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Norfloxacin Promotes Generation of "Spider-like" Formations and SOS Response Induction in *Escherichia coli* K-12 Macrocolony Biofilms in a Regime below of Sub-inhibitory Concentrations

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Quinolones are an important kind of antibiotics employed in the treatment of clinical relevant bacterial infections. It is well known that quinolones causes DNA damage inducing the SOS response system of DNA repair. Many ideas about the effect of these antibiotics on bacterial physiology have been obtained through of treatment of planktonic cultures and sessile biofilms. However, despite these studies, many aspects of how quinolones affect to bacterial metabolism and growth of colonies remains still poorly understood. Here, I report that norfloxacin quinolone antibiotic interferes with the normal development of *Escherichia coli* K-12 old macrocolony biofilms, altering its morphology, abolishing the formation over its surface of characteristic autoaggregative chondrule-like formations observed in macracolonies non treated with norfloxacin; but intriguingly the norfloxacin treatment induces in this kind of biofilms the formation of a new kind of superficial structures that exhibit a "spider-like" morphology, which has not previously been reported. Interestingly, these spider-like formations are also found outside the colony. Most importantly, when macrocolony biofilms carrying a *recA-gfp* transcriptional fusion were ordered sequentially in rows, the norfloxacin promoted the SOS induction of *recA-gfp* activity as well as the alteration of morphology of *E. coli* K-12 macrocolonies in norfloxacin concentrations lower that the sub-inhibitory

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concentrations, in a regime of influence that is not expected to be active. The biological implications of these findings are discussed.

Keywords: Norfloxacin; recA-gfp; SOS response; macrocolony biofilms; spider-like formations; sub-inhibitory concentrations; sub-MICs; b-sub-MIC concentrations; b-sub-MICs.

1. INTRODUCTION

Norfloxacin is a member of the quinolone family of antibiotics, which is rapidly becoming the most important family of antibiotics clinically-relevant fluoroquinolones (FQs) [1]. Quinolones function by inhibition of the activity of two essential type II DNA topoisomerases in bacteria, gyrase and topoisomerase IV which are involved in the control of the DNA topology [2], which is carried out through of the formation of a protein-bridged DNA double strand break (DSB), manipulating the DNA strand topology and finally rejoining the DNA ends [2,3]. It has been demonstrated that for instance ciprofloxacin reversibly binds to the protein-bridged DSB intermediate and inhibits rejoining of the DNA ends [3,4]. It has been traditionally considered that the toxic effects of ciprofloxacin may be the result of topoisomerase subunit dissociation without re-ligation of the DNA ends [3-5], likely producing free double strand ends (DSEs) when the protein-DNA bond is eventually hydrolyzed or the DNA is processed by a nuclease. In addition, covalently bound topoisomerases may also block DNA replication forks, which after processing will also produce DSEs [3]. Recently, it has been proposed that quinolones mediate the death of susceptible bacteria via production of reactive oxygen species ROS [6-8].

The DNA damage originated by the introduction of double-stranded DNA breaks following topoisomerase inhibition by quinolones causes that RecA is activated to form RecA* (i.e. a ssDNA-RecA nucleofilament [7,9]) that promotes auto-cleavage of the LexA repressor protein, allowing the induction of expression of SOS-response genes including DNA repair enzymes and of own *recA* gene [7,9-11]. Notably, several studies have shown that preventing induction of the SOS response serves to enhance killing by quinolone antibiotics (except in the case of the first generation quinolone, nalidixic acid). Resistance to ciprofloxacin requires mutations in the genes that encode the topoisomerases (*gyrA* and *gyrB*, encoding gyrase and *parC* and *parE*, encoding topoisomerase IV) [2,5,7] or in the genes that affect cell permeability or drug export [12].

Interestingly, preventing induction of the SOS response has also been shown to reduce the formation of drug-resistant mutants by blocking the induction of error-prone DNA polymerases [7], homologous recombination and horizontal transfer of drug-resistance elements [7]. Recently, it has been shown that the SOS response is also necessary for persisters formation in response to the fluoroquinolone antibiotic ciprofloxacin [13].

It has been shown that all antibiotics, regardless of their receptors and mode of action, exhibit the phenomenon of hormesis (a term that describes the biological responses to environmental signals or stresses that are characterized by biphasic dose-response relationships, exhibiting low-dose stimulation and high-dose inhibition) and provoke considerable transcription activation at low concentrations [14-16]. Thus, it has been reported that sub-inhibitory concentrations (sub-MICs) of antibiotics can that regulate bacterial gene transcription, physiology and virulence having a strong effect on mutation rates, horizontal gene transfer and biofilms formation, which may all contribute to the emergence and spread of antibiotic resistance [17-23]. However, these studies have been done mainly by treatment of the planktonic cultures or sessile biofilms developed over abiotic surfaces. Many aspects of the effect of quinolones treatment in macrocolony growth and development on semisolid surfaces are still poorly understood.

