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Research Article

An Investigation into the Role of *trkH* Gene Knock-out in the Development of Gentamicin-Resistance in *E. coli*

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Abstract

Atypical gene mutations, such as mutated *trkH* gene, contribute to the worldwide increase of antibiotic resistance in bacteria. *trkH* influences the intracellular concentration of potassium ions by encoding TrkH, an intracellular potassium-ion transporter thus mutations can prevent antibiotic uptake. Since mutated *trkH* is not easily identified as they are not normally involved in the resistance evolution, it is a threat towards the potency of gentamicin. Research into the role of *trkH* in the gentamicin-resistance evolution in *E. coli* may contribute to the development of novel antibiotic therapies to combat resistance. The MIC and resistance induction revealed bacterial growth at high gentamicin concentrations. Growth curve analysis indicated strains exposed to gentamicin were in an extended lag phase; but unexposed strains had a normal growth activity. This was mirrored in the disk-diffusion and reversion rate tests wherein gentamicin-exposed strains were unable to grow in the presence of antibiotics but grew on agars without; meanwhile, all strains unexposed to antibiotic grew sufficiently. The varied results were confirmed by statistical analysis, which revealed the *trkH* gene to pose an influence in the metabolism of *E. coli*. Besides the gene's influence, environmental factors were uncovered to have a possible significant role on the metabolism of *E. coli*. Moreover, resistance evolution may not necessarily progress to complete resistance. Heteroresistance, tolerance, and disruptions in cell composition and/or surface were investigated to be involved in the adaptation to antibiotic presence.

Keywords: *Escherichia coli, Gentamicin, Gentamicin resistance, Gentamicin-resistant E. coli, Antibiotic resistance, Tolerance, Resistance mechanisms*

Introduction

Antibiotic resistance is an increasingly growing global threat to human, animal, and environmental health (Aslam et al., 2018). The rapid emergence and spread of resistant bacteria present serious problems to the potency of the limited number of available antibiotics. These resistant bacteria can cause infections with two-fold greater severe outcomes than those generated by its susceptible counterparts (Friedman et. al, 2016; Malik and Bhattacharyya, 2019). Although bacterial resistance mechanisms are constantly being classified and extensively reviewed at a molecular level, the prediction of its evolutionary processes leading to certain resistance remains unclear. Resistance evolution is a complicated process that could involve multiple mutations in a bacterium. During the preclinical stage of antibiotic development, the evolution of resistance is examined to assess the risks of resistance against an antibiotic; however, the analytical procedures involved present a major drawback as only a fraction of it can be explored thus atypical resistance mutations and combinations are not easily identified. Nevertheless, the manner in which bacteria evolve resistance is still widely used to assess the chances of mutations against an antibiotic (Li and Webster, 2017; Nyerges et al., 2018; Biswas et. al, 2019).

A bacterium notorious for its gradual worldwide increase in resistance to antibiotics is *Escherichia coli*, which is of important concern as it is the most prevalent Gram-negative pathogen in humans (Rasheed et al., 2014; Poirel et al., 2018). It has been found to cause infections in every tissue and organ system in humans—it is responsible for 80% of urinary tract infections (UTI) and is involved in Gram-negative sepsis, diarrhea, nosocomial pneumonia, and neonatal meningitis (Basak and Rajurkar, 2014). Thus, its ability to retain resistance is regarded problematic.

E. coli has been identified to harbour an intrinsic resistance towards β-lactams, such as penicillins and first-generation caphalosporins, by the constitutive low-level expression of the *ampC* gene that encodes β-lactamases (Reygaert, 2017). Although initially minor, mutations in *ampC* stimulate overexpression and consequently, heightened production of β-lactamases that hydrolyze the β-lactam ring in β-lactams, inactivating the drug. This is of great concern as β-lactams, especially penicillins, are widely used to treat bacterial infections (Pfeifer et. al, 2010). Significantly, *ampC* genes can be acquired through horizontal gene transfer (plasmid-born) and has been observed as a more common way of producing high levels of β-lactamase (Bajaj et. al, 2016). Several other plasmid-born β-lactamases were also identified, including the extended-spectrum β-lactamase (ESBL), targeting third-generation cephalosporins that are used for fatal gram-negative infections, and carbapenemase, acting against cabapanems that are utilized for ESBL infections (Bradford, 2001; Rawat and Nair, 2010; Reygaert, 2017). Another method of resistance retained by *E. coli* is drug efflux through various efflux pumps, in which its genes are either constitutively expressed or induced by external factors: MacAB, MefB, EmrAB-TolC, Fsr, and MdfA, which act against macrolides, fluoroquinolones, tetracycline, trimethoprim, and chloramphenicol. *E. coli* can also modify drug target to prevent antibiotics from binding to target proteins; such as in aminoglycosides in which the acquisition of plasmid-born 16s rRNA methyltransferases modify 16s rRNA to decrease the binding ability of aminoglycosides (Reygaert, 2017). These resistance mechanisms are only a few of the numerous methods in which *E. coli* can demonstrate antibiotic-resistance and certainly, its resistance to aminoglycosides is not limited to target modification.

