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Research Article

Effect of Carbon Starvation on *Escherichia coli* Persisted Cells in Exponential Phase Grown in Media with Different Carbon Sources

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A B S T R A C T

Introduction: Persisters represent a protective and transient metabolic state resulting in antimicrobial tolerance rather than resistance.

Method: Phenotyping testing was used in this study to determine persister cell growth in 112 *Escherichia coli* isolates obtained from various sources. The influence of *E. coli* growth rate on sensitivity to ciprofloxacin was studied using bacteria cultured on varied carbon sources for a week and a 1-hour carbon deprivation before the antibiotic was added.

Results: Five E. coli isolates displayed persistent formation. A 1-week treatment with 10 µg/mL of ciprofloxacin decreased the number of surviving cells (CFU/mL) by 395 and 149 times in bacterial strains cultured on glucose and glycerol MOPS minimal medium, respectively. The deprived cell was more tolerant to 10 µg/mL of ciprofloxacin than the non-starved cell. This effect was especially obvious for the strain produced on a glucose medium, which resulted in slowed death rates and a greater survivor fraction during the initial killing phase.

Conclusion: The different *E. coli* growth rates have a great impact on its susceptibility to ciprofloxacin when grown on different carbon sources. Drug tolerance and persistence rise with declining growth rates and are almost non-existent in cultures with rapid growth. Carbon starvation causes a decline in the killing rate with the log reduction decreased to approximately half due to persistence in the killing dynamics process.

Keywords: Growth Rate, Ciprofloxacin, Exponential Growth Phase, *E. coli*

Introduction

It was discovered more than 70 years ago that medications that are thought to be bactericidal actually fail to sterilise cultures.[1] Bigger came to understand that the few bacteria that are able to endure prolonged antibiotic therapy belong to a certain subpopulation of bacteria which he called "persisters". These cells are believed to be one of the causes of treatment failures in patients with chronic infections and make these illnesses challenging to treat since they display tolerance to high doses of bactericidal agents.[2] Antibiotic persistence, resistance, tolerance, and hetero-resistance allow the bacteria to withstand exposure to antibiotic

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treatments that could kill the cells.

Increases in minimum inhibitory concentration (MIC) and minimum duration for killing 99% of the population (MDK99) were used to characterise tolerance and resistance, respectively. These criteria, so far, describe a population that is uniform. A subset of cells with antibiotic persistence has a greater MDK99 than the bulk of the population.[3] Understanding how bacteria are killed when exposed to antibiotics, particularly those with longer survival durations, is necessary to determine the ideal treatment duration. "Persister cells" is a term used to define the long-time survivors which are a subpopulation of cells that withstand antibiotics for a longer amount of time than other members of the population but these cells do not acquire mutations that enable them to resist the antibiotic.[4] In 2004, Keren et al. discovered that fast logarithmic growth in a rich medium did not lead to the formation of persister cells *(E. coli)*.[5] In order to provide a well-defined physiology of the logarithmic growth phase that would help to change the growth physiology in a controlled manner, many equations that characterise the macromolecular composition of an average cell in a logarithmic culture as a function of culture doubling time, have been published.[6] In particular, changing the growth rate by cultivating bacteria in media of different nutrient quality would change the growth physiology. The quantitative claim that faster-growing bacteria are eliminated more quickly is supported by previous research that found a linear relationship between growth rates and B-lactam-mediated lysis rates.[7] The biphasic killing curve, which shows that not all bacteria in a clonal culture are killed at the same rate, and in which the persisters are the subpopulation with a second, slower killing rate than the primary population, was the defining feature of antibiotic persistence.[3] A complex of multiphase death curves is seen, which has been supported by earlier studies. These findings highlight the significance of researching the long-term persistence of the antibiotic-tolerant population.[8–10] The bacterial growth rate was varied by cultivating E. coli in media with different carbon substrates, each of which supports a specific growth rate, in order to investigate how growing conditions affect cell survival. The purpose of the present study is to find out how the persisted cells will act against a high dose of ciprofloxacin for 7 days on media with different carbon sources.