In this study, it is reported the results obtained when *Escherichia coli* K-12 old macrocolony biofilms [24,25] were treated with norfloxacin quinolone antibiotic. The morphological changes caused by this treatment are described, also the results presented here indicating that the influence of norfloxacin on the SOS induction apparently extend beyond of concentration that in principle could be expected to be active.

2. MATERIALS AND METHODS

2.1 Bacterial Strains, Media and Growth

The *Escherichia coli* K-12 strain used in this study was the MG1655 (p*recA-gfp*) strain bearing a low-copy plasmid p*recA-gfp* harbouring a *recA-gfp* transcriptional fusion, a *gfp*-(green fluorescent protein) promoter fusion for *recA* gene [26]. The experiments were conducted using the following protocol: Cells obtained from a colony of these strains grown in Luria-Bertani (LB) medium [24,25]: 1.0% (10 g/L) Difco® Bacto-Trypone, 0.5% (5 g/L) Difco® Yeast Extract and NaCl 0.5% (5 g/L) harnessed with 1.5% (15 g/L) of *Agar Bacteriológico Europeo* (ABE) were inoculated with toothpicks in 8.5 cm or 12 cm Petri dishes made of polystyrene plastic containing 30 ml or 100 ml of LB medium jellified with the indicated ABE concentrations. The plates were sealed with parafilm® to prevent loss of water. After indicated days of incubation in each case at 37ºC, the plates were photographed with reflected light with a digital Kodak EasyShare Z710 camera. Kanamycin (Km) antibiotic (10 µg/ml) obtained from Sigma-Aldrich was added to LB medium when indicated. The norfloxacin (Nor) was obtained from Sigma-Aldrich.

2.2 Microscopy Techniques

The microscopic images were taken with an Ultralyt ULNM-90-10000 microscope (made by Brown & Crown Company). The images of the Figs. were framed with Microsoft Photo Editor software and composed using the Powerpoint software program.

2.3 Visualization of *recA-gfp* Activity

The activity of *recA-gfp* fusion was visualized by situating directly the plates under ultra-violet (UV) irradiation in an Invitrogen safe imager 2.0 transiluminator. The Fig. 3F was taken with a Leika M205 FA stereoscopic fluorescence microscope coupled with a DFC350FX digital camera.

3. RESULTS AND DISCUSSION

To obtain information about as the norfloxacin affects to development of old MG1655 (p*recA-gfp*) macrocolony biofilms, firstly, it was necessary to know the size of inhibition halo of norfloxacin and the induction halo expected under typical disk-plate diffusion assays of antibiotic activity [19,27]. To determinate this, it was carried out a diffusion experiment in Petri dish under the following conditions of assay: 100 µl of an overnight culture of MG1655 (p*recA-gfp*) strain was smeared over a 0.6% LB-ABE semisolid surface and the induction of SOS response was visualized through the induction of activity of *recA-gfp* transcriptional fusion after a day of growth at 37ºC. As it is shown in Fig. 1A, the size of the inhibition halo caused by the bacterial killing mediated by norfloxacin was approximately of 2.0 cm, while that the halo of induction of the fluorescence activity of *recA-gfp* fusion that surrounded the inhibition halo was approximately 0.5 cm wide and observed in the regime of sub-inhibitory concentrations [19,27]. After of seven additional days of incubation at 37ºC, the plate showed a similar phenotypical appearance (data not shown). Similar results were obtained in agar surfaces prepared with 1.5% (15 g/L) ABE agar (data not shown), indicating that the size of inhibition halo of norfloxacin as well as the size of induction halo of *recA-gfp* activity are both independent of agar concentration used. Therefore, under the experimental conditions described here, it is considered that ~2.5 cm is the distance limit to observe the effect of norfloxacin on the activity of the *recA-gfp* transcriptional fusion. In others words, this distance marks an “event horizon” of influence of norfloxacin antibiotic under the condition described previously.

