Review Article

Understanding How Ciprofloxacin Affects Bacteria

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Abstract
Ciprofloxacin is an antibiotic that kills bacteria quickly, but exactly how it works is still unclear. In this study, researchers looked at how ciprofloxacin affects *Escherichia coli* using both traditional lab methods and advanced flow cytometry. They treated growing bacterial cells with different concentrations of ciprofloxacin (from 0.1 to 100 times the minimum inhibitory concentration or MIC) and examined them over time. Although high doses of ciprofloxacin significantly reduced the number of bacteria that could grow on plates, most of the cells remained intact and active under the microscope. These bacteria still had functioning membranes, showed normal metabolism, and even continued making proteins up to 5 hours after treatment. This means that many of the bacteria were alive and functioning, even though they couldn't grow in normal culture conditions. The findings suggest that ciprofloxacin-exposed *E. coli* cells can enter a "viable but nonculturable" (VBNC) state—a survival mode where they stay alive but don't divide or form colonies.

Keywords: *E. coli,* Ciprofloxacin, Minimum inhibitory concentration (MIC)

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Introduction

Ciprofloxacin is a broad-spectrum antibiotic belonging to the quinolone class, known for its potent bactericidal activity against a wide range of Gram-negative and Gram-positive bacteria[(1)]. It functions mainly by inhibiting bacterial DNA gyrase and topoisomerase IV, enzymes essential for DNA replication and transcription [(12,14,32)]. While ciprofloxacin is considered rapidly bactericidal, the exact sequence of cellular events that leads to bacterial death is still not completely understood [(29)]. Previous studies have proposed that ciprofloxacin may induce structural damage to bacterial cell membranes, leading to leakage of cytoplasmic content and death[(4,7)]. However, other evidence indicates that many bacteria exposed to the drug remain metabolically active, even though they are unable to form colonies[(9)]. This observation challenges the traditional reliance on colony-forming unit (CFU) counts to assess bacterial viability.

In this context, the present study by Mason *et al*. (1995)[(18)] aimed to investigate the effects of ciprofloxacin on *Escherichia coli* using both conventional and modern methods. These included light microscopy and advanced flow cytometry, allowing for a detailed assessment of bacterial membrane potential, membrane integrity, metabolic activity, and cell morphology. This multifaceted approach helps to clarify whether bacteria truly die or enter a metabolically active but non-growing state following exposure to ciprofloxacin [(31)].

The primary goal of this study was to investigate how ciprofloxacin affects *Escherichia coli* cells at both physical and metabolic levels. Specifically, the researchers aimed to determine whether bacteria that lose the ability to form colonies after antibiotic exposure are still viable in terms of membrane integrity, energy production, and protein synthesis. Another important objective was to understand how bacterial morphology and respiration are influenced by different concentrations of ciprofloxacin [(13)]. By using flow cytometry with specific fluorescent dyes, the study sought to measure membrane potential, cell permeability, and metabolic activity in real time [(5)]. Additionally, the researchers aimed to examine stress responses such as SOS induction by tracking protein synthesis through enzymatic assays. These combined goals helped evaluate whether ciprofloxacin merely stops bacterial growth or also causes immediate and irreversible cellular death.

2.Materials and Methods

2.1.Bacterial Strains and Culture Conditions: Two *Escherichia coli* strains were used: KL16 and PQ37 (harboring *sulA::lacZ* SOS fusion). Cultures were grown at 37 °C in Iso-Sensitest or Brain Heart Infusion (BHI) broth (filtered through 0.2 µm filters for flow cytometry) with shaking at 200 rpm.

2.2.Antibiotic Preparation and MIC Determination:

Ciprofloxacin (gifted by Bayer, UK) was dissolved in 0.01 N NaOH and diluted in sterile deionized water. The minimum inhibitory concentration (MIC) was

determined using broth dilution in Iso-Sensitest broth, with a starting inoculum of 10^5 CFU/ml. MICs were 0.06 µg/ml for KL16 and 0.03 µg/ml for PQ37. Ciprofloxacin, a fluoroquinolone antibiotic, was generously provided by Bayer, United Kingdom. For experimental use, the compound was first dissolved in 0.01 N sodium hydroxide (NaOH) to ensure complete solubilization. This stock solution was then further diluted with sterile deionized water to achieve the desired working concentrations suitable for microbiological assays [22,16].

