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Effect of RecA inactivation and detoxification systems on the evolution of ciprofloxacin resistance in *Escherichia coli*

S. Diaz-Diaz[1,2,3,4], E. Recacha [1,2,3,4], A. García-Duque[1], F. Docobo-Pérez [2,3,4], J. Blázquez[5], A. Pascual [1,2,3,4] and J. M. Rodríguez-Martínez [2,3,4]*

[1]*Unidad de Enfermedades Infecciosas, Microbiología y Medicina Preventiva, Hospital Universitario Virgen Macarena, Seville, Spain;* [2]*Departamento de Microbiología, Facultad de Medicina, Universidad de Sevilla, Sevilla, Spain;* [3]*Red Española de Investigación en Patología Infecciosa (REIPI), Instituto de Salud Carlos III, Madrid, Spain;* [4]*Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Sevilla, Spain;* [5]*Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Madrid, Spain*

*Corresponding author. E-mail: jmrodriguez@us.es

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Background: Suppression of SOS response and overproduction of reactive oxygen species (ROS) through detoxification system suppression enhance the activity of fluoroquinolones.

Objectives: To evaluate the role of both systems in the evolution of resistance to ciprofloxacin in an isogenic model of *Escherichia coli*.

Methods: Single-gene deletion mutants of *E. coli* BW25113 (wild-type) (Δ*recA*, Δ*katG*, Δ*katE*, Δ*sodA*, Δ*sodB*), double-gene (Δ*recA*-Δ*katG*, Δ*recA*-Δ*katE*, Δ*recA*-Δ*sodA*, Δ*recA*-Δ*sodB*, Δ*katG*-Δ*katE*, Δ*sodB*-Δ*sodA*) and triple-gene (Δ*recA*-Δ*katG*-Δ*katE*) mutants were included. The response to sudden high ciprofloxacin pressure was evaluated by mutant prevention concentration (MPC). The gradual antimicrobial pressure response was evaluated through experimental evolution and antibiotic resistance assays.

Results: For *E. coli* BW25113 strain, Δ*katE*, Δ*sodB* and Δ*sodB*/Δ*sodA* mutants, MPC values were 0.25 mg/L. The Δ*katG*, Δ*sodA*, Δ*katG/katE* and Δ*recA* mutants showed 2-fold reductions (0.125 mg/L). The Δ*katG*/Δ*recA*, Δ*katE*/Δ*recA*, Δ*sodA*/Δ*recA*, Δ*sodB*/Δ*recA* and Δ*katG*/Δ*katE*/Δ*recA* strains showed 4–8-fold reductions (0.03–0.06 mg/L) relative to the wild-type. Gradual antimicrobial pressure increased growth capacity for Δ*sodA* and Δ*sodB* and Δ*sodB*/Δ*sodA* mutants (no growth in 4 mg/L) compared with the wild-type (no growth in the range of 0.5–2 mg/L). Accordingly, increased growth was observed with the mutants Δ*recA*/Δ*katG* (no growth in 2 mg/L), Δ*recA*/Δ*katE* (no growth in 2 mg/L), Δ*recA*/Δ*sodA* (no growth in 0.06 mg/L), Δ*recA*/Δ*sodB* (no growth in 0.25 mg/L) and Δ*recA*/Δ*katG*/Δ*katE* (no growth in 0.5 mg/L) compared with Δ*recA* (no growth in the range of 0.002–0.015 mg/L).

Conclusions: After RecA inactivation, gradual exposure to ciprofloxacin reduces the evolution of resistance. After suppression of RecA and detoxification systems, sudden high exposure to ciprofloxacin reduces the evolution of resistance in *E. coli*.

