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EPIGENETIC AND GENETIC RESPONSES OF *ESCHERICHIA COLI* PERSISTER CELLS TO CIPROFLOXACIN STRESS: INSIGHTS INTO THE ROLE OF NUCLEOID-ASSOCIATED PROTEINS

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ABSTRACT

Persister cells in *Escherichia coli* (*E. coli*) represent a transient, non-mutational form of antibiotic tolerance that contributes to treatment failure and chronic infections. This study investigated the genetic and epigenetic responses of *E. coli* persister cells exposed to ciprofloxacin-induced stress, with a focus on the role of nucleoid-associated proteins (NAPs) in promoting DNA protection and persistence. Fifty clinical *E. coli* isolates were screened for persister cells using the Rapid Killing and Replica Plating Tolerance Isolation System (REPTIS). The expression of selected NAP genes (*hns, hupA, hupB,* and *dps*) was analyzed using real-time PCR (RT-qPCR), while *hns* gene methylation was assessed via qMethyl PCR. DNA integrity was evaluated by the neutral comet assay, and Dps protein expression was measured by Western blotting. Persister cells were detected in 4% of isolates, with isolate E3 confirmed by REPTIS. RT-qPCR showed significant upregulation of NAP genes in persisters, with *hupB* showing the highest fold increase (685-fold). qMethyl PCR revealed elevated *hns* methylation levels (61.55%), suggesting epigenetic regulation. The comet assay showed reduced DNA damage in persister cells, while Western blotting indicated a 1.2-fold increase in Dps expression under ciprofloxacin stress. These findings suggest that *E. coli* persistence involves a multifaceted mechanism, including transcriptional activation, epigenetic modifications, and protein-level regulation of NAPs. The enhanced expression of Dps under antibiotic stress highlights its protective role and supports the potential of targeting NAPs in developing novel therapeutic strategies against persistent and recurrent *E. coli* infections.

Keywords: Antibiotic resistance, Comet assay, DNA methylation, Persister cells, Western blotting

INTRODUCTION

Antibiotic resistance is a rising global issue, and *Escherichia coli* (*E. coli*) is one of the bacterial species at the very forefront of this problem. It is a common gut inhabitant and has the disturbing capacity to develop simultaneous resistance to several antibiotics **(Saeed *et al*., 2024; Santamarina *et al*., 2022).** The emergence of antibiotic-resistant subtypes of *E. coli* is due to a combination of factors, such as years of improper and excessive use of antibiotics and the horizontal transfer of resistance genes encoded in the bacterium's genome (Ali et al., 2021).
The rise of antibiotic-resistant bacteria has also led to the formation of persister cells, a subtype of bacteria with increased tolerance to antimicrobial agents that cause serious public health problems. These resistant bacteria can undergo genetic alterations that cause them to be capable of growth in conditions in which antibiotics are present, while persister cells have an inactive state that makes them less vulnerable to the effects of those drugs **(Lanni *et al*., 2023)**.
Unlike resistant bacteria, persisters do not carry genetic mutations that confer resistance to antibiotics. As a result, they transition into a temporary non-dividing state that allows them to endure lethal levels of antimicrobials **(Van den Bergh *et al*., 2017)**. Formation of persister cells is believed to be a risk-spreading strategy whereby a fraction of the bacterial population survives environmental insults such as antibiotic treatment (Lanni et al., 2023). These cells have decreased metabolism, and they become dormant, which protects them from the effects of antibiotics that kill actively growing cells (Miyaue et al., 2018). More recent work has shown that dormancy alone cannot explain bacterial persistence, but other states (mainly physiological mechanisms) are likely also involved in this phenomenon **(Gollan *et al*., 2019)**.

Ever since, it has become widely accepted that persister cells are heterogeneous, which has important implications for effective antimicrobial treatment strategies. Assessment of the diverse mechanisms that underlie the formation and regulation of persisters transitioning from a dormant to an active state may give rise to novel therapeutic targets **(Wilmaerts *et al*., 2019)**. Although the exact mechanisms of persistence remain an active subject of research, recent evidence indicates that nucleoid-associated proteins (NAPs) are important in this process. NAPs are a group of highly diverse DNA-binding proteins that organize the bacterial chromosome and modulate gene expression in response to environmental cues **(Serebreni & Stark, 2021).** These proteins play roles in establishing and maintaining dormant or slow-growing persister cells that are less sensitive to antibiotic killing **(Jaishankar & Srivastava, 2017)**.
A prominent system involved in bacterial persistence is the toxin-antitoxin (TA) system, a widespread genetic module in numerous bacterial taxa. Following stress exposure, the expression of toxins proteins gets upregulated, forming a dormant state that promotes survival in the face of antibiotic treatment **(Singh *et al*., 2021)**. Some characterized nucleoid-associated proteins have also been shown to interact with and regulate the activity of toxin-antitoxin systems, linking chromosome organization to the persistence phenotype even closer **(Harms *et al*., 2016)**. In addition to toxin-antitoxin modules, a number of nucleoid-associated proteins have been linked to persister cell formation as well. Such repression requires the protein Hns, a histone-like protein that represses growth and metabolism-related gene expression, promoting a quiescent state typically associated with persisters. Likewise, the nucleoid-associated protein Dps (DNA protecting protein from starved cell) allows *E. coli* to survive oxidative stress and antibiotic treatment by stabilizing the bacterial chromosome **(Radzikowski *et al*., 2017)**. The Hu protein (Histone-like protein from *E. coli* strain U93), known for its roles in DNA compaction, transcriptional regulation and DNA damage response, has also been associated with antibiotic persistence, enabling cells to withstand antibiotics and potentially reacquire an infection **(Bose *et al*., 2017; Zhang *et al*., 2018)**.
Entailing a quest for therapeutic targets against such alarming antibiotic resistances, the present study investigates epigenetic and genetic responses of *E. coli* persister cells to ciprofloxacin during periods of cell growth and stress, with an emphasis on the importance of nucleoid-associated proteins (NAPs) in maintaining persistence.