To assess the antibiotic susceptibility of the two *E. coli* strains used in the study (KL16 and PQ37), the minimum inhibitory concentration (MIC) of ciprofloxacin was determined using the standard broth dilution method. Iso-Sensitest broth served as the growth medium for the assay. An overnight culture of each strain was diluted to a final inoculum of approximately 10^5 colony-forming units per milliliter (CFU/ml) in each test well or tube.

The cultures were then incubated at 37 °C for 18 hours. Following incubation, the MIC was defined as the lowest concentration of ciprofloxacin that completely inhibited visible bacterial growth. The determined MIC values were:

- 0.06 µg/ml for *E. coli* KL16
- 0.03 µg/ml for *E. coli* PQ37

These MIC values were used as a reference to prepare ciprofloxacin treatments at various multiples of the MIC (e.g., 0.1×, 1×, 10×, 100×) for further experiments assessing bacterial viability, morphology, membrane integrity, and metabolic activity.

2.3.Experimental Setup:

Early-log phase cultures (10^7 CFU/ml) of KL16 were divided into five groups: control and ciprofloxacin-treated (0.1×, 1×, 10×, and 100× MIC). Cultures were incubated for 120 min, and samples (1 ml) were collected at 0, 15, 30, 60, 90, and 120 min. Samples were centrifuged, washed, and resuspended in fresh broth. Aliquots were stained with DiBAC4(3), propidium iodide (PI), or CTC for flow cytometry; the rest was used for viable plate counts.To investigate the effects of ciprofloxacin on *Escherichia coli* KL16, cultures were first grown to the **e**arly logarithmic phase, reaching a concentration of approximately 10^7 colony-forming units per milliliter (CFU/ml). This growth phase was chosen because bacteria are metabolically active and highly responsive to antibiotics during exponential growth.

The culture was then divided into five equal parts, each containing 25 ml of bacterial suspension: One group served as the untreated control, which was not exposed to ciprofloxacin. Four groups were treated with ciprofloxacin at concentrations corresponding to 0.1×, 1×, 10×, and 100× the minimum inhibitory concentration (MIC), based on the previously determined MIC for KL16.

All control and ciprofloxacin-treated *E. coli* KL16 cultures were incubated at 37 °C with shaking for a total duration of 120 minutes. To assess the bacterial response over time, 1 ml samples were aseptically collected at six time points: 0 minutes (prior to antibiotic exposure), and at 15, 30, 60, 90, and 120 minutes post-exposure. Each collected sample was processed systematically. First, samples were centrifuged at 13,000 rpm for 1 minute using an Eppendorf microcentrifuge to pellet the bacterial cells. The pellets were then washed once with fresh Iso-Sensitest broth to eliminate any residual antibiotic and resuspended in the same medium to ensure consistency across samples.

To evaluate various physiological parameters, aliquots of the resuspended sample were stained with specific dyes for flow cytometry. DiBAC4(3) was used to assess membrane potential, as depolarized cells take up this dye. Propidium iodide (PI), a nucleic acid-binding dye, was employed to evaluate membrane integrity, since it penetrates only cells with compromised membranes. CTC (cyanoditolyltetrazolium chloride), a redox-sensitive dye, was used to detect metabolic activity by identifying actively respiring cells. Each of these dyes was added to separate 0.2 ml aliquots of the sample, which were then subjected to flow cytometric analysis to measure single-cell physiological responses.

The remaining portion of each sample was utilized for viable plate counts by plating on nutrient agar to determine colony-forming units (CFUs), providing an independent assessment of bacterial viability. This multi-parameter experimental approach enabled simultaneous analysis of bacterial viability (through CFU enumeration), membrane integrity and potential (via PI and DiBAC4(3) staining), and metabolic activity (via CTC reduction) across various ciprofloxacin concentrations and time intervals.

