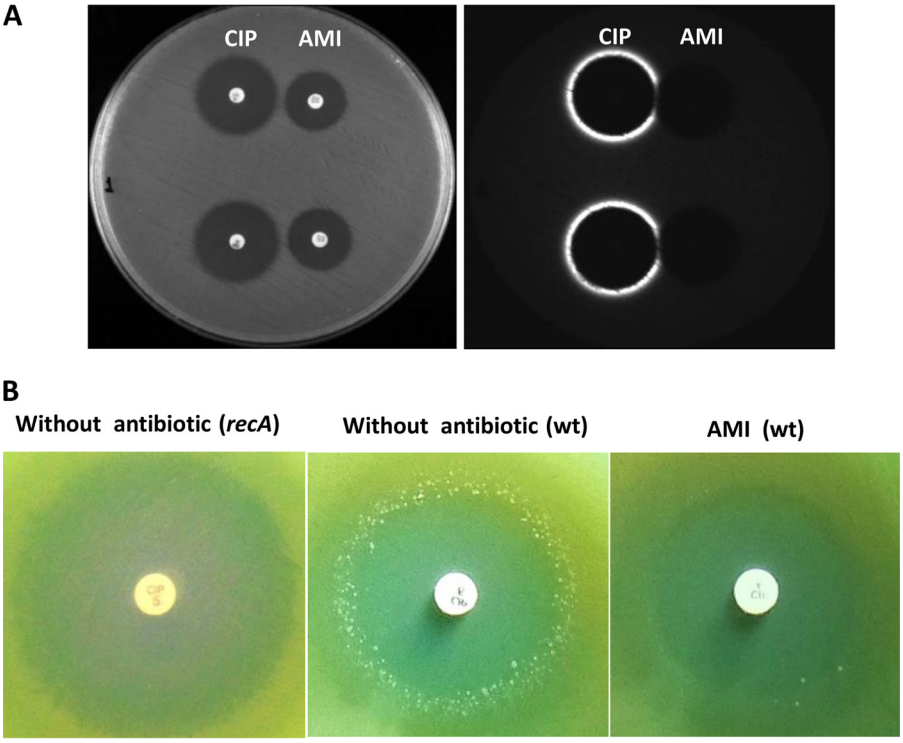

FIG 1 Effect of amikacin on the transcription of P*recA-lux* and mutagenesis on a solid medium. (A) Sub-MICs of ciprofloxacin induce the expression of the P*recA-lux* reporter, and when the amikacin disks are in proximity to the ciprofloxacin disk, a decrease in the light ring is observed, indicating inhibition of *recA* induction. (B) A large number of Cip^r mutants in the clearing zone can be observed when the PAO1 strain is plated on MH agar but not when the *recA* derivative is analyzed. Addition of a sub-MIC amikacin concentration (0.4 μg/ml) to the medium decreases the appearance of Cip^r mutants. A representative result is shown for plates incubated for 72 h.

We tested whether this effect on SOS induction reflects a general antagonistic effect of both drugs, using the checkerboard assay (16), in the presence of CIP (0.0078 to 0.5 μg/ml) and AMI (0.003 to 4 μg/ml). A fractional inhibitory concentration (FIC) index of 0.6 (CIP+AMI) was observed, indicating no antagonism. Accordingly, no apparent antagonism is seen in MH agar (Fig. 1A). The MIC of amikacin (0.5 μg/ml) and ciprofloxacin (0.06 μg/ml) was determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) (17).

The appearance of Cip^r mutants in cells exposed to ciprofloxacin in solid medium was monitored using a previously established assay (18). Fifty microliters of cultures (OD_{600} = 0.1) was seeded on MH agar, and a ciprofloxacin disk (5 μg) was placed in the center of the plate and incubated at 37°C for 72 h. The wild-type strain presents a high number of resistant colonies in the clearing zone. However, when sub-MIC amikacin was added to the medium (0.4 μg/ml), a dramatic reduction in the appearance of Cip^r mutants occurred (Fig. 1B). Eleven Cip^r mutants were grown on plates containing 0.125 μg/ml CIP, with or without 0.4 μg/ml of amikacin, and all formed visible colonies after 24 h in both conditions (data not shown). These results rule out an inability of Cip^r mutants to grow in the presence of subinhibitory levels of amikacin, suggesting that the acquisition of mutations is inhibited.