Introduction

The SOS response is a coordinated cellular response to genotoxic damage that can contribute to antimicrobial resistance evolution.[1] Fluoroquinolones are potent inducers of the SOS response.[2] When DNA synthesis inhibition and DNA damage occur (primary damage), RecA proteins form nucleofilaments around single-stranded DNA and promote self-cleavage of LexA,[3,4] which causes activation of more than 50 genes, notably related to DNA repair and recombination.[1,5,6] At the same time, bactericidal antimicrobials play an important role in reactive oxygen species (ROS) accumulation under aerobic conditions,[7,8] inducing complex redox reactions that contribute to cellular damage and death (secondary damage).[8–10] Bacteria have multiple oxidative detoxification systems to combat oxidative stress, such as three types of superoxide dismutase (SodA, SodB and SodC) and two types of catalase (KatG and KatE), which remove O^{2-} and H_2O_2, respectively.[11,12]

Recently, we showed that suppression of both the SOS response (by *recA* gene deletion) and overproduction of ROS (by multiple detoxification system gene deletion) enhances the activity and

lethality of fluoroquinolones against *E. coli*.[13] Previous studies have shown that antimicrobial treatments can lead to the emergence of resistant bacteria due to SOS-induced mutagenesis[14–16] or ROS production.[17] In this study therefore, we used *E. coli* mutants with a *recA* gene deletion in combination with suppressed ROS detoxification system genes (Δ*sodA*, Δ*sodB*, Δ*katG* and Δ*katE*) to evaluate the interplay of DNA repair, recombination processes and detoxification systems in the evolution of resistance to ciprofloxacin under gradual or sudden antibiotic pressure.

Materials and methods

Strains, growth conditions and antimicrobial agents

Wild-type *E. coli* BW25113 was used as the starting strain (Table S1, available as Supplementary data at *JAC* Online). Single-gene deletion mutants of *E. coli* BW25113 (wild-type) (Δ*recA*, Δ*katG*, Δ*katE*, Δ*sodA*, Δ*sodB*) were selected from the KEIO collection.[18] The double-gene deletion mutants (Δ*katG*/Δ*recA*, Δ*katE*/Δ*recA*, Δ*sodA*/Δ*recA*, Δ*sodB*/Δ*recA*, Δ*katG*/Δ*katE*, Δ*sodB*/Δ*sodA*) and the triple-gene deletion mutant (Δ*katG*/Δ*katE*/Δ*recA*) were generated by P1vir phage transduction.[19,20] Some triple knockouts such as Δ*sodA*/Δ*sodB*/Δ*recA* were planned at the start of the study, but proved not to be viable after several attempts.

Liquid or solid lysogeny broth (LB) and Mueller-Hinton broth (MHB) media were used. Strains were grown at 37°C. The antibiotic used for the different assays was ciprofloxacin (Sigma–Aldrich, Madrid, Spain).

Minimum inhibitory concentrations

MICs were determined in triplicate for each bacterial strain, using two different techniques: the gradient strip test and broth microdilution, following CLSI reference methods.[21]

Mutant prevention concentration (MPC)

MPC was defined as the antibiotic concentration that prevents the growth of any resistant mutants following an inoculum of 10^{10} cells on LB plates containing dilutions of antibiotic, simulating an infectious focus.[22] It was determined as previously described by Machuca *et al.*[23] For each strain used in the study, a 0.5 McFarland culture density was obtained, a 10^{-5} dilution was performed (starting inoculum of $\sim10^3$ cfu/mL) and incubated at 37°C overnight. One millilitre of overnight culture was inoculated into 100 mL of MHB, then incubated for ∼6 h at 37°C with aeration until an OD_{540nm} of ∼1.0 was reached (Genesys 20, Thermo, Barcelona, Spain), corresponding to $\sim10^9$ cfu/mL. Cultures were then centrifuged at 4200 rpm for 20 min. The supernatant was discarded and the pellet containing $\sim10^{11}$ cells was resuspended in 1 mL of MHB. One hundred microlitres ($\sim10^{10}$ cells) was spread onto a MH agar plate containing a specific concentration of quinolones. Each strain was tested against 2-fold-increasing concentrations of ciprofloxacin (ranging from 0.001 to 1 mg/L). Drug-free MH agar plates were inoculated with 100 μL of serial dilutions as an inoculum control. The plates were incubated for a total of 96 h at 37°C and examined every 24 h for the appearance of colonies. The MPC recorded was the lowest quinolone concentration at which no colonies grew on an agar plate at 96 h in at least three independent experiments. The time in hours when the MPC was determined (MPC time window) was also recorded, and the mutant selection window (MSW, defined as the antimicrobial concentration range extending from the minimum concentration required to block the growth of wild-type bacteria up to that required to inhibit the growth of the least susceptible single-step mutant)[22] was calculated as the ratio of MIC to MPC.