2.4.Protein Synthesis Assay:

PQ37 cultures treated with ciprofloxacin (10× MIC) were sampled every 60 min for 300 min. β-galactosidase activity was measured using the Quillardet and Hofnung method and calculated using Miller's formula.[25]

To assess protein synthesis activity in response to ciprofloxacin exposure, *Escherichia coli* PQ37, a reporter strain containing the SOS gene fusion (*sulA::lacZ*), was used. Cultures were grown to early log phase and then treated with ciprofloxacin at a concentration equivalent to 10 times the minimum inhibitory concentration (MIC). Following antibiotic treatment, samples of 0.3 ml were aseptically collected at 60-minute intervals over a period of 300 minutes (i.e., at 60, 120, 180, 240, and 300 minutes post-treatment). The β-galactosidase activity, indicative of *sulA* expression and hence the induction of the SOS response, was measured using the colorimetric method described by Quillardet and Hofnung. This assay involves the enzymatic hydrolysis of the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG), which produces a yellow-colored product, o-nitrophenol, that can be quantified spectrophotometrically. The absorbance was measured at 420 nm (A_{420}) to detect the reaction product, and at 550 nm (A_{550}) to correct for cell debris and turbidity. The initial cell density was monitored at 600 nm (A_{600}).

The β-galactosidase activity was then calculated using Miller's standard formula: **Units = 1,000 × [A_{420} – (1.75 × A_{550})] / (t × v × A_{600})**, where *t* is the reaction time in minutes, *v* is the volume of culture used in milliliters, and A_{600} is the optical density of the culture before the assay. This

method provided a quantitative measure of protein synthesis activity over time in response to high-dose ciprofloxacin treatment. All experiments were performed in triplicate to ensure reproducibility, and representative data are reported.

2.5. Viable Counts and Microscopy:

CFU counts were performed using the Miles method on nutrient agar. For microscopy, bacterial numbers were counted using a Neubauer chamber under a Nikon Diaphot microscope. Cell lengths were estimated by image analysis using a calibrated string measurement method on ≥25 cells per group.[19]

To independently assess bacterial viability following ciprofloxacin treatment, colony-forming unit (CFU) counts were performed using a modified version of the Miles and Misra method. Aliquots of appropriately diluted bacterial cultures were plated onto nutrient agar and incubated to allow for colony development. The number of colonies was then counted and used to calculate the CFU per milliliter, providing a reliable estimate of viable bacterial cells capable of growth and division.

In parallel, light microscopy was employed to directly observe and quantify bacterial cells and assess morphological changes. For this, bacterial samples were loaded into a Neubauer counting chamber and examined under a Nikon Diaphot inverted microscope equipped with a camera. Bacterial cell numbers were counted in five different microscopic fields using a 40× objective under bright-field illumination to determine the average bacterial concentration per milliliter.

Additionally, bacterial cell length was measured as an indicator of morphological changes in response to ciprofloxacin. To achieve this, microscope images were transferred to a monitor via a JVC TK870E video camera. A calibrated string method was employed, where a piece of string was laid along the longitudinal axis of each bacterium to measure its length. This string length was then converted into micrometers using a known scale. The mean cell length and standard deviation were calculated from measurements of at least 25 individual cells per treatment group at the 120-minute time point, allowing for quantitative analysis of antibiotic-induced cell elongation and filamentation.

Reproducibility:

All experiments were performed in triplicate; representative results are presented.