Material and Method

Sample

Overall, 210 samples of *E. coli* were collected from different sources (blood, urine and stool), which were obtained from patients admitted to AL-Elwia Pediatrics Teaching Hospital and Teaching Laboratories in Medical City in Baghdad between January and May 2021.

Rapidly Killing Method

The process for persister isolation was conducted by rapidly killing naturally developing cells with a mixture of lytic solutions.[11] Bacterial cells were cultured in Luria-Bertani (LB) broth overnight at 37 °C. The culture was increased to $1.5*10^8$ CFU/mL the next day. One mL of bacterial suspension was put in a 10 mL test tube along with 200 µL of the lysis solution. The mixture was then vortexed for 10 seconds before being incubated at room temperature for 10 minutes. Following the incubation period, 200 µL of the enzymatic lysis solution was introduced to the mixture and gently homogenised by inverting the tube. The preparation was incubated at 37 °C for 15 minutes in a shaker incubator at 200 rpm. Ten µl of the mixture was poured on LB agar medium. It was then completely spread on the agar and the plates were incubated for 24 h at 37 °C. Finally, persister cells were determined via overnight growing cells.

Long-Term Killing Assay

Cells that were selected for the experiments were grown in a MOPS minimal medium (first the carbon supply was glucose, after which then the carbon supply was glycerol).[12,13] Cells were then diluted up to 10^9 in MOPS minimum liquid medium. A shaker was used to continually shake the flask at 160 rpm in a 37 °C environment. The growth was monitored by measuring the optical density at 436 nm (OD_{436}) since the logarithmic phase refers to cultures with a 0.3 to 0.5 OD_{436}. The back dilutions were to promote balanced growth, and the OD_{436} of the cultures never exceeded 0.3. The growth rates were calculated using at least five measurements in the 0.03 to 0.3 OD_{436} intervals.[13]

Starvation Method

For starvation experiments, a full MOPS minimal medium contained 20% glucose, and another MOPS minimal medium contained 8% glycerol as the carbon source. Cells were grown in the mid-logarithmic phase in MOPS minimal medium and then they underwent filtration of the culture followed by a quick re-suspension on a filter in a pre-warmed MOPS minimum liquid medium which was divided into two media, one with carbon source and the other without carbon source, for one hour. Starvation was confirmed by observing a stable OD_{436}, and the addition of the missing carbon source in various forms (glucose and glycerol) was sufficient for the resumption of growth. A sample was obtained before the addition of ciprofloxacin (10 µg/mL), and the carbon supply, which was added to finish the downshift. Most of the samples were taken at times 2, 4, 6, and 21 + 24·n hours followed by centrifugation of the sample at 4 °C for 10 minutes at 10,000 g. After removing the supernatant, the cell sediment was re-suspended at room temperature in MOPS buffer with no additives. The sample was appropriately diluted and did

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not exceed 1:10^2 per step, equating to 10 in 990 µL. The sample was plated with the target medium (100 µL per plate). All colonies were counted after the plates had been stored at 37 °C for at least one week.

Statistical Analysis

The data results of this study were analysed using Graph Pad Prism 8 software and Microsoft Excel 2007 for each biological replicate. The level of probability at p value ≤ 0.05 was used to identify a significant difference.

Ethical Approval

The research received approval from the Research Ethics Committee of the Department of Biology Sciences at Mustansiriya University. The isolates were gathered and identified in microbiology laboratories at AL-Elwia Paediatric Teaching Hospital and Teaching Laboratories in Medical City, after the acquisition of approval from the Ministry of Health and Environment to collect samples for the present investigation. Informed consent was obtained from the research participants to gather sociodemographic data and conduct a study on the collected samples while ensuring the protection of patients' privacy.

Results

Detection of Persister Cells Formation

Using a rapidly killing approach, all *E. coli* isolates were examined for the phenotypic development of persister cells. The results revealed that out of 112 *E. coli* isolates, five isolates were able to do so. As demonstrated in Figure 1, this approach to persister cell separation is substantially quicker than more conventional approaches that rely on antibiotic concentrations.