Experimental evolution of antibiotic resistance

The experimental evolution study, as described by Escudero *et al.*,[24] started with the inoculation of 2 μL (∼10^6 cells) of the LB overnight culture into 96-well plates with 198 μL of LB containing a subinhibitory concentration of ciprofloxacin. For this approach, we started with 6.25×10^{-5} mg/L of ciprofloxacin, which is between 1/16 and 1/256 of the MIC (determined previously) for the selected strains. This approach maximizes the chances of populations acquiring resistance mutations.[24] Plates were incubated for 20 h at 37°C, without agitation. After overnight culture, bacterial cultures were quantified daily by spectrophotometry at OD_{595nm} of each population, using a plate reader (Infinite 200 PRO plate reader; Tecan, Madrid, Spain). Each bacterial culture was then transferred to a new 96-well plate with double the antibiotic concentration of the day before. The plates were incubated again for 20 h at 37°C. In parallel, we performed control assays for each strain under the same conditions but in the absence of antibiotics. Optical density values <0.1 indicate extinction of the population. The percentage of cultures showing growth was calculated each day as the number of cultures showing OD values higher than 0.1. At least sixteen biological replicates were measured.

Statistical analysis

All statistical analyses were performed using Graphpad Prism 6 software (https://www.graphpad.com). The log-rank (Mantel-Cox) test was used for statistical evaluation. Differences were considered significant when *P* values were <0.05.

Results

Sensitization was confirmed first using MIC values (Table S1). No reductions in ciprofloxacin MIC were observed when any of the detoxification system genes were inactivated, either alone (Δ*katG*, Δ*katE*, Δ*sodA* and Δ*sodB*) or in combination (Δ*katG*/Δ*katE*, Δ*sodB*/Δ*sodA*), compared with wild-type BW25113. For the Δ*recA* mutant, ciprofloxacin MICs were 3.75-fold lower than for wild-type BW25113. Finally, for the combinations (Δ*recA* gene with detoxification system deletion), sensitization increased 7.5–15-fold relative to the wild-type and 2–4-fold relative to Δ*recA* mutant. This confirmed our previous results on synergistic sensitization to ciprofloxacin by *recA* gene suppression in combination with inactivated detoxification system genes.[13]

In this study, two different approaches were used. First, bacterial populations were exposed to a single antibiotic pressure that was constant and high (MPC above the MIC), referred to as sudden high ciprofloxacin pressure, and second, to incremental exposures to the antimicrobial (starting with sub-MIC concentrations), referred to as gradual antimicrobial pressure.

To evaluate the response to sudden high antimicrobial pressure, MPC assays were performed (this approach was aimed at simulating physiological conditions in response to antibiotic treatment pressure at the site of infection). The MPC values of ciprofloxacin (Figure 1, Table S1) for the *E. coli* BW25113 wild-type strain, Δ*katE*, Δ*sodB* single mutants and the Δ*sodB*/Δ*sodA* double mutant were 0.25 mg/L. The Δ*katG*, Δ*sodA* single mutants, the Δ*katG/katE* double mutant and the Δ*recA* single mutant showed 2-fold MIC reductions (0.125 mg/L) relative to the wild-type. The Δ*katG*/Δ*recA*, Δ*katE*/Δ*recA*, Δ*sodA*/Δ*recA*, Δ*sodB*/Δ*recA* double mutants and Δ*katG*/Δ*katE*/Δ*recA* triple mutant showed 4–8-fold reductions (0.03–0.06 mg/L) compared with the wild-type.