3.Results

3.1.Viable Counts:

Exposure of *E. coli* KL16 to ciprofloxacin at 10× and 100× the MIC resulted in a rapid and significant decline in bacterial viability. Within the first 30 minutes, the CFU count decreased by approximately 2 log units, indicating substantial bacterial killing [6,9]. By 120 minutes, the reduction had reached 3 log units. In contrast, cultures exposed to the MIC of ciprofloxacin showed no significant change in viable counts over the same period. The control culture and the culture treated with 0.1× MIC exhibited an increase in CFU, reflecting normal bacterial growth in the absence of lethal antibiotic stress [14,22]

3.2. Fluorescence Shifts in Fixed Cells:

Flow cytometric analysis of ethanol-fixed *E. coli* KL16 cells stained with DiBAC4(3), propidium iodide (PI), and CTC revealed distinct changes in fluorescence intensity. Fixation, which disrupts membrane integrity and halts respiration, led to a marked increase—by at least 1 log unit—in fluorescence associated with DiBAC4(3) and PI, indicating loss of membrane potential and integrity, respectively [14,26]. Conversely, the fluorescence intensity of CTC-stained cells decreased by approximately 1 log unit, reflecting a loss of respiratory activity. These shifts confirm that the dyes effectively report on physiological status: increased DiBAC4(3) and PI fluorescence correlates with membrane damage and depolarization, while decreased CTC fluorescence indicates metabolic inactivity [11,17]

Table.1 showing viable counts over 120 min

A. Viable Counts (CFU Changes over 120 min)

Ciprofloxacin Concentration	CFU Change at 30 min	CFU Change at 120 min
Control (0× MIC)	Increase	Further increase
0.1× MIC	Increase	Further increase
1× MIC	No significant change	No significant change
10× MIC	↓ 2 log units	↓ 3 log units
100× MIC	↓ 2 log units	↓ 3 log units

Table.2 showing viable Fluorescence Shifts in Ethanol-Fixed Cells

B. Fluorescence Shifts in Ethanol-Fixed Cells

Fluorescent Dye	Cellular Target	Change in Fluorescence (Fixed Cells)
DiBAC4(3)	Membrane potential	↑ 1 log unit
Propidium iodide (PI)	Membrane integrity	↑ 1 log unit
CTC	Respiratory (metabolic) activity	↓ 1 log unit
Fluorescent Dye	Cellular Target	Change in Fluorescence (Fixed Cells)
DiBAC4(3)	Membrane potential	↑ 1 log unit

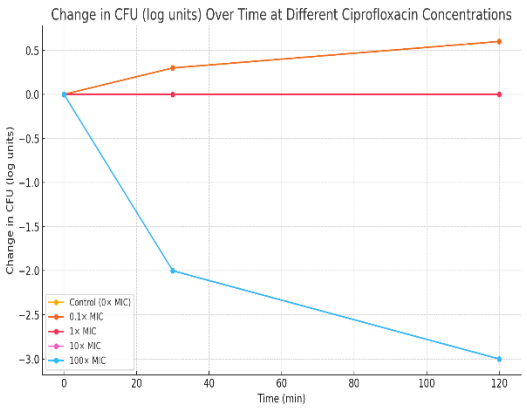

Fig. 2 Figure: Change in CFU (log units) of *E. coli* KL16 over time in response to ciprofloxacin at varying concentrations.

3.3. Shifts in Bacterial Fluorescence Following Ciprofloxacin Exposure

To evaluate the physiological changes induced by ciprofloxacin, the membrane potential, membrane integrity, and respiratory activity of *E. coli* KL16 were assessed using fluorescent dyes and flow cytometry.

(i) Membrane Potential ($DiBAC_4(3)$ Staining):

The membrane potential was analyzed by staining cells with $DiBAC_4(3)$, a dye that accumulates in depolarized cells. Exposure to 0.1× and 10× MIC of ciprofloxacin did not significantly alter membrane potential, with only 5–12% of cells showing fluorescence—comparable to untreated control samples [1,7]. At 1× MIC, fluorescence also remained low (5–12%) up to 90 minutes, but a slight, non-significant increase to 20% was observed at 120 minutes. However, at 100× MIC, more than 95% of the cells were $DiBAC_4(3)$-positive, indicating substantial membrane depolarization at this high concentration [5].