Aminoglycosides are bactericidal antibiotics that integrate mistranslated proteins into the membranes of bacteria—mostly aerobic Gram-negative bacteria, such as *E. coli* (facultative anaerobe)— to increase its cellular uptake. Its entry into cells leads to the inhibition of bacterial protein synthesis through irreversibly binding to the 30S ribosome subunit of the 16s rRNA (Kohanski et. al, 2010). Gentamicin is the most widely used aminoglycoside due to its potency and low-cost (Gonzales III and Spencer, 1998). However, because of its extensive use, cases of gentamicin-resistant *E. coli* strains in humans and animals have continuously been reported (Ho et. al., 2010; Tadesse et al., 2012). This signifies a serious threat to public health and to the potency of the drug. A variety of mechanisms have been identified that contribute to resistance to gentamicin. Antibiotic-producing bacteria, such as *Streptomyces*, produce enzymes to protect them from the antibiotics they produce. This method is particularly relevant to aminoglycosides, in which the bacteria *Streptomyces* produce a large variety of Aminoglycoside Modification Enzymes (AMEs) that render aminoglycosides ineffective. Additionally, target modifications through the 16S rRNA methyltransferase, used by *Mycobacterium tuberculosis* and *M. smegmatis*, can work jointly with AMEs, by the methylation of the antibiotic and removing them from target sites. Modification of the antibiotic is also another common method of aminoglycoside-resistance found in various bacteria, including *Providencia* and *Acinetobacter spp.* (Vester and Long, 2013; Peterson and Kaur, 2018). These modes of resistance are only a few to the growing list of mutations, which include atypical gene mutations.

Atypical genetic mutations are mutations in genes not normally identified in resistance evolution (Wozniak et. al, 2012; Wistrand-Yuen et al., 2018), in contrast to those mentioned above. Mutations in these genes are likely to confer only minor effects on resistance; but, when different atypical genes mutate and are combined in one bacterium, it is probable for high levels of resistance to be produced. Therefore, these atypical genes can push the resistance evolution of an organism to elevated levels. One of the genes recognized to contribute to increased resistance is the *trkH* gene (Wistrand -Yuen et al., 2018).

The *trkH* gene poses a significant role in the bacterial resistance to aminoglycosides (Apjok et. al, 2019). It encodes the TrkH protein, a potassium-ion uptake permease of the Trk system (Alkhuder et al., 2010). Potassium ions are the main intracellular cations responsible for various metabolic processes in bacteria, including the regulation of its cellular homeostasis and turgidity, adaptation to osmolarity, and stimulation of certain cellular enzymes (Epstein, 2003), suggesting a capacity to influence virulence. Meanwhile, the Trk system, which is the main potassium-ion transporter at neutral pH, maintains constant levels of intracellular potassium ions for vital cellular functions through the transport of ions into the cell—this sustains the proton-motive force (PMF) or membrane potential (Su et al., 2009). Therefore, loss or mutation of *trkH* may affect the general metabolism of bacteria.

In relation to gentamicin, an aminoglycoside which requires cellular uptake to target intracellular 30S ribosomal subunit, mutation in the *trkH* can prevent its entry into the cell. Cellular uptake is a respiration-dependent process, which relies on cytochrome activity and the maintenance of the PMF through the quinone pool (Kohanski et. al, 2010; Lázár et. al, 2013). Thus, in addition to the effect of *trkH* on bacterial metabolism, the loss or mutation of this gene has a potential to gentamicin-resistance. Although, because *trkH* is an atypical resistance gene, its effects may be minor and only confer slight resistance. Although, because of the gene's influence on membrane permeability, its mutations could induce a secondary consequence being the inhibition of the action of efflux pumps. This can cause intracellular accumulation of certain antibiotics leading to increased sensitivity thereby (*trkH*) exhibiting a part in the collateral sensitivity—resistance to one antibiotic cause increased sensitivity to another of a different class. Due to the reduced membrane potential in the cell, the activity of the efflux pumps, such as AcrAB-TolC, are also reduced. This increases the accumulation of certain antibiotics intracellularly, which is observed with β-lactams (Lázár et. al, 2013; Pál et. al, 2015; Wistrand-Yuen et al., 2018; Apjok et al., 2019; Roemhild et. al, 2020).

In comparison to the well-studied genes that encode AMEs, the *trkH* gene is largely overlooked, especially since its mutated form (individually) can only cause minor resistance therefore cannot easily be identified in clinical isolates; standard procedure involving transformation or transduction, and then reconstruction will be ineffective. However, researching more into the mechanisms of *trkH* may be valuable as it could provide means of better understanding the mechanisms behind hypersensitivity and/or hyper-resistance, leading to the development of novel therapies (Wistrand-Yuen et al., 2018).

Significantly, the increasing cases of gentamicin-resistant *E. coli* present a huge problem, especially amongst children. UTI is the most common severe bacterial infection in early childhood with *E. coli* as the main pathogen, causing 70-90% of its cases. Gentamicin, along with ampicillin, is a first-line drug for its treatment; however, considering the emerging gentamicin-resistant strains, there could be a possible further decrease of already limited antibiotic treatment options for children (Allen et al., 1999; Nickavar and Sotoudeh, 2011; Salas-Mera et al., 2017). In addition, gentamicin is also the first line of choice for treatment for sepsis in neonates. Sepsis is a primary cause of neonatal morbidity and mortality, with *E. coli* as the most common cause of mortality. It accounts for 40% of deaths in ages under five worldwide, with the survivors being more susceptible to short- and long-term brain development deficiency (Darmstadt et al., 2009; Simonsen et al., 2014; Shehab El-Din et al., 2015).

Justification

Considering the worldwide crisis of rising gentamicin-resistant strains and the potential severity of the consequences of atypical resistance, an attempt to investigate the mechanism was carried out through the atypical *trkH* gene in mutant *E. coli*. A mutant knock-out *trkH E. coli* will be compared to parent wildtype *E. coli*; the loss of *trkH* in mutant *E. coli* will be examined as whether its loss will still influence the development of resistance to gentamicin. If so, it may suggest the involvement of other genes in gentamicin resistance with the *trkH*; but if resistance in the mutant is achieved, although at lower levels compared to the parent, then it may imply the *trkH* to play a role as a driver of heightened resistance.