MATERIALS AND METHODS

Bacterial Isolation and Identification

Between October 2023 and February 2024, 121 clinical samples (urine, blood, stool, burn, and wound) were collected from Elwia Pediatric and Alkindy Hospitals in Baghdad. Fifty *E. coli* isolates were identified using colony morphology on selective media (MacConkey, Blood, Eosin Methylene Blue EMB, and CHROM agar), Gram staining, and biochemical tests, including oxidase, catalase, and the IMViC series (Indole, Methyl Red, Voges-Proskauer, Citrate). Species-level

confirmation was performed using the VITEK-2 compact system (bioMérieux/France) with ID-GNB cards, ensuring accurate identification.

Detection of the existence of persister cells forming *E. coli*

A rapid killing method

A rapid killing method, as described by **Canas-Duarte *et al*. (2014)**, was employed to identify *E. coli* persister cells by selectively eliminating actively dividing cells. Bacterial strains were cultured overnight in LB broth at 37°C and standardized to 0.5 McFarland (1.5×10^8 CFU/ml). A 1 ml aliquot of the bacterial suspension was treated with 200 µL genomic lysis buffer (Promega, USA), vortexed for 10 seconds, and incubated at room temperature for 10 minutes. This was followed by the addition of 200 µL lysozyme solution, gentle mixing, and incubation at 37°C with shaking for 15 minutes. After treatment, 10 µL of the mixture was plated on Luria-Bertani (LB) agar and incubated at 37°C for 24 hours. Persister cells were identified based on their ability to survive lysis and regrow into colonies on the LB medium.

Validation of Persister Phenotype Using REPTIS

The Replica Plating Tolerance Isolation System (REPTIS) was used to validate the findings of the rapid killing method and confirm the persister phenotype of the two suspected isolates following Matuso (2019) method. A suspension of the parent strain, standardized to 0.5 McFarland standard, was spread on brain heart infusion (BHI) agar plates containing 800 µg/ml ciprofloxacin (10× MIC). These master plates were incubated for 72 hours to eliminate actively dividing cells, leaving only nongrowing, potential persister cells.
Colonies from the master plates were transferred onto antibiotic-free Brain Heart Infusion (BHI) agar plates (replica plates) using a replica plating technique. The replica plates were incubated for another 72 hours, allowing any surviving persister cells to regrow. Any colonies observed on the replica plates confirm the presence of persisters, representing the subpopulation that endured ciprofloxacin stress **(Matsuo *et al*., 2019)**.

Antibacterial susceptibility assay

Antibiotic susceptibility testing was performed using the disc diffusion method (DDT) according to CLSI guidelines (2023) (Rai et al., 2023). Overnight cultures of *E. coli* were suspended in 5 ml of normal saline and adjusted to match a 0.5 McFarland standard. Using a sterile cotton swab, a 0.1 ml aliquot of the standardized suspension was uniformly spread onto Mueller-Hinton agar (MHA) plates. Antibiotic discs were placed on the inoculated plates at equal distances using sterile forceps. The tested antibiotics included Imipenem IPM (10 µg), Meropenem MEM (10 µg), Ceftazidime CAZ (30 µg), Ceftriaxone CRO (30 µg), Cefotaxime CTX (30 µg), Cefepime FEP (30 µg), Ciprofloxacin CIP (5 µg), Levofloxacin LEV (5 µg), Doxycycline DOX (30 µg), Gentamicin GEN (10 µg), Tobramycin TOB (10 µg), Streptomycin STR (10 µg), Amikacin AMK (30 µg), Amoxicillin-clavulanate AMC (30 µg), and Azithromycin AZM (15 µg). Plates were incubated at 37°C for 24 hours. After incubation, the diameters of the inhibition zones were measured in millimeters and compared with MM294 (Carolina/USA) as the standard control strain.

PCR for the Detection of Nucleoid-Associated Proteins (NAPs)

DNA extraction by boiling method and primer design

DNA templates were prepared with the boiling method described by **Ruppé *et al*. (2009)**. The bacterial isolates were cultured on Brain Heart Infusion (BHI) agar and incubated at 37°C for 24 h. Individual colonies were resuspended in 0.5 mL of distilled water in Eppendorf tubes and thoroughly mixed in the vortex. The tubes were boiled in a water bath for 5 minutes and then centrifuged (10,000 rpm, 10 min). The supernatant was then harvested and used as a DNA template for PCR.
Specific primers used for PCR amplification of nucleoid-associated protein (NAP) genes (*hns, hupA, hupB,* and *dps*) and the 16s rRNA (used as a reference gene) were designed using the Primer3 web tool and synthesized from Macrogen, South Korea, as shown in (Tab1). The OligoCalc web tool was used to check for self-complementarity and dimer formation for optimal performance. The NAP genes were selected based on their roles in DNA protection and gene regulation.

Polymerase Chain Reaction (PCR)

The PCR reaction mixture was prepared at a 200 µL Eppendorf tube containing 12.5 µL of master mix, 1 µL of both forward and reverse primers, 6 µL DNA template, and 4.5 µL DNase free water for a final volume of 25 µL. The PCR program was initiated with an initial denaturation at 95°C for 5 minutes, then followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and extension at 72°C for 30 seconds. The target DNA amplification was confirmed by a final extension cycle of 10 minutes at 72°C.