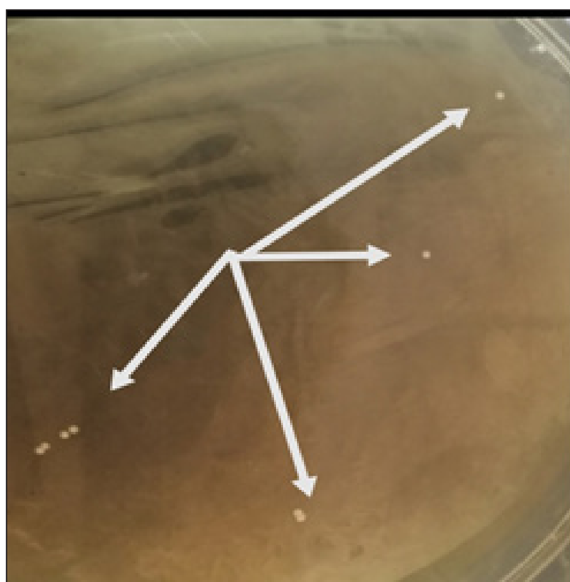

Figure 1.Development of Persister Cells via Rapidly Killing Technique

Growth Rate and Doubling Time on Different Carbon Sources

Prior to the downshift and the experiment, the doubling time was derived from the exponential growth. Table 1 contains the data of the doubling time with glycerol and glucose as carbon sources.

Table 1.Doubling Time with Glycerol and Glucose as Carbon Sources

E.coli Isolate Number	Carbon Source	Doubling Time (Min)
30	Glucose	63
82	Glucose	86.6
102	Glucose	57.7
107	Glucose	115
108	Glucose	57.7
30	Glycerol	99
82	Glycerol	115
102	Glycerol	77
107	Glycerol	138.6
108	Glycerol	99

The doubling time throughout the log phase ranged between 57 and 115 minutes for cells grown on glucose. The cells grown on glycerol exhibited a significant lag phase and their doubling time varied between 77 and 138.6 minutes. During the early phase, the population growth rate was found to be positively associated with the killing rate. However, the link was weaker in terms of long-term survival and was lost in the third phase of killing.

Ciprofloxacin Susceptibility of *E. coli* Grown with Different Carbon Sources

In MOPS minimal medium, E. coli was grown using either glucose or glycerol as the carbon source. In these conditions, bacterial growth rates were 0.80 and 0.61 h^{-1}, respectively. The colony forming unit (CFU) significantly dropped after the logarithmic developed cultures were treated with ciprofloxacin. The different substrate-grown cultures reacted to the antibiotic in quite diverse ways. During a 6-hour exposure to 10 µg/mL of ciprofloxacin, the surviving cells were decreased by 5.65 times (glucose) and 2.8 times (glycerol) during growth (Figure 2a). Figure 2b demonstrated that after 7 days of experimentation, the number of surviving cells that were growing on glucose and glycerol was reduced by 395 and 149 times, respectively. In both situations, the rate of bacterial death decreased with exposure time; the log reduction was 1.6 and 1 log in the first 7 hours, compared to 0.9 and 0.8 log in the next 7 days while the bacteria were growing on glucose and glycerol, respectively. Ciprofloxacin had a substantially greater antibacterial impact on *E. coli* cultured on glucose than it did on cells grown on glycerol. Hence, before the

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addition of the antibiotic, the rate of cell death rose as the rate of culture growth increased.

Effect of the Downshift of the Carbon Source on the Ciprofloxacin Susceptibility of *E. coli* Pesister Cells

A carbon source downshift was induced for one hour after the balanced exponential growth, and this was in contrast with a control culture that did not undergo a downshift. OD data supported the downshifts observed in this study. The goal was to demonstrate that there was no growth in the cultures without a carbon supply while there was growth in the control cultures with a carbon source. The OD was assessed twice during the downshift within an hour to verify whether biomass increased or decreased (Table 2). The information in Table 2 demonstrates the verification of the downshift of the cell during starvation. Additionally, it confirms that the control cultures saw growth with a doubling time that was roughly close to the predicted value. The optical absorbance was measured twice during the downshift, to confirm that the starvation took place.