Aims

a) This research attempts to gain more insight into the role of *trkH* in the development gentamicin resistance in *E. coli*;

b) to examine whether the loss of *trkH* can affect the development of resistance;

c) to investigate the influence of the loss of *trkH* on the metabolic activity of *E. coli*.

Hypothesis

- Knock-out *trkH* will have an effect in the development of gentamicin resistance in *E. coli*.
- Knock-out *trkH* will have no effect in the development of gentamicin resistance in *E. coli*.

Methods

Strain and Growth Conditions

The parental and mutant knockout *trkH* strain used in this study is derived from *E. coli* K-12 BW25113 (Keio Collection, Dharmacon). Cultures were grown in Luria-Bertani (LB) broth or on LB agar unless otherwise stated, and the Muller-Hinton (MH) agar was used in disk diffusion assay.

Antibiotic stock solutions and media were prepared shown in the table below (Table 1). Standard growth conditions were used throughout (37°C overnight or 35°C over the weekend).

Table 1. Stocks and media used in the experiment with its constituents.

Gentamicin stocks	Constituents
2mL of 1024µg/mL	0.002g of Gentamicin sulfate powder
	2mL of water
5mL of 1024µg/mL	0.005g of Gentamicin sulfate powder
	5mL of water
10mL of 1024µg/mL	0.010 of Gentamicin sulfate powder
	10mL of water
10.1mL of 50,000 µg/mL	0.51g of Gentamicin sulfate powder
	10.1mL of water
LB broth:	
1x concentration of LB broth	10g of LB broth powder
	500mL of water
2x concentration of LB broth	20g of LB broth powder
	500mL of water
LB agar:	
500mL of pure LB agar	17.5g of LB broth agar powder
	500mL of water
200mL of LB agar with 750µg/mL of gentamicin	3mL of 50,000 µg/mL gentamicin stock
	197mL of LB agar
200mL of LB agar with 512µg/mL of gentamicin	2.048mL of 50,000 µg/mL gentamicin stock
	197.95mL of LB agar
MH agar	8.5g of MH agar powder
	500mL of water
Overnight *E. coli* cultures	1 colony from original parent or mutant knockout *trkH* strains on LB agar
	20mL of LB broth
	20µL of broth with parent or mutant strains
	20mL of LB broth
Peptone water	4g of Peptone powder
	200mL of water

Broth MIC

MIC was determined using the broth microdilution method with slight modifications (Andrews, 2001). Gentamicin was diluted in 150µL of distilled water in all wells to produce top concentrations (µg/mL) of 512, 256, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25. 200µL of 1024µg/mL gentamicin stock was added to the first well and serially diluted across by 100µL until the fifth well. This was repeated in the next five columns and the remaining two columns were reserved for control—bacteria only for maximal growth, and a contamination control with LB broth media only. 1024µg/mL of stock was added to A1. 200µL of antibiotic stock (1024µg/mL) was added to the first well on the 96-well microtiter plate (A1). 100µL of distilled water was added to columns 2-5 of row A and to columns 1-5 of rows B-H. From A1, 100µL of the stock was serially diluted across the following wells until A5; and 50µL from A1 was serially diluted down to H1.

The latter step was replicated in wells A2-A5 down to H2-H5. This step was repeated in column 6-10 and rows A-H. 50µL of the overnight cultures of the E. coli parent strain were then added onto each well. Column 11 represented maximal growth and so only contains culture in distilled water; column 12 displayed no growth so contains 2x concentration of LB media and gentamicin. The plate plan is illustrated on Figure 1.

Plates were covered with breathable membranes and incubated at 37ºC, overnight; absorbance was read at 600nm.

MIC was defined as the concentration with growth below 90% of the maximal growth (column 11 of the plates).

	1	2	3	4	5	6	7	8	9	10	11	12
A	512	256	128	64	32	512	256	128	64	32	150µL of water with 50µL of bacteria	100µL of 2x LB media and 100µL of gentamicin
B	256	128	64	32	16	256	128	64	32	16		
C	128	64	32	16	8	128	64	32	16	8		
D	64	32	16	8	4	64	32	16	8	4		
E	32	16	8	4	2	32	16	8	4	2		
F	16	8	4	2	1	16	8	4	2	1		
G	8	4	2	1	0.5	8	4	2	1	0.5		
H	4	2	1	0.5	0.25	4	2	1	0.5	0.25		

Figure 1. *Image of the 96-microwell titer plates (parental and mutant E. coli strains) with the appropriate concentrations after two-fold serial dilution.*

Induction of Resistance to Gentamicin in *E. coli* strains

Overnight cultures of parent and mutant *E. coli* were grown in LB broth. A solution of gentamicin (at the determined MIC) and LB broth was prepared using the stocks. 10µL of the cultures were propagated into two separate 96-well microtiter plates: the first five columns contained 90µL of broth with gentamicin and the last five columns contained only 90µL of broth. The two middle columns were left as barrier between the two groups and as control, representing absence of growth (Figure 2).

The plates were covered with breathable membranes and incubated at either 37ºC overnight, or 35ºC over the weekend, and were read at 600nm after 16-22 hours of incubation. Following reading, the cultures were transferred into a new plate with fresh LB broth (with and without gentamicin) (Figure 2). This was reproduced for 15 days with gentamicin concentration doubling every 2-3 days, depending on growth.