Table 1 Primers employed for the identification of NAPS genes, all designed during the present study

Target Gene	Oligo 5→3	Product size (bp)
hns	F:TGCTGCTGAAGTTGAAGAGC R: CCAGGTTTTAGTTTCGCCGT	187
hupA	F: ACTGTCCAAAACCCAGGCTA R: TTCAGTGCCTTGCCAGAAAC	213
hupB	F: AGGGGATGATGTAGCACTGG R: TTTACCGCGTCTTTCAGTGC	156
dps	F: GCGCTAACTTCATTGCCGTA R: CCTGAACGTTGTGGATGTCC	180
16s *rRNA*	F: TAGGCCCGAAACTGACGATT R: AACCAGACGATTCACCACCT	206
hns for qMethyl PCR	F: CGTCGAGGGATTTACCTTGCT R: ACGCTGGAAGAAATGCTGGA	187

DNA Methylation Level Detection

DNA methylation levels were assessed using the OneStep qMethyl™ Kit (ZYMO/ USA), a bisulfite-free method, that combines methylation-sensitive restriction enzyme (MSRE) digestion with real-time PCR for precise analysis of the *hns* gene. Custom primers for the *hns* gene were redesigned to avoid MSRE-specific restriction sites, ensuring primer stability and accurate amplification, as detailed in (Tab1).
For each DNA sample, two reactions were prepared: a test reaction (including MSREs) and a reference reaction (excluding MSREs). Each reaction mixture contained 10 µL of 2× Reaction PreMix, 2 µL of 10 µM primers, 5 µL of DNA template, and 3 µL of RNase/DNase-free water. The digestion step with MSRE was optimized at 30°C for 12 hours, providing sufficient time for complete DNA cleavage before proceeding to qPCR. The qPCR cycle included denaturation at 95°C for 8 minutes, followed by amplification cycles of 97°C for 20 seconds, annealing at 57°C for 1 minute and amplification at 72°C for 20 seconds, repeated for 40 cycles, and a final holding step at 4°C. The methylation levels for the experimental persister isolate and the non-persister control were determined using the following equation
Percent methylation = $100*2^{-\Delta Ct}$
Where ΔCt = Ct (Test reaction) - Ct (Reference reaction)
The resulting difference in methylation levels provides a quantitative measure of the variation between the experimental persister isolate and the non-persister control.

Detection of NAPs gene expression

Preparation of selective isolate

RT-qPCR analysis via (Esco/ Singapore) was performed on both the persister isolate (E3) and the non-persister isolate (E7) to investigate their responses under stress conditions. For stress induction, bacterial cultures were exposed to ciprofloxacin, a DNA-damaging antibiotic, at a sub-minimum inhibitory concentration (sub-MIC). The optimal sub-MIC concentration of ciprofloxacin was determined using a plate serial dilution method. Bacterial cultures were subjected to this stress condition for 4 hours to evaluate gene expression changes.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from the stressed bacteria using the Wizprep™ Total RNA Mini Kit (cell) according to the manufacturer's instructions (South Korea). For cDNA synthesis, 3 µL of the extracted RNA template was mixed with 0.75 µL each of forward and reverse primers (1.5 µL total), 10 µL of RT master mix, and RNase-free water to a final volume of 20 µL in a 0.2 mL PCR microtube, following the guidelines of the Wizprep™ protocol.

Quantitative Real-Time PCR (RT-qPCR)

The RT-qPCR reaction mixture was prepared in a 200 µL Eppendorf tube containing 10 µL of 2X RT-qPCR master mix, 1 µL each of forward and reverse primers, 3 µL of cDNA template, and 5 µL of nuclease-free water, resulting in a total reaction volume of 20 µL. The thermocycling protocol started with an initial denaturation at 95°C for 12 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 20 seconds. The *16S rRNA* gene was used as a housekeeping gene to normalize mRNA levels and calculate fold changes. Gene expression was quantified using the $2^{(-\Delta\Delta CT)}$ method, ensuring accurate and reliable analysis of mRNA levels.

Comet assay (Neutral)

The neutral comet assay, adapted from **Singh *et al*. (1988)**, involved coating slides with 1% agarose gel, embedding cells in melted agarose, and lysing them at 4°C for 1 hour in a Triton X and Dimethyl sulfoxide (DMSO) buffer (pH 10). After equilibration in EDTA-NaOH (pH 9) for 20 minutes, electrophoresis was

performed at 24 V and 300 mA for 30 minutes. Slides were neutralized with tris-base (pH 7.5), DNA was stained with ethidium bromide, and comet images were captured and analyzed using CASP software. DNA damage, including olive tail moment and apoptotic comets, was quantified for 50 nuclei per sample using the method described by **(Collins, 2004)**.

Western Blotting for Detection of Dps Protein in Ciprofloxacin-Stressed *E. coli* Persister Cells

To specifically assess Dps protein expression induced by ciprofloxacin stress, excluding Dps naturally produced during the stationary phase a clinical *Escherichia coli* isolate (E3) was cultured in Luria-Bertani (LB) broth at 37°C for 18 hours and then sub-cultured to an initial OD_{600} of 0.01 to ensure growth initiation from the lag phase. Cells were allowed to grow only until the mid-log phase (OD_{600} of 0.4–0.5), deliberately avoiding progression into the stationary phase. At this point, ciprofloxacin was added at a sub-minimum inhibitory concentration (sub-MIC), and cultures were incubated for an additional 4 hours at 37°C to induce stress without triggering stationary phase-associated Dps expression.

After stress induction, cells were pelleted (12,000 rpm, 10 min, 4°C), lysed in SDS loading buffer, heated at 95°C for 10 minutes, and centrifuged (12,000 rpm, 2 min, 4°C). Supernatants were collected for protein analysis. Proteins were separated on 12% Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using a semi-dry system. Membranes were blocked with 5% skim milk, probed with anti-Dps primary antibody and HRP-conjugated secondary antibody, and visualized using enhanced chemiluminescence after substrate addition (Elabscience, Catalog No E-IR-R305).

Statistical analysis

Statistical analysis was conducted with GraphPad Prism 8.1.0. Data are expressed as mean ± SD, and differences between treated and untreated samples were evaluated using one-way ANOVA, with $p < 0.05$ being statistically significant.

RESULTS

Isolation and identification of *E. coli* isolates

The study collected 50 *E. coli* isolates, predominantly from urinary tract infections (80%, 40/50), with additional isolates from blood (10%, 5/50), wounds (6%, 3/50), and stool samples (4%, 2/50). Biochemical identification revealed positive catalase activity, negative oxidase activity, and typical IMViC test results: positive for Indole and Methyl Red, and negative for Voges-Proskauer and Citrate utilization. Species confirmation was achieved using the Vitek-2 Compact system, which analyzed 64 biochemical reactions, identifying all isolates with 97–99% accuracy.