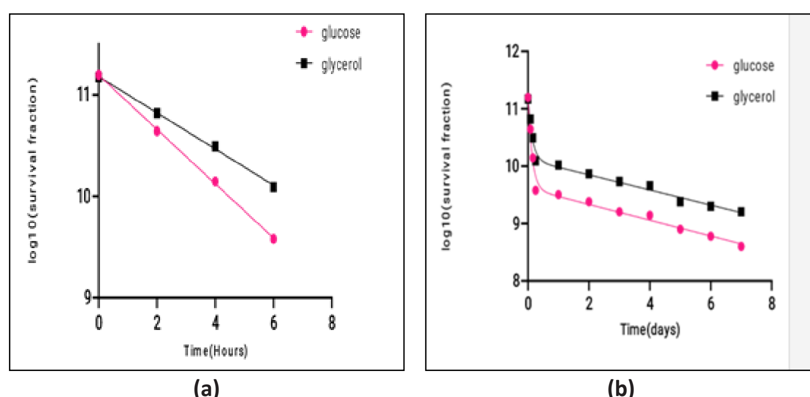

Figure 2.Killing Dynamics of Exponential-Phase *Escherichia coli* Persisters in Glycerol and Glucose Minimum Media (a).Up to 6 Hours when the First and the Second Killing Phases Took Place (b).For 7 days when the Third Killing Phase Took Place which had a Slower Killing Rate than the First Phase

Table 2.Verification of the Downshift

E. coli Isolate Number	T_1 (Min)	OD_{436}	T_2 (Min)	OD_{436}		T_D (Min)
30 (with downshift) glucose 30 (without downshift)	15 15	0.055 0.080	55 55	0.060 0.141		438 67
82 (with downshift) 82 (without downshift)	18 18	0.065 0.075	48 48	0.065 0.115		∞ 77
102 (with downshift) 102 (without downshift)	13 13	0.048 0.053	46 46	0.050 0.097		780 52
107 (with downshift) 107 (without downshift)	12 12	0.037 0.041	42 42	0.039 0.053		560 113
108 (with downshift) 108 (without downshift)	16 16	0.058 0.054	46 46	0.060 0.096		940 55
30 (with downshift) glycerol 30 (without downshift)	14 14	0.057 0.081	40 40	0.055 0.110		-776 93
82 (with downshift) 82 (without downshift)	19 19	0.043 0.037	50 50	0.043 0.052		∞ 101
102 (with downshift) 102 (without downshift)	10 10	0.049 0.065	47 47	0.044 0.101		-302 73
107 (with downshift) 107 (without downshift)	11 11	0.056 0.045	35 35	0.056 0.054		∞ 133
108 (with downshift) 108 (without downshift)	17 17	0.034 0.056	41 41	0.037 0.075		336 97

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The log reduction for the glucose-starved cells was 0.6 log which means that the killing rate was much lower than the non-starved cells (1.6 log). When the two circumstances are evaluated, it can be seen that glucose-starved cells survive longer after 6 hours of ciprofloxacin exposure than non-starved cells (Figure 3a), and there was also an increase in the survival fraction of glucose-starved cells in the 7 days exposure to the antibiotic (for at least 4 days) more than the non-starved cells (Figure 3b). As a result, the starved bacterial community has a higher percentage of persisters than the unstressed bacterial population.