Resistance induction was considered complete when there were only five wells left showing growth of 0.05OD or higher in the wells containing gentamicin.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10µL of overnight cultures of either parent or mutant *E. coli* in 90µL of **LB broth with gentamicin concentration**					Wells left empty		10µL of overnight cultures of either parent or mutant *E. coli* in 90µL of **only LB broth**				
B												
C												
D												
E												
F												
G												
H												

Figure 2. *Image of the 96-microwell titer plates in induction of resistance to gentamicin in the parent and mutant E. coli.*

Growth Curves

The growth characteristics of the parent and knockout strains were assessed using spectrophotometry at 600nm. Cultures were grown under standard conditions and absorbance was measured every 2 minutes for 24 hours. Figure 3 illustrates the 96-microwell titer plate for the detection of OD growth in the plate reader.

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="3" rowspan="3">5µL of mutant E. coli (exposed to gentamicin) from resistance induction in 195µL of LB broth</td><td rowspan="3"></td><td colspan="3" rowspan="3">5µL of parent E. coli (exposed to gentamicin) from resistance induction in 195µL of LB broth</td><td rowspan="3"></td><td colspan="3" rowspan="3">5µL of mutant E. coli from fresh cultures in 195µL of LB broth</td></tr>
<tr><td>B</td></tr>
<tr><td>C</td></tr>
<tr><td>D</td><td colspan="11" rowspan="2"></td></tr>
<tr><td>E</td></tr>
<tr><td>F</td><td colspan="3" rowspan="3">5µL of mutant E. coli (not exposed to gentamicin) from resistance induction in 195µL of LB broth</td><td rowspan="3"></td><td colspan="3" rowspan="3">5µL of parent E. coli (not exposed to gentamicin) from resistance induction in 195µL of LB broth</td><td rowspan="3"></td><td colspan="3" rowspan="3">5µL of parent E. coli from fresh cultures in 195µL of LB broth</td></tr>
<tr><td>G</td></tr>
<tr><td>H</td></tr>
</table>

Figure 3. *Image of the 96-microwell titer plate for the growth curves analysis. Three strains of each mutant and parent E. coli exhibiting the most OD growth are propagated into columns 1-3 and 5-6 respectively. Columns 10-12 were added with three fresh cultures of parent and mutant E. coli; rows A-C were of mutant strains and rows F-H were of parent strains. Columns 4 and 8-9, and rows D-E were left as barriers.*

Statistical Analysis

Analysis of the growth curves was done using Microsoft Excel. Two points within the log phase of the growth curves (of the parent and mutant *E. coli* strains from the original and post-resistance induction cultures) were identified to determine the doubling and generation times, and the subsequent fitness costs. The absorbance values were converted to logcolony count (log CFU/mL), and the difference between these two values were divided by to obtain the doubling time. The generation time was calculated by dividing the difference of the time periods of the identified absorbance values by the doubling time. Using the parent strain from the original *E. coli* culture as a 100% fitness, the percentage change formula was employed on the remaining strains.

The unpaired Student's t-test was then carried out on the generation times to determine the significance of the hypothesis—to accept or reject it—and significance level was set at 0.05. Levene's test was utilized to check if the variance between two groups of strains are equal or unequal, which will indicate the t-test to be used; if values are greater than 0.05, then variances in the data are equal, but less than 0.05 will indicate variances in the data are unequal.

Kirby-Bauer (disk diffusion) test

Antibiotic resistance profiles for the parent and mutant strains from the resistance induction: LB broth-cultured and gentamicin-exposed strains, and from the original culture were determined using a disk diffusion assay with M14 rings carried out in triplicate (Table 2) (Gram negative disk, MAST). A bacterial lawn was grown on MH agar in the presence of the antibiotic disk under standard conditions. After incubation the diameter of the zone of clearance was recorded.

Table 2. M14 gram-negative antibiotic rings with the respective concentrations of each antibiotic present.

M14 gram-negative antibiotic rings
10µg of ampicillin
5µg of cephalothin
25µg of colistin sulphate
10µg of gentamicin
10µg of streptomycin
200µg of sulphatriad
25µg of tetracycline
25µg of cotrimoxazole

Reversion Rates

Overnight cultures of three strains of gentamicin-exposed parent and mutant *E. coli* were grown in LB broth. After incubation, the cultures were serially diluted down to 10^{-6} in peptone water. LB agar plates with the final gentamicin concentrations reached in the resistance induction were prepared: 512 µg/mL for mutant strains and 750 µg/mL for parent strains; pure LB agar plates were also prepared. LB plates with gentamicin were divided into three areas: Neat, 10^{-1}, and 10^{-2}; plain LB plates were also divided into three areas: 10^{-4}, 10^{-5}, and 10^{-6} (Figure 4). The areas indicate the serial dilution of the cultures to be plated. The plates were incubated at 37 ºC for 18–24 hours and counted after. This was carried out for two days.

Reversion rate was determined by calculating the percentage of growth of the colony count on the gentamicin plates over the count on the LB only plates.