MSW (Table S1) values were as follows: in the *E. coli* BW25113 wild-type strain, the Δ*katE*, Δ*sodB* single mutants and the Δ*sodB*/

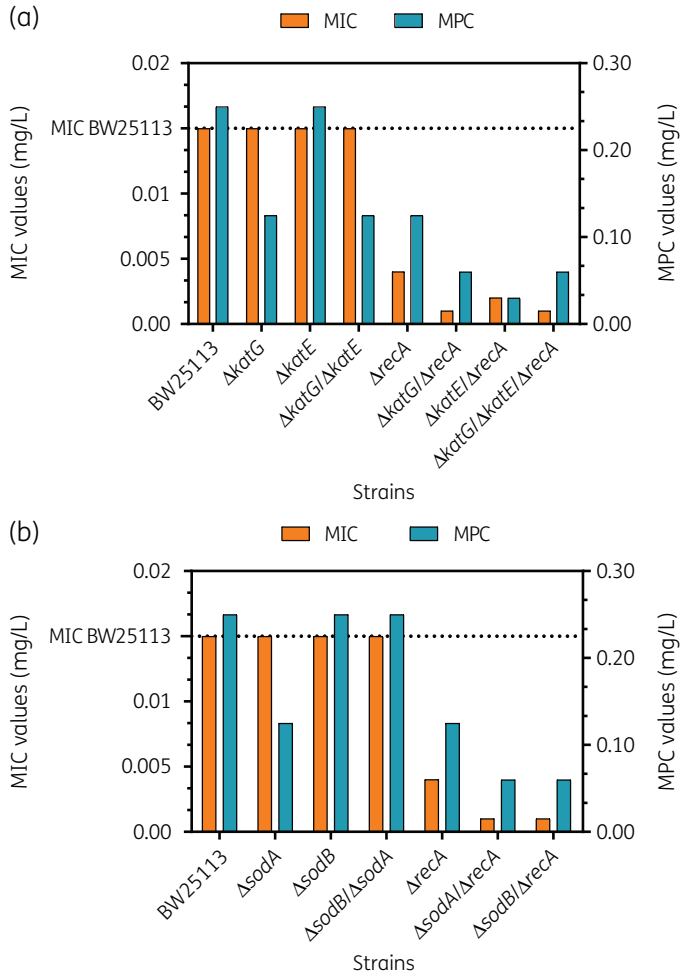

Figure 1. MIC (orange) and MPC (blue) of ciprofloxacin for the catalase mutants group (a) and superoxide dismutase mutants group (b) at 96 h. The wild-type (*E. coli* BW25113) MIC of ciprofloxacin is 0.015 mg/L. CIP, ciprofloxacin. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