With this initial information in mind it was carried out the following experiment: Three inoculation points from an overnight culture of the MG1655 (p*recA-gfp*) were carried out using a toothpick in a serial arrangement on semisolid 0.6% ABE agar surface in a Petri dish. The Fig. 1B shows the morphotype exhibited by the macrocolonies developed from these inoculation points after five days of growth and development at 37ºC. Then, a paper disk containing norfloxacin (100 µg/µl) was deposited on semisolid surface of the Petri dish (Fig. 1C) situated next to one of macrocolonies. After of seven days in incubation of this plate at 37ºC, the induction of the *recA-gfp* fusion was visualized in these macrocolony biofilms (Fig. 1D). Unexpectedly, it was observed that the activity of *recA-gfp* transcriptional fusion was induced in the macrocolony biofilm situated far away of disk with the norfloxacin antibiotic (Fig. 1E). Similar inductive phenomenon was observed when the intermediary colony was removed (data not shown).

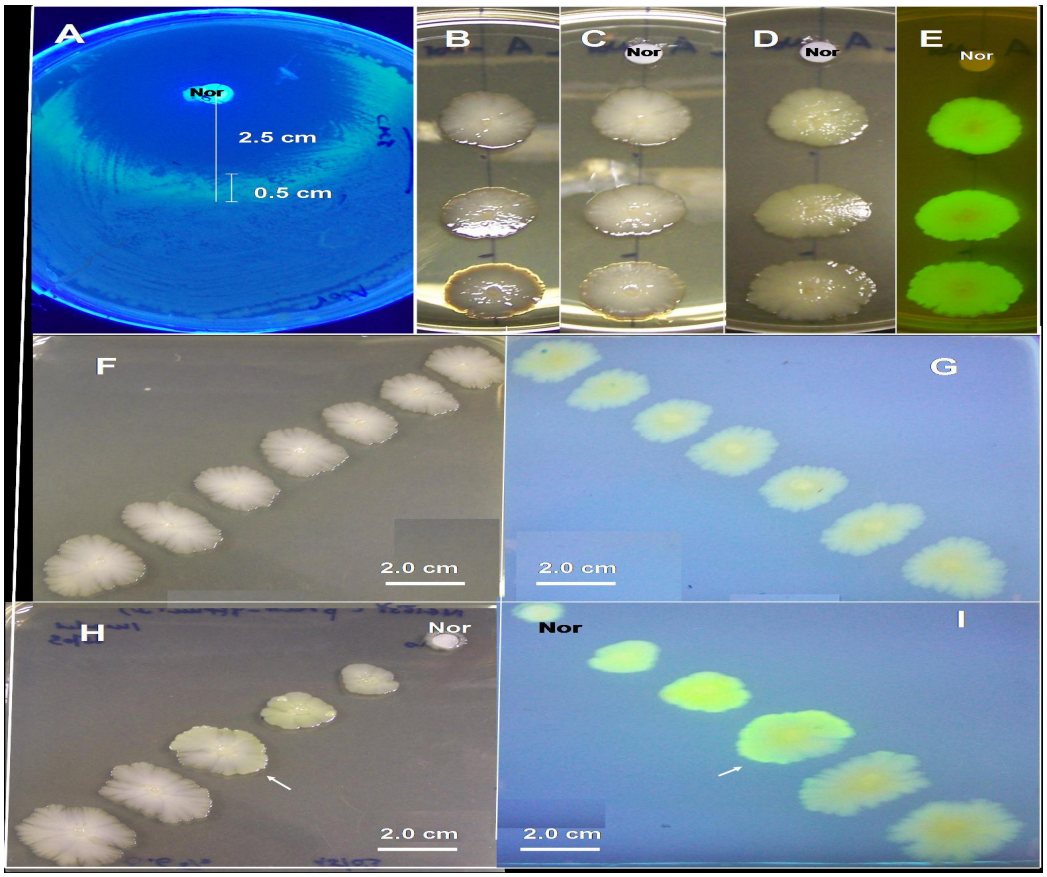

Fig. 1. The norfloxacin "event horizon" and the induction of *recA-gfp* transcriptional fusion in old *E. coli* K-12 MG1655 strain macrocolony biofilms in different arrangements of inoculation