Persister Cell Detection

Rapid Killing assay

The presence of persister cells in all *E. coli* isolates was determined using the rapid-killing approach. The findings revealed that 4% (2/50) of the 50 isolates (E3, E44) could produce persister cells. In this context, the emergence of one to a few colonies (sub-population) was identified as persisters for each isolate.

Replica plating tolerance isolation test

The two isolates identified as persisters using the rapid killing method were further evaluated using the REPTIS technique to confirm their persister phenotype. Among these, only isolate E3 demonstrated the ability to survive and regrow on the replica plate where the E44 failed to do so, thereby validating its classification as a persister isolate based on the REPTIS criteria.

Antimicrobial Susceptibility of *E. coli* Isolates

According to the Clinical Laboratory Standards Institute (CLSI) guidelines (2023). The isolates showed variable levels of resistance to different antibiotic classes, by showing resistance to aminoglycoside class Amikacin (2%), Gentamicin (4%), Streptomycin (12%), and Tobramycin (14%), βeta-Lactam class represented by Augmentin (16%), Cephalosporins 3rd generation class Cefotaxime (90%), Ceftriaxone (82%), and Ceftazidime (66%), Cephalosporins 4th generation including Cefepime (52%), Carbapenem class including Meropenem (0%) and Imipenem (0%), Tetracycline class represented by Doxycycline (54%), Fluroquinolone class including Ciprofloxacin (30%) and Levofloxacin (26%), and Macrolides represented by Azithromycin (46%) as shown in figure 1 and illustrated in details in (Tab 2). Out of all the used antibiotics, only one isolate was found to be sensitive to all (E46), and 23 isolates (46%) were classified as MDRs.

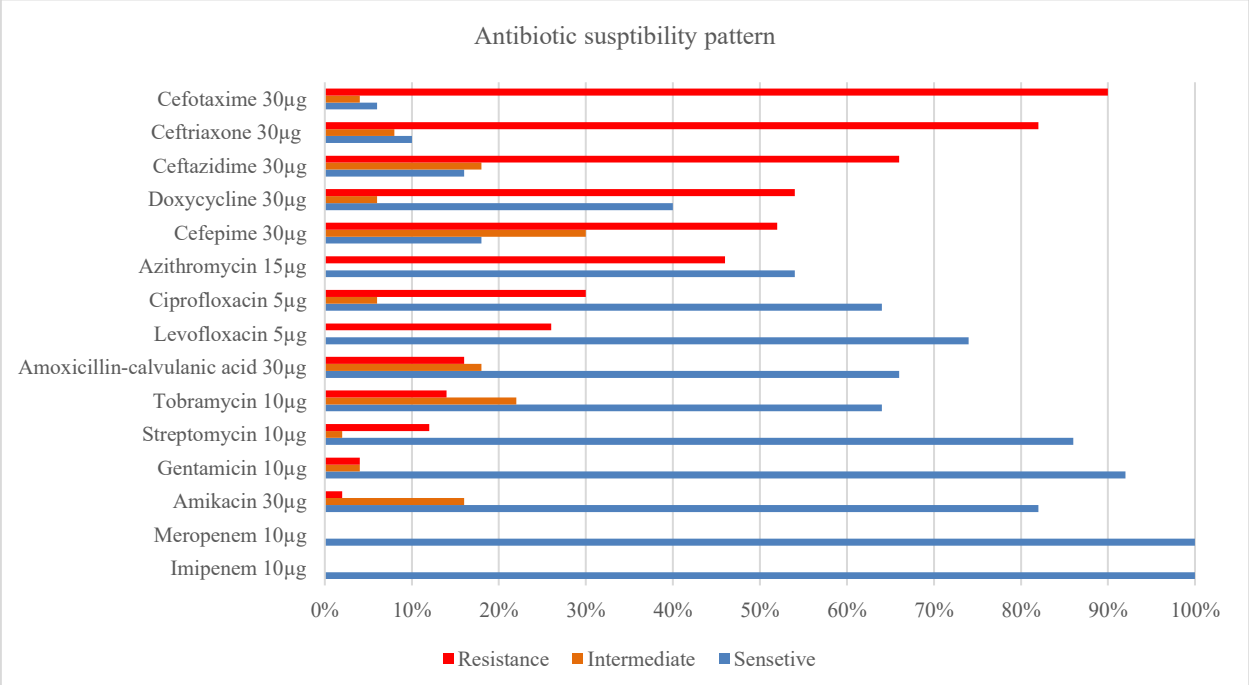


Figure 1 Antibiotic susceptibility pattern

Bar graph showing the antibiotic susceptibility profiles of 50 *Escherichia coli* isolates against 15 antimicrobial agents. Antibiotics tested include: AK (Amikacin), GM (Gentamicin), TOB (Tobramycin), ST (Streptomycin), AUG (Amoxicillin-clavulanate), CTX (Cefotaxime), CRO (Ceftriaxone), CAZ (Ceftazidime), FEP (Cefepime), IMI (Imipenem), MRP (Meropenem), CIP (Ciprofloxacin), LEV (Levofloxacin), DXT (Doxycycline), and AZM (Azithromycin). Bars represent the number of isolates categorized as sensitive, intermediate, or resistant based on CLSI guidelines (2023).

Table 2 Antibiotic Susceptibility of *E. coli* Isolates Against Various Antimicrobials

	AK	GM	TOB	ST	AUG	CTX	CRO	CAZ	FEP	IMI	MRP	CIP	LEV	DXT	AZM
Sensitive	41	46	32	43	33	3	5	8	9	50	50	32	37	20	27
Intermediate	8	2	11	1	9	2	4	9	15	0	0	3	0	3	0
Resistance	1	2	7	6	8	45	41	33	26	0	0	15	13	27	23

PCR for the Detection of Nucleoid-Associated Proteins (NAPs)

In the current study, DNA was isolated from a total of 10 *E. coli* isolates, encompassing both persister and non-persister phenotypes (designated as E1, E2, E3, E4, E5, E6, E7, E8, E9, and E44). The extracted DNA was subjected to PCR amplification using specific primers to detect the presence of the *hns*, *hupA*, *hupB*, and *dps* genes. The PCR products were subsequently validated by comparing their respective base pair sizes 187 bp, 213 bp, 156 bp, and 180 bp to those of a DNA marker. The results conclusively demonstrated that all the tested *E. coli* isolates possess the four genes encoding the targeted nucleoid-associated proteins (NAPs), as shown in figure 2.