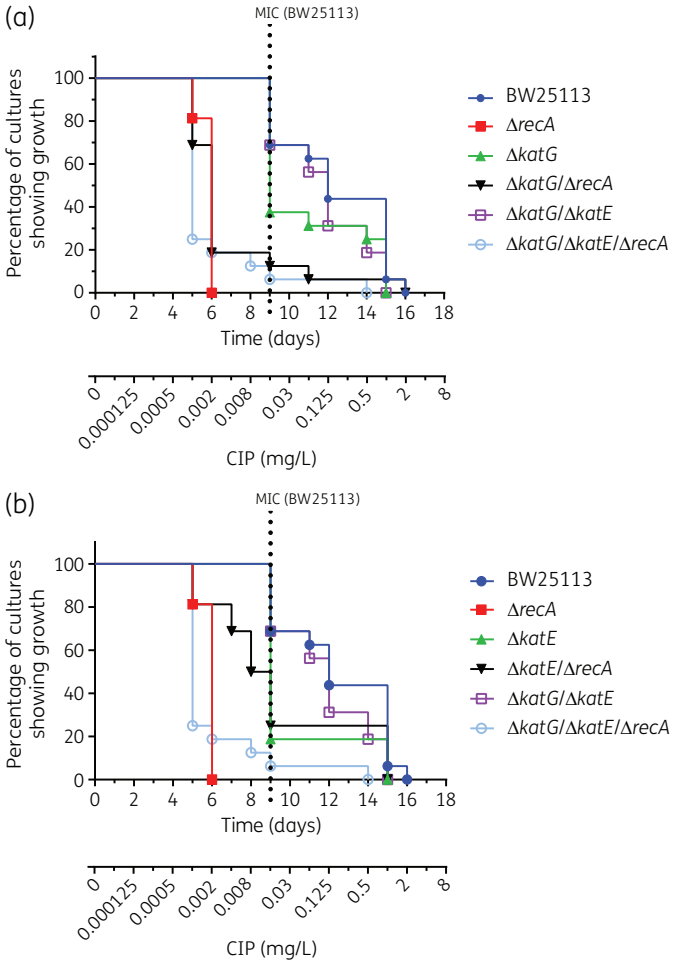

Figure 2. Curves showing culture growth of (a) the *katG* gene group, and (b) the *katE* gene group with increasing concentrations of antibiotics. Representation of the number of viable cultures over time. Sixteen populations of each strain were propagated under increasing concentrations of ciprofloxacin, starting with 6.25×10^{-5} mg/L of antibiotic on day 1 and doubling the antibiotic concentration each day. The dashed vertical line represents the wild-type (BW25113) MIC of ciprofloxacin. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Δ*sodA* double mutant, 17; in the Δ*katG* and Δ*sodA* single mutants and the Δ*katG*/*katE* double mutant, 8; in the Δ*recA* single mutant, 31; in the Δ*katE*/Δ*recA* double mutant, 15; and in the Δ*katG*/Δ*recA*, Δ*sodA*/Δ*recA*, Δ*sodB*/Δ*recA* double mutants and the Δ*katG*/Δ*katE*/Δ*recA* triple mutant, the MSW was 60.

These results indicate that suppression of the SOS response and the detoxification systems (both separately and, more markedly, in combination), reduces the MPC of ciprofloxacin under sudden high antimicrobial pressure. The MPC reduction was always greater when the SOS response was suppressed. Additionally, due to the initial reductions in MIC values, the MSW was wider and the values higher when the *recA* gene, alone or in combination with one or two detoxification systems, was suppressed.

In order to evaluate the response to gradual antimicrobial pressure, experimental evolution of was also performed and measured using antibiotic resistance assays (the aim of this approach was to simulate ecological conditions under environmental antibiotic pressure). Experimental evolution of antibiotic resistance (Figures 2 and 3), in which ciprofloxacin concentrations were gradually increased on a daily basis, showed that all mutants, except for the Δ*recA* mutant, grew at concentrations above the MIC of the *E. coli* BW25113 wild-type strain (>0.015 mg/L). The wild-type strain showed no growth at ciprofloxacin concentrations in the range of 0.5–2 mg/L (according to data from two different inter-day assays), and no differences in growth capacity were observed against the Δ*katG*, Δ*katE* single mutants and the Δ*katG*/Δ*katE* double mutant (no growth in 1 mg/L). Increased capacity for growth was observed with the Δ*sodA* and Δ*sodB* single mutants and the Δ*sodB*/Δ*sodA* double mutant (no growth in 4 mg/L). On the other hand, the Δ*recA* single mutant showed no growth at ciprofloxacin concentrations in the range of 0.002–0.015 mg/L (according to data from two different inter-day assays), and increased capacity for growth was observed with the Δ*recA*/Δ*katG* (no growth in 2 mg/L), Δ*recA*/Δ*katE* (no growth in 2 mg/L), Δ*recA*/Δ*sodA* (no growth in 0.06 mg/L), Δ*recA*/Δ*sodB* (no growth in 0.25 mg/L) double mutants,