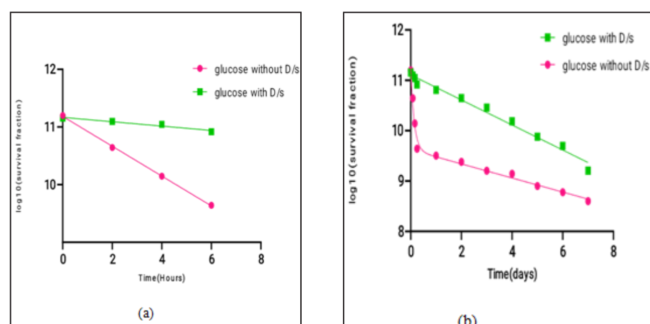

Figure 3.Killing Dynamic of *E. coli* With and Without the Carbon Source (Glucose) (a).Up to 6 Hours (b).Through 7 Days

In glycerol-starved MOPS minimal media, the log reduction of the cell was 0.7 in the first 6 hours which is slightly different from the non-starved MOPS minimal media cells. The downshift had a smaller effect in the glycerol medium (Figures 4a and 4b). The average persister level with downshift was elevated up to 4 days, but the statistical significance of the differentiation was recognised merely until 6 hours due to larger data sparse.

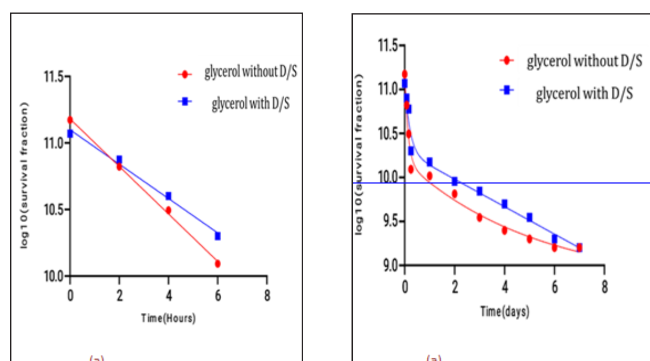

Figure 4.Killing Dynamic of *E. coli* with and Without the Carbon Source (Glycerol) (a).Up to 6 Hours (b).Through 7 Days

Discussion

It is challenging to treat infections caused by persistent bacteria. Clinically, persisters may cause persistent and latent infections as well as post-treatment recurrence, leading to considerable difficulties in treating many bacterial infections caused by various bacterial species like *Klebsiella pneumonia, Pseudomonas aeruginosa* and *E. coli.* In Iraq, two local investigations stated that only two *Klebsiella pneumonia* isolates out of 50 were able to form persisted cells by the rapidly killing approach,[14] while seven P. aeruginosa isolates out of 100 were able to create persister cells[15] and the results of this study demonstrate that only 5% of collected *E. coli* was persisters. The mechanics of persisters are poorly known even though their findings were reported more than eight decades ago. Numerous genes and pathways that shed light on the mechanics of persistence or survival have been discovered in recent investigations.[16]

Bacterial persisted rebels do not grow or die when exposed to bactericidal agents, and they awaken from their metabolically dormant state when the drugs are not present, displaying a high level of multidrug resistance. A new population can go from presister cells to a sensitive stage after the impacts of the stress are over.[17] However, the primary intent of the name persister cell was to make a clear distinction between them and resistant mutants. Persistence differs from resistance in that persisters have phenotypic variations rather than mutations, and they are unable to pass on their tolerance to their offspring in the way that resistant mutants may. As a result, populations with similar persister numbers are obtained through re-inoculation of surviving persisters.[5]

E. coli, like other bacteria, may use a variety of carbon and energy sources, with the growth rate varying according to the substrate. In the current investigation, *E. coli* was cultivated in MOPS minimum medium using one of the two carbon sources: glycerol and glucose. The doubling time was longer in glycerol which led to a much higher growth rate in glucose than glycerol. These observations are consistent with many studies that used glycerol as the sole carbon source and reported a longer doubling time and eventually a lower growth rate.[18–20] According to one study; glycerol stimulated all key enzymes involved in its metabolism. This might explain the prolonged lag phase observed in cell development on glycerol, as cells need this time to stimulate the production of enzymes required for effective glycerolysis.[20]

Many studies have proven the activity of ciprofloxacin against gram-negative bacteria, especially against E. coli,[21] and as previously documented, phenotypic resistance to antibiotics has generally been associated with sluggish growth, minimal metabolism, and bacterial dormancy.[22–24]

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Consistent with this theory, this study showed that ciprofloxacin had the greatest bactericidal impact on E. coli grown on glucose, but it had a significantly lesser effect on glycerol-grown cells. Consequently, prior to the administration of the antibiotic, the rate of cell death rose with increasing culture growth rate.