Inactivation of *recA* in *Escherichia coli* reduces mutagenicity (11) and increases the activity of a large number of antimicrobials in other bacteria (19, 20). To determine if mutations arise in a *recA*-dependent manner, a *recA* mutant strain was constructed by insertional mutagenesis. An internal region of the *recA* gene (from bases 90 to 594 of the coding region) was cloned into the pKNOCK-Tc suicide plasmid (21), transferred to *P. aeruginosa* by conjugation using the *E. coli* S17-1 λpir, and the integration was confirmed by PCR. The *recA* mutant strain shows a substantial reduction in the number

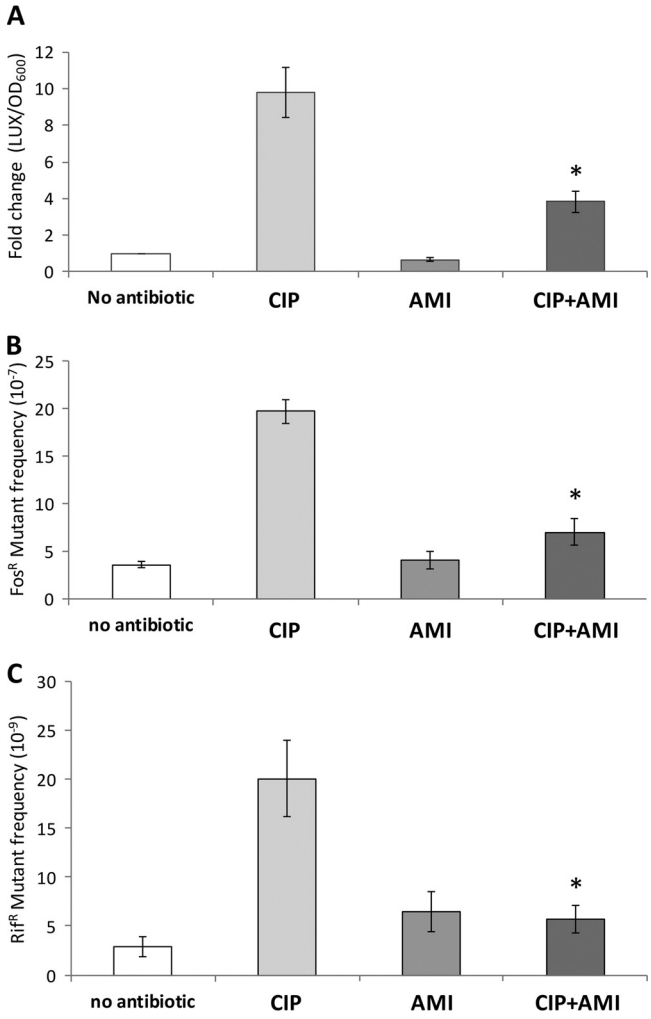

FIG 2 Quantitative analysis of amikacin's effect on the SOS response. (A) The luminescence activity of P*recA-lux* values was normalized by the optical density of the cultures (Lux/OD_{600}), and the results shown represent the fold change compared to control (no antibiotic) after 4 h of treatment. *, significant difference between CIP and CIP+AMI (*t* test; $P < 0.05$). Error bars represent the mean ± standard deviation of three independent determinations. Fos^r (B) and Rif^r (C) mutant frequencies after treatment with different antibiotics. Data shown represent the mean mutant frequencies of 30 cultures from three independent experiments, and the error bars show the standard error. Asterisks indicate the significant difference between CIP and CIP+AMI treatments determined by the Mann-Whitney rank sum test with a *P* value of ≤0.05.

of Cip^r mutants, as well as a larger zone of growth inhibition. MIC determination showed that the *recA* strain is more sensitive to ciprofloxacin (MIC = 0.007 μg/ml) than the wild-type strain.