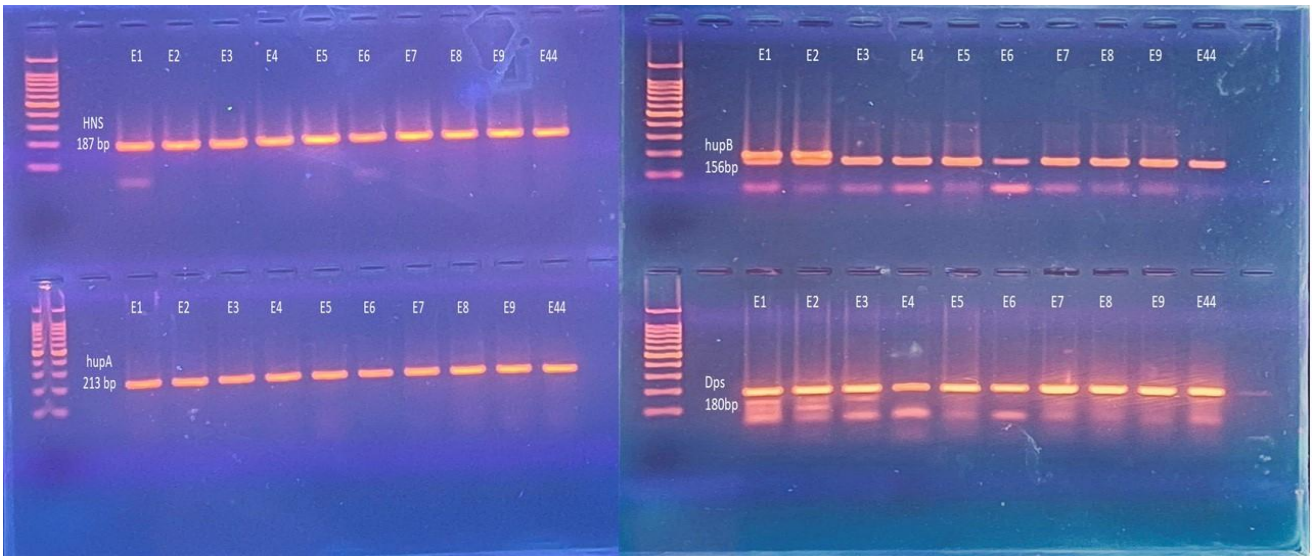


Figure 2 NAPs genes Visualization through electrophoresed gel.

qMethyl PCR

The DNA methylation analysis of the *hns* gene using the OneStep qMethyl™ Kit revealed significant differences in methylation levels between persister isolate under ciprofloxacin sub-MIC stress and non-persister *E. coli* isolate. For the persister isolate (Sample A1, reference reaction, does not contain methyl-sensitive restriction enzyme MSRE), the Ct value was 26.0, while the test reaction (Sample A2, with methyl-sensitive restriction enzyme) exhibited a Ct value of 26.7 as illustrated in (Tab 3) and shown in figure 3.

ΔCT was calculated as the Ct value of the test reaction minus the Ct value of the reference reaction. The persister isolate showed a ΔCt of 0.7, resulting in a methylation level of 61.55% after formula application. Conversely, for the non-persister control isolate (Samples A3 and A4, serving as reference and test controls respectively), the ΔCt was 2.3 (29.0 - 26.7), leading to a significantly lower methylation level of 20.30%, which resembles the methylation level under normal, not stressed conditions. Therefore, the difference in methylation levels between the persister and non-persister isolates was (41.25%).

Table 3 CT Values and Methylation Analysis for Persister and Non-Persister *E. coli* Isolates Using qMethyl PCR

Hole number/ isolate mixture type	Mixture type content	CT
A1/ Persister isolate reference sample	Reference reaction mixture (isolate DNA under antibiotic stress, without restriction enzyme)	26.0
A2/ Persister isolate test sample	Test reaction mixture (isolate DNA under antibiotic stress, with restriction enzyme)	26.7
A3/ Non persister isolate reference sample	Reference reaction mixture (isolate DNA without antibiotic stress, without restriction enzyme)	26.7
A4/ non persister isolate test sample	Test reaction mixture (isolate DNA with no antibiotic stress, with restriction enzyme)	29.0
H1/ positive control reference sample	Supplied by the manufacturer	16.1
H2/ positive control test sample	Supplied by the manufacturer	15.2

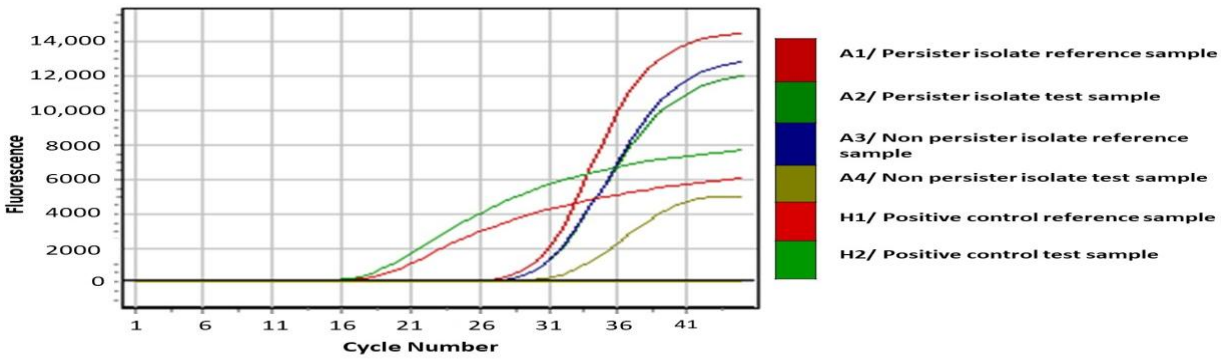


Figure 3 Real-Time PCR Amplification Profiles Depicting Methylation Status of *hns* Gene in Persister and Non-Persister *E. coli* Isolates.