(A) *The E. coli K-12 MG1655 strain carrying the recA-gfp transcriptional fusion was grown in LB-Km liquid medium at 37ºC overnight. 100 µl of this culture was smeared on a semisolid 0.6% ABE agar (6g/L) surface containing kanamycin (Km) antibiotic and LB as nutrient (LB-Km) and then a disk containing norfloxacin (Nor) in a concentration of 100 µg/µl was deposited over it. The plates were inoculated at 37ºC overnight. After this time the recA-gfp was visualized under UV and photographed.* ***(B)*** *three E. coli K-12 MG1655 (precA-gfp) strain macrocolony biofilm 5-day-old grown in LB-Km at 37ºC on 0.6% semisolid ABE agar surface.* ***(C)*** *A disk containing norfloxacin (Nor) antibiotic (100 µg/µl) was deposited on this surface.* ***(D)*** *Appearance of these three colonies after other seven days of additional incubation at 37ºC.* ***(E)*** *Visualization of fluorescence recA-gfp activity after of this time.* ***(F-G)*** *macrocolony biofilms not treated with norfloxacin as a control experiment* ***(F)*** *seven E. coli K-12 MG1655 (precA-gfp) strain macrocolony biofilm 7-day-old grown at 37ºC in LB-Km medium over 0.6% semisolid ABE agar surface visualized with reflected light.* ***(G)*** *theses same colonies visualized under UV irradiation.* ***(H)*** *In this plate a disk containing norfloxacin (Nor, 100 µg/µL) was deposited on the 0.6% ABE semisolid agar surface after of that six colonies were inoculated with toothpick. appearance of these colonies after seven days of growth and development at 37ºC visualized with reflected light.* ***(I)*** *activity of fluorescence's of recA-gfp fusion under UV irradiation. the arrows indicate the macrocolony biofilms farthest where the induction of recA-gfp was observed*

To gain idea about the maximal distance the norfloxacin action on macrocolony biofilms, an experiment similar to described previously was carried out in Petri dish of 12 cm with seven and six inoculations points. The result of this experiment is detailed in Fig. 1F-I. From this Fig. is concluded that approximately 8.6 cm is the maximal distance where induction of *recA-gfp* fusion after seven days of norfloxacin treatment at 37ºC can be observed in macrocolony biofilms developed on semisolid 0.6% ABE agar surfaces.

Due to that the activity of *recA-gfp* fusion in the macrocolony beyond the event horizon was observed after seven days of incubation, an

important question to respond was if there was a sequence of "firing" of this activity in macrocolonies serially arranged, i.e. whether the induction of *recA*-*gfp* activity was progressive, begun in the macrocolony closest to the disk containing the norfloxacin and then in the next colony and so on to finally to be observed this induction in farthest colony. The Fig. 2A shows the disposition in a Petri dish of an array of four of MG1655 *(*p*recA*-*gfp*) strain 5-day-old macrocolony biofilms previous to receive the norfloxacin treatment. The Fig. 2B shows the same colonies after they were treated with norfloxacin, in this case the dish with norfloxacin was situated over the surface of a macrocolony. The induction of *recA*-*gfp* and growth of these norfloxacin treated colonies was monitored for five days; from this experiment was possible to conclude that the induction of this SOS induced fusion follows a determinate pattern of firing. Thus, after of the first day an "induction wave" of *recA*-*gfp* activity can be observed (Fig. 2D-E) which even can be visualized under naked eye (the macrocolony appear yellow-green coloured when the *recA*-*gfp* is expressed, Fig. 2C) in the macrocolony next to disk of norfloxacin (Fig. 2C), after other two days at 37ºC the *recA*-*gfp* activity is initiated in the next macrocoloy in such way that after a week of treatment with norfloxacin the induction of *recA*-*gfp* activity is observed in the macrocolony that is beyond of 2.5 cm limit, in regime of concentration that is not expected to be able to trigger SOS induction (Fig. 2E). Interestingly, as it was observed in Fig. 1D and in Fig. 2C, the macrocolony biofilms situated next to or directly with the norfloxacin disk in its surface showed a complete arrest of its growth and development, exhibiting a different morphological aspect of those whose macrocolony biofilms were not treated with norfloxacin. On the other hand, the macrocolony situated far away of the norfloxacin disk was able to growth increasing its size during the seven days of monitoring. However, these macrocolonies that shows finally SOS induction of fusion *recA*-*gfp* are also affected in its microscopic morphology (data not shown).

An additional experiment was designed to confirm the previous results and additionally to obtain information about the behaviour of inductive phenomenon when new additional colonies were inoculated in the Petri dish. As it is shown in Fig. 3, firstly, two colonies were cultured on the semisolid agar surface, then a disk of norfloxacin (100 µg/µl) was situated over a macrocolony and then two new inoculations of MG1655 (p*recA*-*gfp*) strain were carried out on the plate in the disposition indicated. The evolution of this plate was followed during a week at 37ºC.

Several important conclusions can be extracted of these results:

i) The induction of the *recA*-*gfp* activity in a macrocolony next to norfloxacin disk follows a particular pattern. Thus, the inductive phenomenon starts by the periphery of the colony and then advances by the perimeter of this, continuing toward the center of the macrocolony (Fig. 2D-E and Fig. 3C-E).
ii) The norfloxacin inhibited completely the development of the new inoculation situated next to norfloxacin dish but not of macrocolony biofilms situated far away of the norfloxacin disk (Fig. 3D). In this last the induction of the *recA*-*gfp* could be observed after seven days of incubation at 37ºC.
iii) The norfloxacin treatment cause a "shrinkage" of the macrocolonies, affecting to the phenotypical appearance of the macrocolony biofilms (compared Fig. 3D *versus* 3E) that appear exhibiting a "wrinkled" aspect.