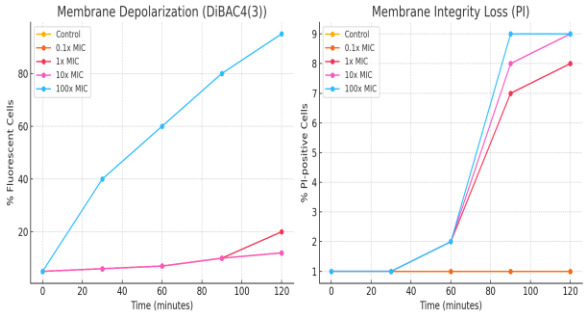

Figure: Effects of ciprofloxacin on membrane depolarization and integrity in *E. coli* KL16. Left: Membrane depolarization was assessed using DiBAC4(3) staining over 120 minutes. A dose-dependent increase in fluorescent cells was observed, with the highest depolarization at 100× MIC.

Right: Membrane integrity loss, measured by propidium iodide (PI) uptake, showed a sharp increase after 60 minutes at 10× and 100× MIC, indicating membrane damage. No significant PI uptake was seen in control, 0.1×, or 1× MIC groups. These results suggest that ciprofloxacin induces early membrane depolarization followed by membrane integrity loss at high concentrations.

Table 3. Membrane Depolarization - DiBAC4(3) (% Fluorescent Cells)

Time (min)	Control	0.1× MIC	1× MIC	10× MIC	100× MIC
0	5	5	5	5	5
30	6	6	6	6	40
60	7	7	7	7	60
90	10	10	10	10	80
120	12	12	20	12	95

Table.4. Membrane Integrity Loss - PI (% PI-positive Cells)

Time (min)	Control	0.1× MIC	1× MIC	10× MIC	100× MIC
0	1	1	1	1	1
30	1	1	1	1	1
60	1	1	2	2	2
90	1	1	7	8	9
120	1	1	8	9	9

1. Respiratory Activity - CTC (% Active Cells at 120 min)

Table.5. % Respiratory Active Cells

Condition	% Respiratory Active Cells
Control	67
0.1× MIC	90
1× MIC	90
10× MIC	90
100× MIC	57
Control	67
0.1× MIC	90
1× MIC	90

(ii) Membrane Integrity (Propidium Iodide Staining):

Membrane integrity was measured using propidium iodide (PI), which penetrates cells with compromised membranes. In both the control group and the 0.1× MIC-treated cells, PI staining remained low (0.7–1.5%) throughout the 120-minute period. Similarly, exposure to 1×, 10×, and 100× MIC resulted in minimal PI uptake (0.6–2.5%) during the first 60 minutes. However, by 90 minutes, cells treated with 1×, 10×, and 100× MIC showed an increased PI-positive population, ranging between 7–9%, suggesting a gradual loss of membrane integrity at higher concentrations and longer exposure [6,9].

(iii) Respiratory Activity (CTC Reduction):

The metabolic activity of the cells was assessed by the reduction of the tetrazolium dye CTC, which fluoresces in actively respiring cells. Up to 10× MIC, ciprofloxacin did not affect respiratory activity, with around 90% of cells remaining metabolically active after 120 minutes [27]. Interestingly, a decline in fluorescence was observed in both the control group (67%) and the 100× MIC group (57%) at the 120-minute mark, indicating a general reduction in respiratory activity over time, particularly at very high drug concentrations[21,28].

3.4. Bacterial Cell Counts:

Flow cytometry-based total bacterial counts showed that cultures treated with 0.1× MIC and the control group exhibited an increase in cell numbers over time, indicating ongoing growth. In contrast, the bacterial counts for cultures exposed to 1×, 10×, and 100× MIC remained unchanged throughout the 120-minute incubation, reflecting a growth arrest despite sustained cell presence. [10,18]

3.5. Cell Morphology:

Microscopic examination revealed that the average cell length remained consistent (3.2 ± 1.1 µm) in both control and 0.1× MIC-treated cultures, suggesting that sub-MIC exposure did not induce morphological changes in the bacterial population.

3.6. SOS Induction: Ciprofloxacin at 10× MIC triggered strong SOS induction in *E. coli* PQ37, as indicated by elevated expression of the *sulA::lacZ* gene fusion. β-Galactosidase activity, a reporter for SOS response, rose exponentially during the first 120 minutes and continued to increase linearly over the following 180 minutes. This sustained increase reflects ongoing de novo protein synthesis despite continuous antibiotic exposure, highlighting the bacteria's active physiological state under stress.