Detection of NAPs gene expression

Stress induction in persister cells was performed for isolate E3 by exposing it to ciprofloxacin at a sub-MIC concentration, determined through a plate double serial dilution assay figure 4. The sub-MIC level was confirmed using resazurin dye and the MM294 strain (Carolina/USA) served as the control. Following this, RNA extraction from the persister isolate under 39 µg/ml ciprofloxacin stress was carried out according to the manufacturer's protocol (Wizprep™). The extracted RNA was then reverse-transcribed into cDNA for subsequent analysis. Finally, qPCR was conducted to evaluate the expression levels of various genes under stress, with the differences in gene expression levels quantified and presented in (Tab 4).

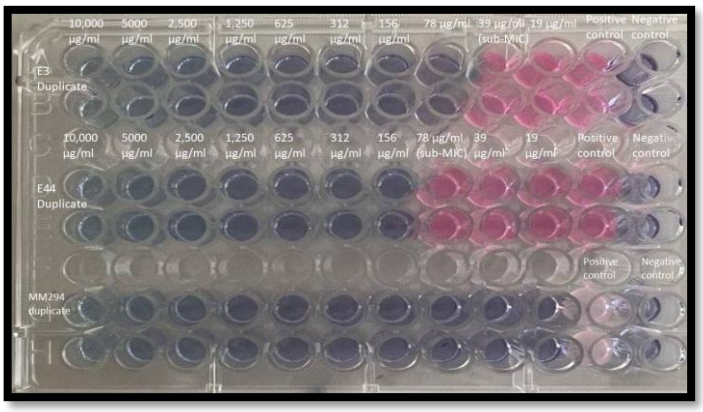


Figure 4 Antibiotic double serial dilution for sub-MIC (stress) detection

Upon completing qPCR, different levels of gene expression and accordingly fold change in expression were acquired as shown in figure 5**,** Bar graph showing relative fold changes in expression levels of nucleoid-associated protein (NAP) genes **(*hns, hupA, hupB, dps*)** in persister (E3) versus non-persister (E7) *E. coli* isolates following ciprofloxacin sub-MIC stress. Gene expression was measured by RT-qPCR and normalized using the 16S rRNA housekeeping gene. Expression was quantified using the 2^–ΔΔCt method. Persister isolate (E3) demonstrated significant upregulation, with *hupB* showing the highest increase (685-fold).

Table 4 qPCR results for NAPs genes

GENE	CT*		ΔCT*		ΔΔCT	Fold change
	NON-Persister	Persister	NON-Persister	Persister		
hns	24.82	15.86	1.6	-7.54	-9.14	564.17
hupA	24.53	15.9	1.31	-7.32	-8.63	396.17
hupB	24.83	15.41	1.61	-7.81	-9.42	685.01
dps	24.67	15.63	1.45	-7.59	-9.04	526.39

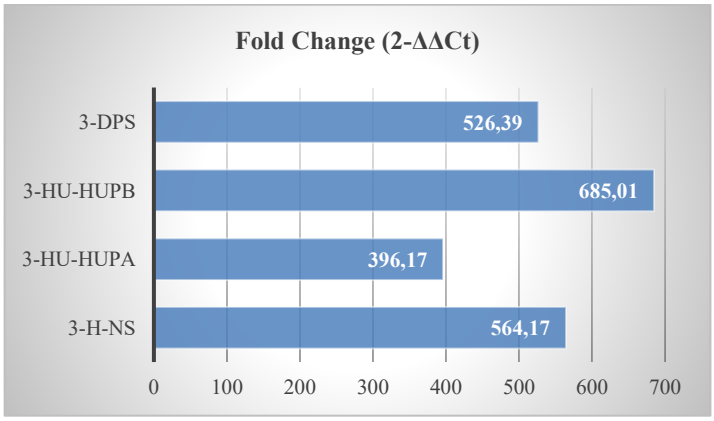
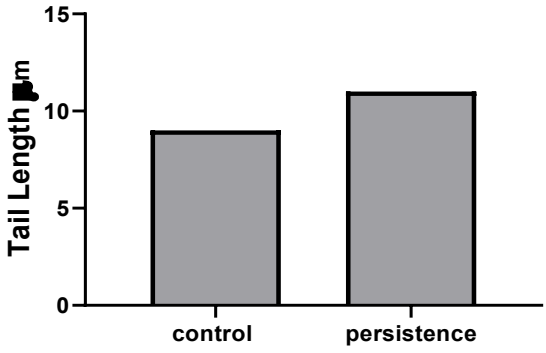


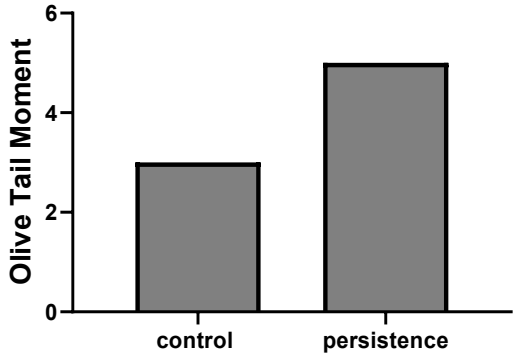
Figure 5 Fold change differences for the selected NAPs genes.

Comet Assay

The neutral comet assay was used to evaluate DNA damage in *E. coli* persister cells treated with antibiotics and control non-treated cells. Various parameters were analyzed, including tail DNA percentage, tail length, and olive tail moment, to quantify DNA damage at the single-cell level. Results showed a significantly higher DNA tail percentage in antibiotic-treated persister cells compared to untreated controls ($p < 0.05$) figure 6, indicating increased DNA damage under antibiotic stress



(A)



(B)

Figure 6 (A) Tail length and (B) Olive tail moment in antibiotic-treated persister cells compared to untreated controls.
Comet assay results showing DNA damage parameters in *E. coli* persister cells exposed to ciprofloxacin sub-MIC stress versus untreated controls. (A) Tail length and (B) Olive tail moment were measured using CASP software for 50 cells per group.