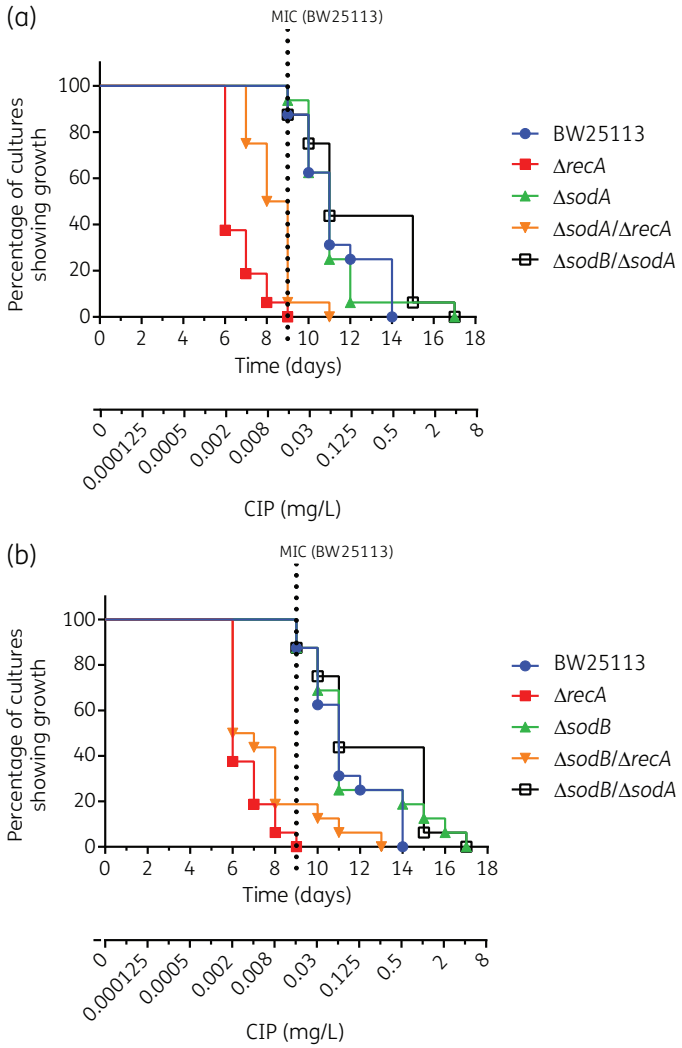

Figure 3. Curves showing culture growth of (a) the *sodA* gene group, and (b) the *sodB* gene group with increasing concentrations of antibiotics. Representation of the number of viable cultures over time. Sixteen populations of each strain were propagated under increasing concentrations of ciprofloxacin, starting with 6.25×10^{-5} mg/L of antibiotic on day 1 and doubling the antibiotic concentration each day. The dashed vertical line represents the wild-type (BW25113) MIC of ciprofloxacin. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

and the Δ*recA*/Δ*katG*/Δ*katE* triple mutant (no growth in 0.5 mg/L). The log-rank test showed significant differences for Δ*recA* ($P < 0.0001$), Δ*katE* ($P < 0.05$), Δ*katG*/Δ*recA* ($P < 0.01$), Δ*katE*/Δ*recA* ($P < 0.05$), Δ*sodB*/Δ*sodA* ($P < 0.05$), Δ*sodA*/Δ*recA* ($P < 0.0001$), Δ*sodB*/Δ*recA* ($P < 0.0001$), and Δ*katG*/Δ*katE*/Δ*recA* ($P < 0.0001$) compared with the wild-type. There were also significant differences for Δ*katE*/Δ*recA* ($P < 0.001$) and Δ*sodA*/Δ*recA* ($P < 0.001$) compared with Δ*recA*.