Taken together these observations strongly suggest that norfloxacin is able to induce SOS response beyond of "event horizon" below of sub-inhibitory concentrations (sub-MICs). Hence, this new regime of concentration of norfloxacin able of influence the expression of *recA*-*gfp* promoter fusion is called here b-sub-MICs. Furthermore, the effect of norfloxacin on macrocolony biofilms that previously had been developed before to be treated with norfloxacin is progressive, following a sequential order and pattern, affecting gradually different regions inside macrocolony as is reported by the induction of *recA*-*gfp* activity. In addition, the results indicate that the induction of SOS response in *E. coli* macrocolonies follow apparently a different pattern of induction that is observed in confluent lawn of *E. coli* cells under the conditions used for example in the traditional disk-plate antimicrobial diffusion tests (Fig. 1A) [19,27].

It has been described previously that *Escherichia coli* K-12 "volcano-like" old macrocolonies biofilms shows a massively chondrule-like formation over their surface [23]. The effect of

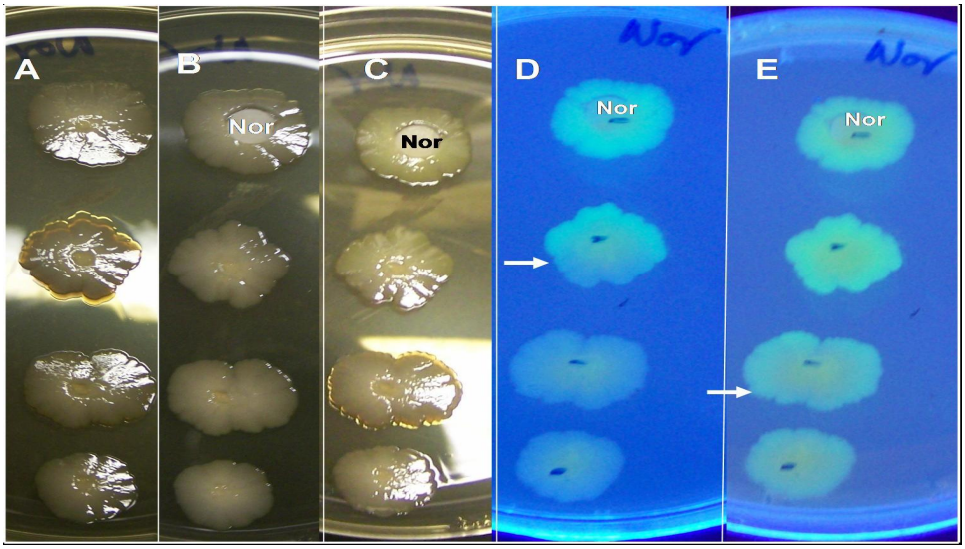

Fig. 2. Sequential "firing" of norfloxacin promoted SOS induction of *recA-gfp* transcriptional fusion in *E. coli* macrocolony biofilms serially ordered

***(A)** Appearance of four E. coli K-12 MG1655 (precA-gfp) macrocolony biofilms 5-day-old developed in LB-Km medium at 37ºC over 0.6% semisolid ABE agar surface before norfloxacin (100 µg/µl) was added in a paper disk. **(B)** One day and **(C)** Five days after of that a disk paper containing norfloxacin was situated over a macrocolony. **(D,E)** activity of recA-gfp visualized of the macrocolonies showed in (B,C) respectively. the arrows indicated the advance of induction of recA-gfp activity*