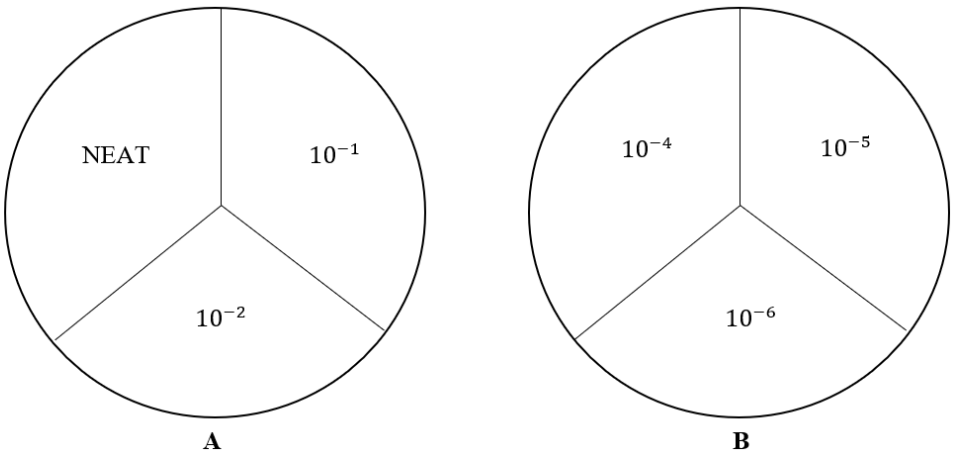


Figure 4. *Plates for reversion rates. A total of 12 plates were prepared. Plate A is of LB agar plate with gentamicin concentrations of either 512µg/mL for mutant strains or 750µg/mL for parent strains; 3 plates contained 512µg/mL of gentamicin and another 3 plates contained 750µg/mL of gentamicin. Plate B is of LB agar plate without gentamicin for mutant and parent strains.*

Results

Determining MIC

MIC determination for the parent and mutant strains was done in three trials, in which its average is displayed on Figure 5 and 6.

The MIC cut-off values for the parent and knockout mutant *E. coli* are below 0.836 and 0.858 (log CFU/mL), respectively. According to their number of cells (log CFU/mL), both the strains in all concentrations were adequately growing as the numbers were greater than or in the same range as their respective control wells of pure LB broth: 8.36 and 8.58; however, none of the values in the trials progressed to below 90% of the maximal growth. Due to time constraint, MIC determination for gentamicin was concluded and set at 2µg/mL as this is a reasonably and sufficiently low value within the range of MICs suggested by Andrews (2001).

Furthermore, the averaged cell-count revealed the mutant *E. coli* as consistently producing more growth (Figure 6) than the parent (Figure 5); although both strains showed a similar growth trend of a decrease from 64µg/mL of gentamicin concentration then an increase from 256µg/mL, and then a steep decline from 512µg/mL to 1024µg/mL. The somewhat elevated growth of the mutant is also apparent in the maximal growth (without gentamicin), in which the mutant has a cell count of 8.58, whereas the parent has a count of 8.36.

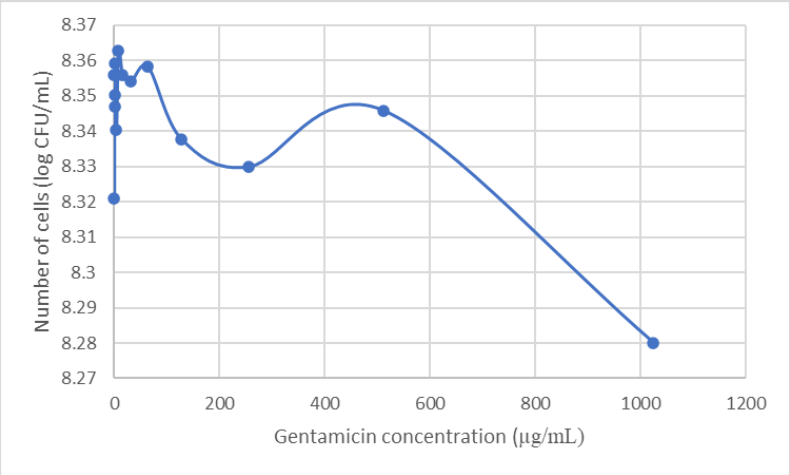


Figure 5. *Average of the three trials for the determination of MIC of the parent strains.*

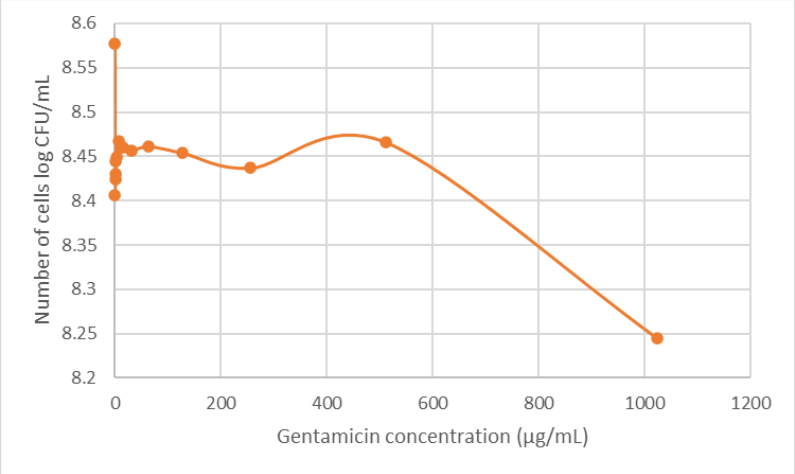


Figure 6. *Average of the three trials for the determination of the MIC of the mutant strains.*

Lengthened lag phase in parent and mutant-knockout *trkH E. coli* strains post-exposure to Gentamicin

The growth curve profiles for the post-resistance induction gentamicin-exposed parent and mutant strains produced a declining, almost linear graph starting from Time 20 minutes proceeding a steep decrease at Time 0 (Figure 7). The mean optical density (OD) growth of parent and mutant strains were calculated to 0.115 and 0.117, respectively; whilst the OD growth of the control wells, produced a mean of 0.110. The mean values of the bacterial strains are 0.05 greater than the control wells, indicating growth. Thus, the strains display an extended lag phase.