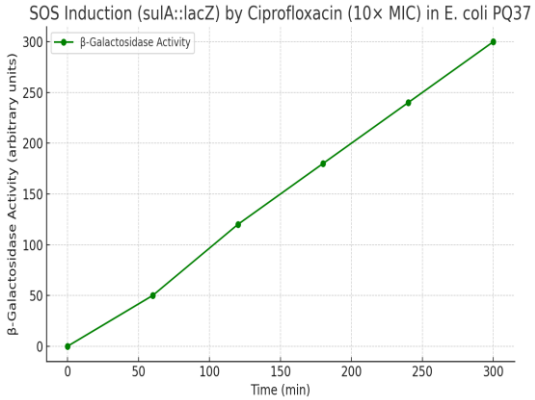

Figure 3: SOS response induction in *E. coli* PQ37 upon ciprofloxacin exposure.

4. Discussion

Ciprofloxacin, a fluoroquinolone antibiotic, is widely known for its potent bactericidal activity, primarily through inhibition of bacterial DNA gyrase and topoisomerase IV, leading to DNA damage and eventual cell death [(8)]. However, the exact sequence of physiological events that constitute ciprofloxacin-mediated killing remains complex and not fully elucidated. In this study, we combined flow cytometry, plate counts, microscopy, and reporter assays to comprehensively analyze the early cellular responses of *E. coli* to ciprofloxacin at varying concentrations.

Despite a 3-log reduction in CFU within 120 minutes at high ciprofloxacin concentrations (10× and 100× MIC), our microscopy and flow cytometry data demonstrated that most bacterial cells retained membrane integrity and metabolic activity, as indicated by exclusion of propidium iodide (PI) and reduction of CTC. Moreover, over 90% of cells maintained membrane potential at all concentrations except 100× MIC [(33)], where a significant drop was observed. These findings challenge the assumption that loss of culturability equates with cell death, aligning instead with the concept of the viable but nonculturable (VBNC) state, where cells are metabolically active yet fail to grow on sqqa WQAwaqtandard media [(21)].

The continuation of protein synthesis, as evidenced by increasing β-galactosidase activity in *E. coli* PQ37, further supports the idea of active physiological processes in ciprofloxacin-treated cells. The *sulA::lacZ* fusion, indicative of SOS response activation, showed progressive induction for up to 300 minutes [(24)]. This suggests that the bacteria are not only sensing DNA damage but also mounting a repair response, consistent with previous studies showing fluoroquinolone-induced SOS gene upregulation [(15)]. Persistent induction of SOS genes can also lead to mutagenesis, potentially contributing to antibiotic resistance development [(2)].

Interestingly, cells exposed to subinhibitory concentrations (0.1× MIC) displayed increased CFU over time, similar to untreated controls, indicating that low-level ciprofloxacin may not impair growth and could potentially prime stress response pathways. In contrast, cells exposed to 1× MIC showed static CFU counts, with no significant killing or growth, suggesting bacteriostatic effects at this threshold.

Our findings also validate the use of multi-parameter flow cytometry to distinguish between viability, membrane integrity, and metabolic function. Ethanol-fixed controls clearly demonstrated that DiBAC4(3) and PI fluorescence increased upon membrane compromise, while CTC signals decreased with metabolic arrest [(30)]. These tools enabled a nuanced understanding of bacterial fate post-antibiotic exposure beyond traditional CFU-based assays[(31)].

In conclusion, ciprofloxacin induces rapid loss of culturability in *E. coli* without immediate loss of metabolic or physical viability, highlighting a transient, stress-adapted state rather than outright cell death[(6)]. This underscores the importance of using multiple viability indicators in antimicrobial studies and suggests that the early effects of fluoroquinolones involve more than just lethal DNA damage. Moreover, persistence of protein synthesis and membrane function points to potential survival strategies that may influence treatment outcomes and resistance evolution[(10,11)].

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