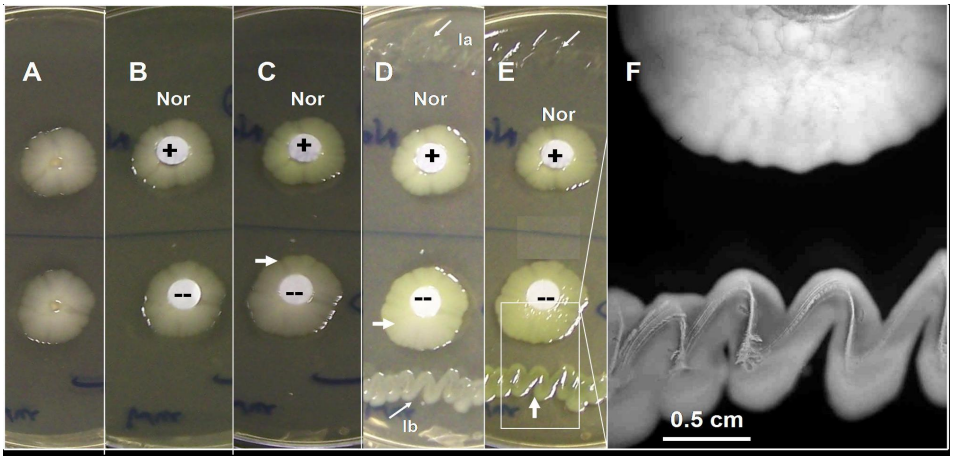

Fig. 3. Sequential SOS Induction of *recA-gfp* transcriptional fusion in *E. coli* K-12 macrocolony biofilms after that a dish containing norfloxacin were added

***(A)** Morphotype exhibited by two E. coli K-12 MG1655 (precA-gfp) strain macrocolony biofilm 5-day-old developed in LB-Km medium at 37ºC over 0.6% semisolid ABE agar surface before that two paper disks with (+) or without (-) norfloxacin (100 µg/µl) were situated on top of these colonies. **(B)** first **(C)** second **(D)** fifth and **(E)** seventh day **(F)** after of addition of norfloxacin disk. enlargement of the box showed in (E) showing the induction of recA-gfp fusion. In **(C)** two new inoculations of E. coli K-12 MG1655 (precA-gfp) strain were carried out in the site indicated in the plate (Ia and Ib), after of an additional day of incubation of petri dish at 37ºC. the norfloxacin inhibited the growth of colony situated next to disk of norfloxacin (marked as Ia, upper slim arrow) but no of the colony situated away of this (marked as Ib, lower slim arrow). the arrows indicated the advance of induction of recA-gfp activity observed to simple vista as a change in the colour of the macrocolony from yellow-brown to yellow-green. **(F)** enlargement of the box in (E), the recA-gfp activity was visualized in an optical fluorescence microscope under the GFP channel. In this image, the upper colony shows the typical "wrinkled" aspect of an E. coli MG1655 (precA-gfp) macrocolony under the influence of norfloxacin treatment*

norfloxacin upon macrocolony morphology of this kind of the macrocolonies was observed directly under the optical microscope. The Fig. 4A shows microscopically the appearance of surface of a 5-day-old macrocolony biofilms previously to be treated with norfloxacin. In its surface is observed the massive apparition of chondrule-like formations (Fig. 4B-F). Then, the same macrocolony was treated with a disk of norfloxacin (100 µg/µl). The Fig. 4G-Q shows the effect of this treatment. In these images can be observed that the chondrule-like formations were partially disrupted exhibiting a reduced size compared with the aspect that was observed before to that the macrocolony was treated with norfloxacin (see and to compare for example Fig. 4D vs Fig. 4J). It was observed also unexpectedly that a different kind of formations emerged on the surface of this norfloxacin-treated macrocolonies, which have not been previously reported. Intriguingly these structures exhibit morphology akin to a "spider" (Fig. 4H-Q); hence these have been called "spider-like" formations. Intriguingly, the spider-like formations were found in two places, inside macrocolony biofilms (Fig. 4K and 4M-Q) as well as outside macrocolonies (Fig. 4I). The possibility of that these structures were expelled outwards in the norfloxacin-treated macrocolony biofilms is suggested here, although additional experiments must be done to clarify this issue.

Several interesting results have been originated in this work which must be discussed.

How the norfloxacin antibiotic promotes SOS induction below of sub-inhibitory concentrations (b-sub-MICs), beyond of the "event horizon"? In other words, how norfloxacin acts in a concentration regime considered be inactive to trigger the SOS response.

Two theoretical scenarios could be visualized which evidently would require of future experiments to be confirmed (Fig. 5).