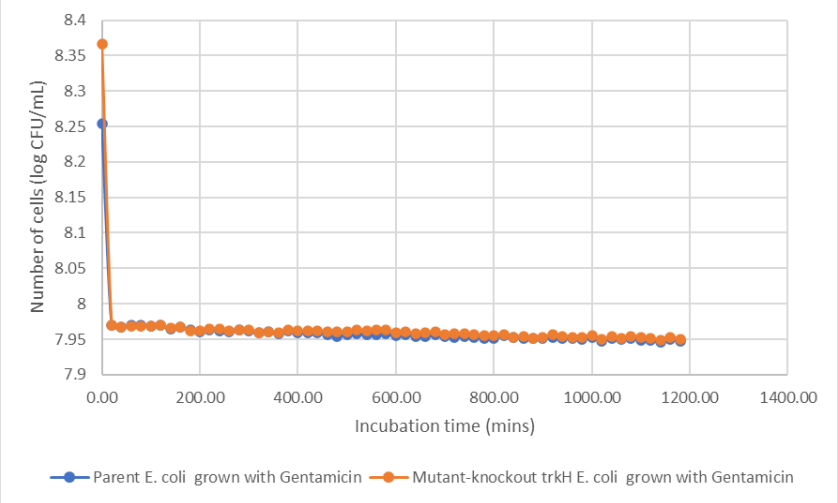


Figure 7. *Growth curve of parent and mutant-knockout trkH E. coli exposed to gentamicin, characterized by the number of cells (log CFU/mL) over time (minutes). There is a steep drop from Time 0 to Time 20 and a decreasing, almost linear graph.*

In contrast, the growth curve profiles of the original parent and mutant strains, and the post-induction (of gentamicin resistance) LB broth-cultured strains did not show an extended lag phase. Instead, the *E. coli* strains displayed exponential curves that are slowly shifting into the stationary phase (Figures 8 and 9). In both the curves, the lag phase appears as a drop in the number of bacterial cells between Time 0 and Time 20, as the cells adjusted to their environment, and lasted for only 20 minutes. Between the parent and mutant strains from the cultures, there is an apparent difference in their metabolic activity, illustrated in both graphs: the parent strains have higher growth curves than the mutant strains (Figures 8 and 9).

The parent from the original culture produced a relatively stabilized stationary phase and at Times 1140 to 1180, the graph appears to flatten (Figure 8). This implies the stationary phase is close to completion and cells will soon enter the death phase. In comparison, the parent from the broth culture from the post-induction embodied a stationary phase that is gradually increasing (Graph 9). Moreover, in the case of the mutant cultures in both graphs, the strains present no expansive differences from each other and show a stationary phase that is progressively increasing; albeit, its metabolic activity is evidently lower than that of the parent strains (Figures 8 and 9).

p-values and doubling and generation times imply knockout *trkH* have a role in the metabolic activity in mutant strains from both cultures

From the growth curve, the metabolic activity of the original and post-induction broth cultures of parent and mutant *E. coli* was analyzed through their doubling and generation times, and fitness costs (Table 3-5). The growth curve of parent and mutant *E. coli* exposed to gentamicin was not analyzed as the bacteria remained in lag phase (Figure 7).

The averages of the doubling times (CFU/mL/min) revealed the strains from post-induction broth cultures to have lower doubling rates than those from the original cultures (Table 3). Although, the mutant strains from both cultures demonstrated considerably reduced doubling rates than their parent counterparts—with the mutant from the original culture having 22.6% less than the parent, and the mutant from the post-induction culture having 25% less than its parent counterpart (Table 3).

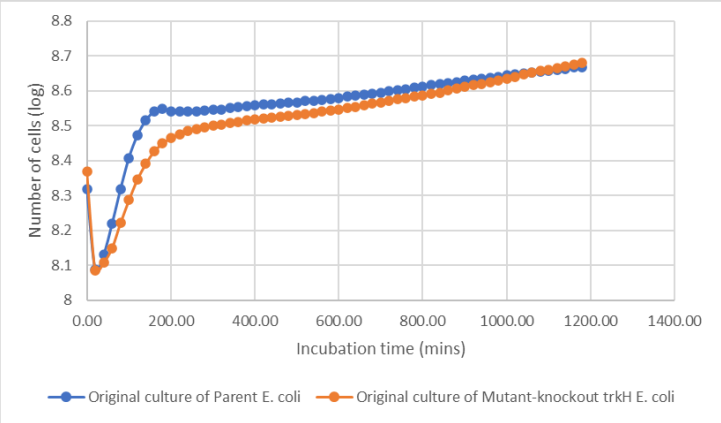


Figure 8. *Growth curve of parent and mutant-knockout trkH E. coli from original cultures, characterized by the number of cells (log CFU/mL) over time (minutes). There is a steep drop from Time 0 to Time 20 and a decreasing, almost linear graph.*

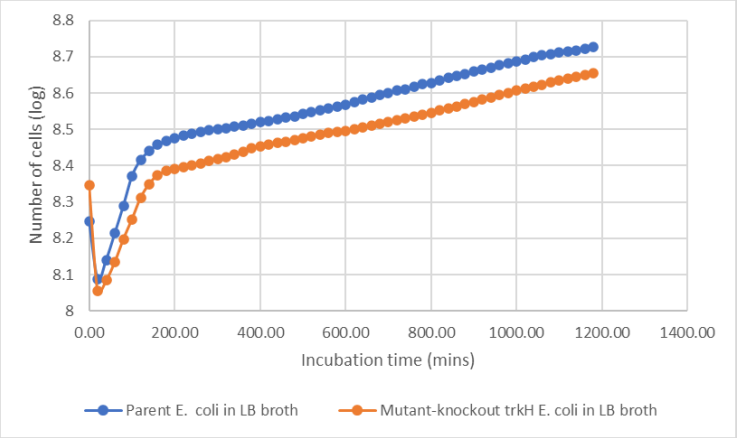


Figure 9. *Growth curve of parent and mutant-knockout trkH E. coli from LB broth cultures in resistance induction, characterized by the number of cells (log) over time (minutes). There is a steep drop from Time 0 to Time 20 and a decreasing, almost linear graph.*

Table 3. Doubling Time (CFU/mL/min) of the original and post-gentamicin induction resistance-LB broth cultures of parent and mutant *E. coli* strains.