Western blotting

Western blot analysis confirmed Dps protein presence in both treated and untreated *E. coli* cells, with a 1.2-fold increase under ciprofloxacin stress. This upregulation was quantified using the ImageJ software program.

DISCUSSION

This study has illustrated widespread genetic, epigenetic, and genomic adaptations involved in forming persister cells in *E. coli* under ciprofloxacin-induced stress. Selected isolates were tested for the persistence phenotype using the rapid killing method, then confirmed by REPTIS analysis, which narrowed the investigation down to a single isolate (E3) for further accurate investigation. The study identified a subpopulation of antibiotic-tolerant bacteria that can survive high concentrations of antibiotics without genetic resistance. These findings are consistent with previous works and suggest that persistence may serve as a survival strategy for *E. coli* to withstand environmental stressors, especially when facing antibiotic exposure **(Gollan *et al*., 2019; Van den Bergh *et al*., 2017)**.
REPTIS method confirmed the existence of the persister cells and demonstrated that during 72 h of exposure to antibiotics, persister cells show a high level of tolerance towards ciprofloxacin. Interestingly, despite persister cells being susceptible to antibiotics when actively growing, their atypical endurance of prolonged antibiotic treatment illustrates an innovative strategy for survival. Whereas resistance can be induced via genomic adaptation or deregulation of gene expression, tolerance is specific to persister cells and provides a means for them to resist/hinder antibiotic exposure and treatment **(Van den Bergh *et al*., 2017)**. The degree of tolerance that was observed in persisters indicates their clinical significance, as routine antibiotic susceptibility testing fails to detect these cells.

The qRT-PCR results indicated that persister cells displayed a significant upregulation of the nucleoid-associated protein (NAP) genes *hns, hupA, hupB,* and *dps* when compared to non-persisters when exposed to ciprofloxacin stress. Notably, *hupB* exhibited the most significant fold change, underscoring its pivotal function in the organization and stability of DNA under stress conditions **(Brambilla & Sclavi, 2015)**. Likewise, *hns* is a gene that encodes a regulator of gene silencing and chromosomal structure and further highlights the role of NAPs in establishing a protective and sedentary state conducive to persistence **(Chiancone & Ceci, 2010)**. These observations underscore the role of NAPs in sustaining dormancy and protecting cells from genotoxic stress **(Bose *et al*., 2017)**. Quantitative Methyl Specific PCR qMethyl PCR showed that epigenetic changes play an important role in transcription regulation which might be important in various bacterial persistence cases. Elevated methylation of the silencing gene *hns* in persister cells further suggests activation of transcriptional repressive mechanisms. These mechanisms probably save energy and improve survival under antibiotic stress. This is consistent with an earlier study that reported that DNA methylation is linked to bacterial adaptation and the persistor phenotype **(Marinus & Løbner-Olesen, 2014)**.

Comet assay data further confirmed that NAPs provide genomic protection, as persister cells had more DNA damage compared to non-persisters. However, parameters like tail length and olive tail moment showed no statistically significant differences, though higher levels were observed in treated cells. These findings highlight the role of antibiotic-induced DNA damage in persister cell survival mechanisms. In particular, DPS protects DNA from oxidative stress and double-strand breaks under its ferroxidase activity and physical protection of DNA **(Karas *et al*., 2015)**.

The upregulation of Dps under ciprofloxacin stress aligns with its known role in protecting bacterial DNA from oxidative damage and other environmental stressors. Traditionally associated with the stationary phase, Dps was also detected in mid-log phase cells exposed to sub-MIC ciprofloxacin, indicating that antibiotic-induced stress can independently trigger its expression. This supports the broader function of Dps as a general stress response protein **(Orban & Finkel, 2022)**.

Notably, differences between mRNA levels (qPCR) and protein abundance (Western blot) may be due to post-transcriptional regulation, protein stability, and oxidative degradation caused by reactive oxygen species generated during antibiotic exposure **(Nie *et al*., 2006)**. Such factors may explain the modest fold increase observed at the protein level despite stronger transcriptional signals. Overall, these findings suggest that Dps contributes to *E. coli* persister survival by enhancing DNA protection under antibiotic stress.

Overall, this research highlights the essential functions of genetic expression, epigenetic regulation, and DNA protection during the generation of *E. coli* persisters and their survival under ciprofloxacin stress. Utilizing qPCR, qMethyl PCR, and comet assay techniques, the research delivers a holistic insight into the phenomenon of persistence. The discoveries underscore the importance of directing toward persistence-related pathways to enhance therapeutic approaches for treating recurrent and chronic bacterial infections. Further studies should also investigate the interplay of persistence mechanisms and NAPs and may reveal new targets for the disruption of this survival strategy.

CONCLUSION

This study defines the role of nucleoid-associated proteins (NAPs) in *E. coli* persistence under ciprofloxacin stress. Persister cells showed significant upregulation of NAP genes (*hns*, *hupA*, *hupB*, *dps*), along with increased *hns* methylation, indicating transcriptional and epigenetic regulation. Dps overexpression, confirmed by Western blotting even at mid-log phase, highlights its induction by environmental stress, not just stationary growth. Reduced DNA damage observed via comet assay further supports the protective role of NAPs. These findings reveal a multifaceted persistence mechanism and suggest that targeting NAPs, particularly Dps, may help disrupt persister formation and improve the treatment of chronic *E. coli* infections.