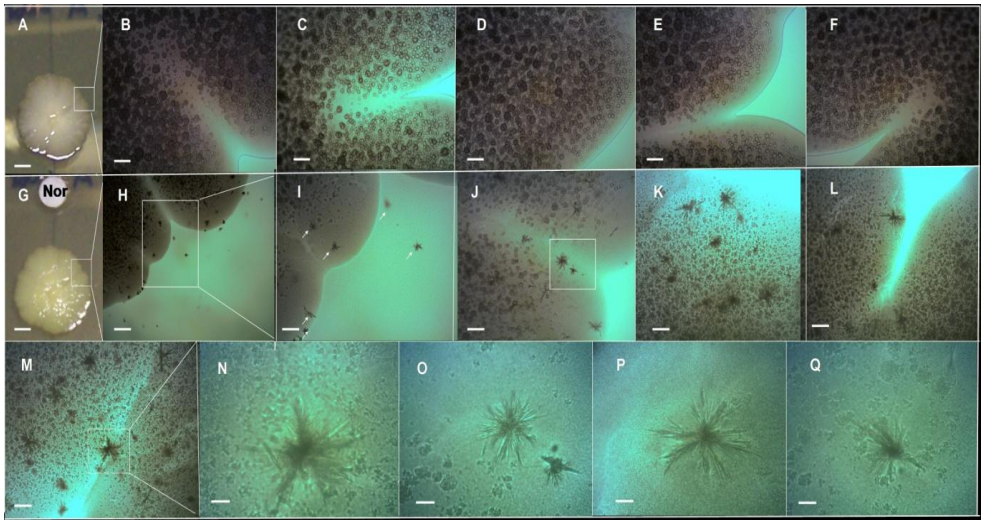

Fig. 4. Norfloxacin induces the production of "spider-like" formations in *E. coli* K-12 MG1655 (p*recA-gfp*) strain old macrocolony biofilms

(A) *Phenotypical appearance of an E. coli K-12 MG1655 (precA-gfp) "volcano-like" 5-day-old macrocolony biofilm developed in LB-Km medium on 0.6% semisolid ABE agar at 37ºC previous to be treated with a norfloxacin paper disk (100 µg/µl).* ***(B-F)*** *typical microscopic appearance of surface of this macrocolony exhibiting massively chondrule-like formations [24].* ***(G)*** *phenotypical appearance of the macrocolony shown in (A) after seven days of be treated with norfloxacin disk. the yellow-green colour stain of the macrocolony marks the recA-gfp expression and it is easily appreciated.* ***(H-M)*** *typical appearance of this treated macrocolony with norfloxacin showing "spider-like" formations on its surface and also outside colony.* ***(I)*** *enlargement of the box in (H), two "spider-like" structures are observed outside macrocolony.* ***(K)*** *enlargement of the box in (J).* ***(N-Q)*** *typical "spider-like" formations observed in situ on the surface at x400 magnification.* ***(N)*** *enlargement of the box in (M). scale bars: (A,G) 0.5 cm; (H,J) 400 µm; (B-F and I, K-M) 200 µm; (N-Q) 40 µm*

3.1 Direct Effect, *E. coli* Macrocolonies Pumping Norfloxacin Outwards?

The effect of norfloxacin in macrocolonies situated beyond of the event horizon would be direct, i.e. the norfloxacin is able to generate DNA damage and SOS induction directly on the cells of these macrocolonies (Fig. 5A), but immediately then in this model, other fundamental question arise, how can be achieved a concentration of norfloxacin active in this situation when this concentration cannot be achieved only as consequence of passive diffusion of norfloxacin (Fig. 5B)? To explain this apparent paradox a hypothetical explicative scenario could be devised. It well known that *E. coli* cells use a diverse array of efflux pumps for the extrusion of antibiotics across of its two membranes (inner and outer) present in the cells of this representative gram-negative bacteria. For instance, it has been described in *E. coli* the existence of fluorquinolones (FQ) multidrug resistance efflux systems (FQ-MDR): AcrAB-TolC, AcrEF-TolC, EmrB, EmrD that mediate FQ resistance [12]. It is tempting to speculate that these FQ-MDR systems could extrude norfloxacin outwards of *E. coli* macrocolonies creating from these a new focus of emission of norfloxacin on the semisolid agar, altering thus the passive diffusion of norfloxacin, modifying in consequence the norfloxacin gradient that could be expected in absence of these norfloxacin "pumping" macrocolonies (Fig. 5C). Hence, this process could work to achieve a sufficient concentration of norfloxacin to affect to the macrocolony situated in the proximity to this new focus, boosting so the activity of norfloxacin beyond of the "event horizon", achieving norfloxacin thus the b-sub-MICs regime of action.