Doubling Time				
	Original culture of parent *E. coli*	**Original culture of mutant *E. coli***	**Post-gentamicin resistance induction-LB broth culture of parent *E. coli***	**Post-gentamicin resistance induction-LB broth culture of mutant *E. coli***
Average of three trials (CFU/mL/min)	0.84	0.65	0.52	0.39

Corresponding to the doubling times, the mutant strains exhibited longer generation times than the parent strains (Table 4), indicating demand for an extended time to undergo binary fission. The standard deviation values of the generation time for the parent strains suggest a relatively varied spread of values around the mean, whilst for the mutant strain from LB broth, a lesser spread of the values. However, the mutant strain from original culture has an elevated standard deviation, implying great disperse of the values (Table 4).

Table 4. Generation time (minutes) of the original and post-gentamicin induction resistance-LB broth cultures of parent and mutant *E. coli* strains.

Generation Times				
	Original culture of parent *E. coli*	**Original culture of mutant *E. coli***	**Post-gentamicin resistance induction-LB broth culture of parent *E. coli***	**Post-gentamicin resistance induction-LB broth culture of mutant *E. coli***
Average of three trials (mins)	71.6	92.6	76.7	102.6
Standard deviation	0.475	1.21	0.569	0.180

Using the unpaired Student's t-test, the generation times were statistically analyzed, comparing the strains from each other to determine whether there are any significant differences. The t-test produced varied p-values; the original cultures of *E. coli* had a p-value of , whilst the original culture and the post-induction broth culture of parent *E. coli* had a higher p-value of . Meanwhile, the t-test of the original culture of parent *E. coli* and the post-induction broth mutant *E. coli* displayed a low p-value of , and the p-values of the post-induction LB broth cultures were even lower: (Table 5).

Based on the *E. coli* strains' statistical data, the hypothesis is supposed to be accepted, but since it cannot be applied to the strains unexposed gentamicin, it is nulled. Nevertheless, the results of the p-values were lower than 0.05 in the comparison between the parent strains from the original culture and the mutant strains from the post-induction broth culture, between the parent and mutant strains from the post-induction LB broth culture, and between the strains from the original culture (Table 5)—indicating significant difference. And the parent *E. coli* strains from the original and the post-induction broth cultures generate a p-value higher than 0.05 (Table 5)—implying insignificance.

Table 5. p-values of the compared strains of *E. coli*

p-values			
Original cultures – Parent *E. coli* vs. Mutant *E. coli*	**Post-gentamicin resistance induction-LB broth cultures – Parent *E. coli* vs. Mutant *E. coli***	**Original-Parent *E. coli* vs. Post-gentamicin resistance induction-LB broth-Parent *E. coli***	**Original-Parent *E. coli* vs. Post-gentamicin resistance induction-LB broth-Mutant *E. coli***
7.77×10^{-3}	4.77×10^{-5}	6.49×10^{-2}	4.25×10^{-5}

Fitness costs indicate another factor influencing the fitness of post-gentamicin resistance induction strains

In reference to the fitness cost of the strains, the mutant from the broth culture displayed the largest cost of -43.4%; the parent strain from the same culture showed a lower, but still relatively large cost of -29.6%, whereas the mutant strain from the original culture demonstrated only a -7.3% cost from the 100% fitness of ancestor parent strain. Comparison of results from mutant strain from original culture and strains from broth culture indicate another factor for the significant increase of the fitness costs in the latter strains.

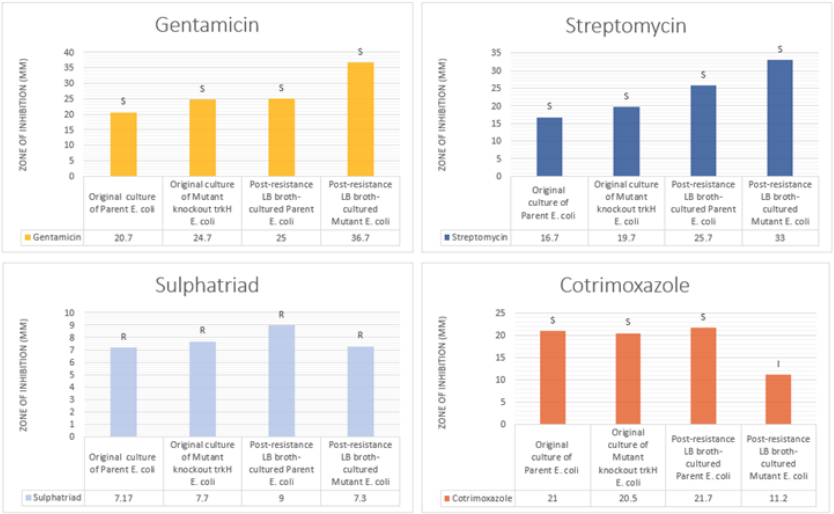
Table 6. Fitness costs of mutant *E. coli* from original culture, and parent and mutant *E. coli* from LB broth cultures. The parent *E. coli* from original culture is considered as the ancestor of the strains, thus, it is theoretically without a fitness cost and is used as basis for 100% fitness.