We conducted a quantitative analysis of the P*recA-lux* expression in the absence (control) and presence of ciprofloxacin and amikacin (Fig. 2A). Saturated cultures were diluted 1:10,000 in MH medium, incubated at 37°C with shaking until reaching an OD_{600} of ~0.3. At this point, antibiotics were added, and cells were incubated for 4 h at 37°C. One-hundred-microliter aliquots of these cultures were withdrawn to measure both luciferase activity and OD_{600} using the GloMax-Multi+ microplate multimode reader detector (Promega, USA). We noted a 9-fold increase in *recA* expression after treatment with ciprofloxacin and no change in the presence of amikacin. Nevertheless, the combined treatment with ciprofloxacin plus amikacin promotes only a 3.8-fold increase in *recA* expression. Therefore, SOS induction by ciprofloxacin is reduced by more than 2-fold due to the presence of amikacin in liquid medium. Additionally, growth was monitored in the same conditions during the 4-h treatment. Growth yields presented

the following OD_{600} values after 4 h of growth: 4.8 (MH without any antibiotic), 3.9 (AMI), 1.9 (CIP), and 1.5 (CIP+AMI) (data not show). The small reduction in growth yield in the presence of CIP+AMI compared to CIP alone indicates that the reduction in *recA* expression was not caused by an effect on growth.

Next, we investigated the mutagenic effects of exposure to ciprofloxacin, amikacin, and a combination of both in *P. aeruginosa* PAO1 by scoring the appearance of rifampin and fosfomycin-resistant mutants (Rif^r and Fos^r) (Fig. 2B and C). After 4 h of treatment as described above, 2.5 ml of these cultures was centrifuged, resuspended in 5 ml of fresh MH medium, and cultivated overnight. Mutant frequencies were calculated as the total number of resistant colonies per viable cells in each culture. Cells treated with ciprofloxacin present 5.5-fold and 6.9-fold increases in the frequency of Fos^r and Rif^r mutants, respectively, compared to the nontreated control. Treatment with amikacin alone did not increase the mutant frequency in both markers. However, when both antibiotics were added, the mutant frequency decreases to 1.9-fold/2-fold (Fos^r/Rif^r) compared to the nontreated control. Thus, mutagenesis was increased by treatment with ciprofloxacin, but this effect is counteracted by the presence of amikacin.

Studies in *E. coli* showed that fluoroquinolones, β-lactams, trimethoprim, and sulfamethoxazole induce *recA* expression, whereas aminoglycosides, tetracycline, and chloramphenicol do not (11). However, in *Vibrio cholerae*, *Klebsiella pneumoniae*, and *Photorhabdus luminescens*, sub-MICs of aminoglycosides induce SOS-dependent promoters (22, 23). Here, we confirmed that ciprofloxacin induces the *recA* promoter activity in *P. aeruginosa* and reported the inhibitory effect of amikacin on this induction, which causes a decrease in mutagenesis.

The search for drugs that can prevent SOS induction is of considerable interest since suppression of this response may reduce the emergence of antibiotic-resistant bacteria. Examples of suppressors of the SOS response induced by fluoroquinolones were reported in Gram-positive and Gram-negative bacteria, such as the polyphenols baicalein (24), curcumin (25), and suramin (polysulphonated naphthylurea) (26), which have the ability to disassemble RecA single-stranded DNA filaments. Novobiocin blocks the ATP-binding site of the GyrB and inhibits ciprofloxacin and UV-induced SOS response (27, 28). The small-molecule N6-(1-naphthyl)-ADP acts as an ATP competitor, which prevents the formation of the RecA-DNA filament that is essential for all RecA-associated functions (29). Interestingly, sublethal concentrations of amikacin prevent FtsZ polymerization in *E. coli* (30), and perhaps the same phenomenon could happen with RecA nucleofilaments. The nature of amikacin-mediated inhibition of *recA* expression and mutagenesis is still not known, and future studies are still needed.

Treatment of *P. aeruginosa* clinical isolates with ciprofloxacin in combination with amikacin or gentamicin showed either synergistic, additive, or indifferent effects (31–33). Our results with the PAO1 strain are consistent with these previous observations since we observed no antagonistic effect of the ciprofloxacin-amikacin combination. In this regard, such a combined therapy may be used with the goal of inhibiting the SOS response and the development of resistance through mutagenesis.

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