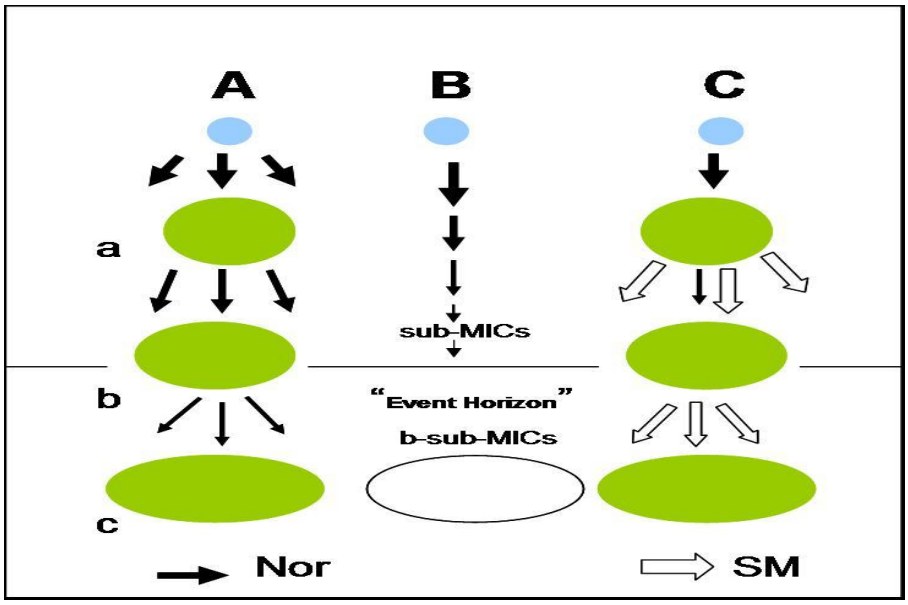

Fig. 5. Two hypothetical scenarios proposed to explain how the norfloxacin quinolone antibiotic promotes SOS induction response in macrocolony biofilms beyond of "event horizon"

(A) Direct effect. *The arrows represent schematically the gradient of concentration of norfloxacin due to diffusion. the thickness of arrows is related to the concentration of norfloxacin. by using flux pumps the macrocolonies treated with norfloxacin may cause re-difussion of the norfloxacin, changing in this manner the norfloxacin diffusive field with respect to that originates in a semisolid agar surface that no contain growing colonies* ***(B)****. the black horizontal line marks the "event horizon" beyond of this, it is not expected any SOS inductive effect of norfloxacin.* ***(C) indirect effect via induction of production of a putative signal(s) inductive molecule that triggers SOS induction****. the open arrows indicated the emission by the macrocolonies treated with norfloxacin of a putative signalling substance (signal molecule(s), SM) that mediates the SOS induction of recA-gfp transcriptional fusion. the intensity of SM field decays with the distance to the dish with norfloxacin. In blue the norfloxacin's disks. the green ovals represent macrocolonies with the recA-gfp fusion induced. the open oval represents a macrocolony where the recA-gfp fusion is not induced. the sub-MICs and b-sub-MICs concentration regimes are indicated*

3.2 Indirect Effect, is Acting a Communicative Process?

In this hypothetical scenario, the effect of norfloxacin is indirect on the SOS induction through of generation of chemical signals that mediate the norfloxacin effect on SOS induction beyond the "event horizon" under the b-sub-MICs regime. Thus, this model (Fig. 5C) takes account the possibility that the quinolones affects the genetic expression under sub-inhibitory concentrations, regulating the expression of different kind of genes (as is it has been described for other antibiotics, see introduction). The products of these genes could promote the production of a signal molecule(s) (SM) which could mediate the SOS induction. Therefore, it is proposed hypothetically that the *E. coli* macrocolony biofilms treated with norfloxacin would generate "communicating" signals that mediate the induction SOS among *E. coli* colonies situated far away from the dish with norfloxacin in a b-sub-MICs regime of concentration.

Other interesting question to be answered in a future investigation is: Which is the origin of spider-like formations? These formations apparently represent a novel product of response of *E. coli* macrocolony biofilms to treatment with norfloxacin. The origin and the chemical material(s) that compose these structures are unknown. In state of the present knowledge, is no possible to determinate if these formations were generate after of norfloxacin treatment in a process of synthesis *de novo* of macrocolony or whether its emergence on the surface of norfloxacin treated-macrocolony biofims was a consequence of disruption or disassembly of the chondrule-like formations. It is expected that future experiments can to clarify this issue. The presence of these formations outside macrocolony represents other enigmatic aspect of its production.

4. CONCLUSION

In conclusion, in this work it has been presented experimental evidence indicating that the norfloxacin antibiotic is able to promote the induction of SOS response (reported by the induction of activity of a *recA-gfp* transcriptional fusion) in old macrocolony biofilms of *E. coli* in a regime of concentration below of sub-inhibitory concentrations (b-sub-MICs) previously considered not active for this induction. Several theoretical scenarios have been proposed to explain this unexpected result that can be tested experimentally. In addition, it has been discovered novel spider-like formations on the surface of *E. coli* macrocolony biofilms treated with norfloxacin. The identification of its chemical nature and whether or not these formations have a role in the adaptation of *E. coli* macrocolony biofilms to the antibiotic challenge will be the focus of future experimental work.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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