Accordingly, when capacity for growth at the MIC for the wild-type (0.015 mg/L, day 9) was compared, three groups were distinguished (Figures 2 and 3). The first one included the wild-type BW25113 (70%–90% of cultures showed growth), Δ*sodA* (95%), Δ*sodB* (90%), Δ*sodB*/Δ*sodA* (90%) and Δ*katG*/Δ*katE* (70%) strains. The second group included Δ*katG* (40%), Δ*katE* (20%), Δ*katE*/Δ*recA* (25%), Δ*katG*/Δ*recA* (15%) and Δ*sodB*/Δ*recA* (20%) strains. The third group included Δ*sodA*/Δ*recA* (5%), Δ*katG*/Δ*katE*/Δ*recA* (5%) and Δ*recA* (0%).

Discussion

Antibiotic-induced bacterial mutagenesis is considered to be partly SOS response dependent.[2,14] When DNA damage occurs as a result of antimicrobials, genes in the SOS regulon related to DNA repair (including translesion synthesis) are expressed, contributing to mutagenesis.[6,25,26] When the SOS response is suppressed, bacteria are unable to evolve resistance beyond the MIC (Figures 2 and 3). One explanation for this effect is that the SOS response is not activated and thus that mutagenesis is drastically reduced. Furthermore, the *recA* gene is also involved in double-strand break repair, caused by the inhibition of DNA gyrase activity, through homologous recombination, so that when the *recA* gene is suppressed, recombination processes do not occur via this pathway.[1] On the other hand, mutagenesis is also ROS-dependent when low doses of antibiotics are used.[9] Nevertheless, ROS have a killing effect at higher antibiotic doses (and hence also a bacteriostatic effect),[27] characterized by DNA base oxidation, causing lethal DNA breaks.[28] Accordingly, changes in MPC values could be related to deletions of detoxification system genes and ROS accumulation.

When ROS are produced at low antibiotic doses,[9] the detoxification systems reduce the oxidative stress. When these systems are suppressed and bacteria are exposed to ciprofloxacin gradually, bacteria such as the wild-type are able to grow at concentrations above their MIC (Figures 2 and 3). This effect could be the consequence of mutagenesis due to ROS production, which leads to the appearance of ciprofloxacin resistance mutations. Of note, there are differences in behaviour between catalase system suppression (Δ*katG* and Δ*katE*) and superoxide dismutase system suppression (Δ*sodA* and Δ*sodB*), which may be the consequence of the different modes of action of the two systems, since superoxide dismutases remove O_2^- and catalases remove H_2O_2.[11,12] Furthermore, when both the SOS response and detoxication systems are suppressed, mutants are able to grow in ciprofloxacin concentrations above their MIC, which is different from what happens when the SOS response alone is suppressed. In this case, ROS production may be sufficient to lead to mutagenesis even though the SOS response is suppressed. A possible explanation for this effect is the activation of DNA damage repair systems independent of the SOS response. There are many systems described in the literature that are involved in DNA damage repair processes, such as the activities described for the Adaptive Response or the 'GO system'.[29]

It is noteworthy that even though BW25113 and the Δ*recA* mutant showed different capacities for growth in the inter-day assays, the overall tendency was similar (Figures 2 and 3). It is also important to mention that there were differences between the two methodologies used. In the experimental evolution assays, bacteria were exposed to gradually increasing concentrations of ciprofloxacin, in which the initial ciprofloxacin concentrations simulated those found in the environment (3×10^{-5}–0.031 mg/L),[30–33] which could also facilitate antibiotic-induced mutagenesis. By way of contrast, sudden high antimicrobial pressure limited adaptation and evolution (as measured by determination of the MPC). Finally, both methodologies were designed to analyse the behaviour of

populations capable of active growth (colonies in the MPC assays and turbidity in the experimental evolution assays). In neither case were the populations of non-replicating surviving bacteria analysed for tolerance or persistence.

In conclusion, suppression of the SOS response, through the deletion of *recA* gene and detoxification systems, helps to reduce the evolution of resistance in *E. coli* after sudden exposure to ciprofloxacin. Suppression of the SOS response helps to reduce the evolution of resistance after gradual exposure to ciprofloxacin. In contrast, under this latter condition, detoxification systems, alone or in combination with SOS response suppression, could favour mutagenesis and the evolution of ciprofloxacin resistance.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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