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REFERENCES

Ali, M., Ijaz, M., Ikram, M., Ul-Hamid, A., Avais, M., & Anjum, A. A. (2021). Biogenic synthesis, characterization and antibacterial potential evaluation of copper oxide nanoparticles against Escherichia coli. Nanoscale Research Letters, 16, 1-13.https://doi.org/10.1186/s11671-021-03605-z

Bose, T., Venkatesh, K., & Mande, S. S. (2017). Computational analysis of host-pathogen protein interactions between humans and different strains of enterohemorrhagic Escherichia coli. Frontiers in cellular and infection microbiology, 7, 128.https://doi.org/10.3389/fcimb.2017.00128

Brambilla, E., & Sclavi, B. (2015). Gene regulation by H-NS as a function of growth conditions depends on chromosomal position in Escherichia coli. G3: Genes, Genomes, Genetics, 5(4), 605-614.https://doi.org/10.1534/g3.114.016139

Canas-Duarte, S. J., Restrepo, S., & Pedraza, J. M. (2014). Novel protocol for persister cells isolation. PloS one, 9(2), e88660.https://doi.org/10.1371/journal.pone.0088660

Chiancone, E., & Ceci, P. (2010). The multifaceted capacity of Dps proteins to combat bacterial stress conditions: detoxification of iron and hydrogen peroxide and DNA binding. Biochimica et Biophysica Acta (BBA)-General Subjects, 1800(8), 798-805.https://doi.org/10.1016/j.bbagen.2010.01.013

Collins, A. R. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. Molecular biotechnology, 26(3), 249-261.https://doi.org/10.1385/MB:26:3:249

Gollan, B., Grabe, G., Michaux, C., & Helaine, S. (2019). Bacterial persisters and infection: past, present, and progressing. Annual review of microbiology, 73, 359-385.https://doi.org/10.1146/annurev-micro-020518-115650

Harms, A., Maisonneuve, E., & Gerdes, K. (2016). Mechanisms of bacterial persistence during stress and antibiotic exposure. Science, 354(6318), aaf4268.https://doi.org/10.1126/science.aaf4268

Jaishankar, J., & Srivastava, P. (2017). Molecular basis of stationary phase survival and applications. Frontiers in Microbiology, 8, 298976.https://doi.org/10.3389/fmicb.2017.02000

Karas, V. O., Westerlaken, I., & Meyer, A. S. (2015). The DNA-binding protein from starved cells (Dps) utilizes dual functions to defend cells against multiple stresses. Journal of Bacteriology, 197(19), 3206-3215.https://doi.org/10.1128/JB.00475-15

Lanni, A., Iacobino, A., Fattorini, L., & Giannoni, F. (2023). Eradication of drug-tolerant Mycobacterium tuberculosis 2022: where we stand. Microorganisms, 11(6), 1511.https://doi.org/10.3390/microorganisms11061511

Marinus, M., & Løbner-Olesen, A. (2014). DNA methylation. EcoSal plus, 6(1), 10.1128/ecosalplus. ESP-0003-2013.https://doi.org/10.1128/ecosalplus.esp-0003-2013

Matsuo, M., Hiramatsu, M., Singh, M., Sasaki, T., Hishinuma, T., Yamamoto, N., . . . Hiramatsu, K. (2019). Genetic and transcriptomic analyses of ciprofloxacin-tolerant Staphylococcus aureus isolated by the replica plating tolerance isolation system (REPTIS). Antimicrobial Agents and Chemotherapy, 63(2), 10.1128/aac. 02019-02018.https://doi.org/10.1128/AAC.02019-18

Miyaue, S., Suzuki, E., Komiyama, Y., Kondo, Y., Morikawa, M., & Maeda, S. (2018). Bacterial memory of persisters: bacterial persister cells can retain their phenotype for days or weeks after withdrawal from colony-biofilm culture. Frontiers in Microbiology, 9, 1396.https://doi.org/10.3389/fmicb.2018.01396

Nie, L., Wu, G., & Zhang, W. (2006). Correlation of mRNA expression and protein abundance affected by multiple sequence features related to translational efficiency in Desulfovibrio vulgaris: a quantitative analysis. Genetics, 174(4), 2229-2243.https://doi.org/10.1534/genetics.106.065862

Orban, K., & Finkel, S. E. (2022). Dps is a universally conserved dual-action DNA-binding and ferritin protein. Journal of Bacteriology, 204(5), e00036-00022.https://doi.org/10.1128/jb.00036-22

Radzikowski, J. L., Schramke, H., & Heinemann, M. (2017). Bacterial persistence from a system-level perspective. Current Opinion in Biotechnology, 46, 98-105.https://doi.org/10.1016/j.copbio.2017.02.012

Ruppé, E., Hem, S., Lath, S., Gautier, V., Ariey, F., Sarthou, J.-L., . . . Arlet, G. (2009). CTX-M β-lactamases in Escherichia coli from community-acquired urinary tract infections, Cambodia. Emerging infectious diseases, 15(5), 741.https://doi.org/10.3201/eid1505.071299

Santamarina, S. C., Heredia, D. A., Durantini, A. M., & Durantini, E. N. (2022). Antimicrobial photosensitizing material based on conjugated Zn (II) porphyrins. Antibiotics, 11(1), 91.https://doi.org/10.3390/antibiotics11010091

Serebreni, L., & Stark, A. (2021). Insights into gene regulation: From regulatory genomic elements to DNA-protein and protein-protein interactions. Current Opinion in Cell Biology, 70, 58-66.https://doi.org/10.1016/j.ceb.2020.11.009

Singh, G., Yadav, M., Ghosh, C., & Rathore, J. S. (2021). Bacterial toxin-antitoxin modules: classification, functions, and association with persistence. Current Research in Microbial Sciences, 2, 100047.https://doi.org/10.1016/j.crmicr.2021.100047

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental cell research, 175(1), 184-191.https://doi.org/10.1016/0014-4827(88)90265-0

Van den Bergh, B., Fauvart, M., & Michiels, J. (2017). Formation, physiology,

ecology, evolution and clinical importance of bacterial persisters. FEMS microbiology reviews, 41(3), 219-251.https://doi.org/10.1093/femsre/fux001
Wilmaerts, D., Windels, E. M., Verstraeten, N., & Michiels, J. (2019). General mechanisms leading to persister formation and awakening. Trends in Genetics, 35(6), 401-411.https://doi.org/10.1016/j.tig.2019.03.007
Zhang, X., Zhao, B., Yan, T., Hao, A., Gao, Y., Li, D., & Sui, G. (2018). G-quadruplex structures at the promoter of HOXC10 regulate its expression. Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms, 1861(11), 1018-1028.https://doi.org/10.1016/j.bbagrm.2018.09.004