It should be noted that in this study, a rather high concentration of ciprofloxacin (10 μg/mL) was used and the rate of killing was much lower than that reported in other studies with lower ciprofloxacin concentrations. In a previous study, the levels of E. coli clearance by ciprofloxacin for bacteria growing on glucose were greater for the 0.3 μg/mL dosage of this antibiotic than for the 3.0 μg/mL dose.[25] Ciprofloxacin and other quinolones' paradoxical impact was apparently due to suppression of RNA synthesis at doses above the optimum bactericidal concentration (OBC),[26,27] while the experiments continue after the 1-h carbon starvation which is one of the most common forms of stress encountered to E. coli in natural environments. The log reduction and the killing rate for the glucose-starved cells were much lower than for the non-starved cells. Other related members of the family Enterobacteriaceae are known to go through considerable modifications in gene expression and physiology as a result of the stress of carbon depletion and help the non-spore-forming cells endure prolonged periods of starvation and exposure to other forms of stress.[28] Previous research has shown that the start of starvation in E. coli follows a temporally organised pattern that leads to the trigger of two groups of genes during carbon deprivation: the cst genes, which require cyclic AMP for activation, and the pex genes, which do not. The cst genes are not responsible for the development of the persistent form and are connected with starving escape, but the activation of the pex genes appears to be related to resistance. Several of them are brought on by different types of malnutrition. These include genes for resistance to heat, shock, and oxidation.[29] Another study on fluoroquinolone-induced tolerance found significant changes in the macromolecular structure and morphology of the bacterial cell during starvation, demonstrating that ofloxacin tolerance was entirely dependent on a functional SOS response on starvation to both amino acids and carbon sources. The results also demonstrated that the molecular mechanism that results in high resistance to fluoroquinolones is the SOS stress response, which is activated in heterogeneous and nutrient-depleted cells.[30]

While the effect of the carbon shortage was very clear in glucose-grown cells, the effect was slower and merely found in glycerol. Many studies have stated that a quick change in the carbon supply is known to generate a spike in the strain's (p)ppGpp level immediately after the shift, and a brief pulse of carbon supply deprivation to the exponentially developing cells prior to the killing assay would also cause a spike. Measurable differences in the strain's long-term persistence would be beneficial if the starving pulse increased the number of persisters.[31] Lack of the effect when glycerol was used as the carbon source gives credibility to this theory. This may be due to the fact that glycerol has a higher basal level of (p)ppGpp in a minimum medium than glucose.[32]

The results of this investigation reveal that the phase of bacterial development frequently has a significant impact on how susceptible bacteria are to antibiotics. Drug tolerance and resistance rise with slower growth rates and are almost non-existent in cultures that develop quickly.[33] It was demonstrated that the rate of bacterial death by antibiotics in glucose-limited samples of *Escherichia coli* is directly related to the rate of bacterial growth.[34] During the early stages, the killing rate was strongly related to the pace of population growth. The link, on the other hand, was weakened during the longer-term surviving phase and lost during the later killing phase. The carbon downshift had a noticeable impact on the strain cultivated in a glucose medium and the result showed a slower death and a higher survivor fraction during the initial killing phase and a decline in the log reduction to approximately half due to the generated persistence in the killing dynamics by toxin-antitoxin activation via the SOS response which is a molecular mechanism leading to high tolerance to antibiotics.[35]

Conclusion

It appears that persistence is a temporal phenomenon, with varied survival strategies accounting for the diversity of bacterial life cycles. The different *Escherichia coli* growth rates have a great impact on its susceptibility to ciprofloxacin when grown on different carbon sources. Drug tolerance and persistence increase with decreasing growth rates and decline to the lowest in rapidly growing cultures. After the carbon source depletion, the strain cultivated in a glucose medium showed a slower death and a higher survivor fraction during the initial killing phase and a decline in the log reduction to approximately half due to generated persistence in the killing dynamics.

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