Fitness Costs			
	Original culture of mutant *E. coli*	**Post-gentamicin resistance induction-LB broth culture of parent *E. coli***	**Post-gentamicin resistance induction-LB broth culture of mutant *E. coli***
Average of three trials	-7.3%	-29.6%	-43.4%

Inoculated gentamicin-exposed cultures in lag phase displayed absence of growth on plates with antibiotic(s)

In the disk diffusion test, the parent and mutant *E. coli* strains previously exposed to gentamicin displayed absence of growth on the MH agar, whilst the strains unexposed to gentamicin showed sufficient growth. LB broth cultures demonstrated growth with sensitivity profiles relatively similar to the original cultures—with some differences in certain antibiotics (Figure 10). In the presence of cotrimoxazole, the broth-cultured mutant *E. coli* demonstrated low intermediate susceptibility at 11.2mm, in contrast to the same cultured parent with susceptibility at 21.7mm and to the mutant *E. coli* from the original culture at 20.5mm. For cephalothin, the mutant displays no difference in its profiles compared to the parent; both strains appear as intermediately susceptible to the antibiotic with the parent being closer to resistance. However, these strains differ to pattern from the original culture, wherein the mutant demonstrated low resistance and the parent, intermediate resistance (Figure 10).

In the case of gentamicin and streptomycin, the broth-cultured mutant demonstrated great differences in its sensitivity pattern for those antibiotics, in comparison to that of the parent from the same culture and of the mutant from the original culture (Figure 10). It showed increasingly high susceptibility for gentamicin at 36.7mm and for Streptomycin at 33mm, which are 46.8% and 28.4% greater than the zones of the parent from the same culture, and 48.5% and 67.5% greater than of the mutant from the original culture (Figure 10).



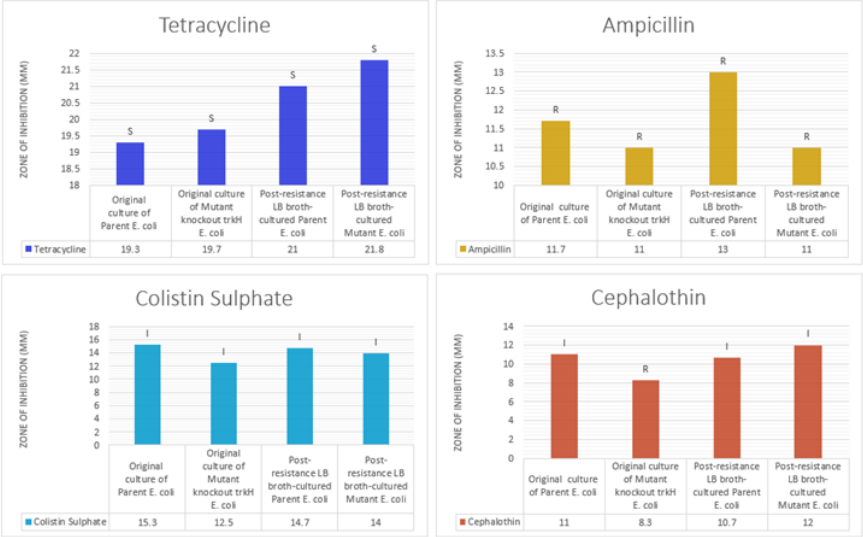


Figure 10. *Averaged antibiotic sensitivity profiles of the parent and mutant strains from the original and post-gentamicin resistance induction cultures.*

Likewise, in the reversion rate test, the parent and mutant strains previously exposed to gentamicin, displayed full sensitivity (no growth) to the antibiotic on LB agars with the concentrations, 750µg/mL for the parent strains and 512µg/mL for the mutant strains, on the first day of exposure (Table 7). This outcome was repeated on the second day (Table 10). The concentrations in the agars were determined according to the resistance induction. Resistance induction was considered completed when only five wells on the plate are left showing growth of 0.05OD or higher than the control wells. However, due to time constraint, induction was finalized when there were still twenty-three wells left in the plate of the parent strains at a gentamicin concentration of 1024µg/mL and forty wells of the mutant strains at a gentamicin concentration of 512µg/mL. Although the final concentration reached by the parent *E. coli* in the resistance induction was 1024µg/mL, only 750µg/mL was added to the LB agar in the reversion rate test due to inadequate gentamicin sulfate powder.

Meanwhile, strains plated on pure LB agars exhibited substantial colony growth, on the first and second days—indicating bacterial cells were not dead—with the mutant consistently demonstrating higher colony growth (Table 8 and 11). The absence of growth at Days 1 and 2 (Tables 7 and 10) in the plates with gentamicin concentrations consequently led to the lack of percentage growth (Tables 9 and 12). All strains retained their forms until Day 2 of antibiotic exposure (Tables 10 -11).

Table 7. Day 1 of reversion rate test showed absence of growth (CFU/mL) by parent and mutant *E. coli* strains previously exposed to gentamicin on LB agars with gentamicin concentrations.

1st Day	Parent *E. coli* on LB agar with 750µg/mL of Gentamicin			Mutant *E. coli* on LB agar with 512µg/mL of Gentamicin		
	Strain 1	Strain 2	Strain 3	Strain 1	Strain 2	Strain 3
Average of three trials (CFU/mL)	No growth was observed at all dilutions (Neat, 10^{-1}, and 10^{-2}).			No growth was observed for all dilutions (Neat, 10^{-1}, and 10^{-2})		

Table 8. Day 1 of reversion rate test displayed growth (CFU/mL) by parent and mutant *E. coli* strains previously exposed to gentamicin on pure LB agars.

1st Day	Parent *E. coli* on pure LB agar			Mutant *E. coli* on pure LB agar		
	Strain 1	Strain 2	Strain 3	Strain 1	Strain 2	Strain 3
	10^{-6}	10^{-5}	10^{-6}	10^{-6}	10^{-6}	10^{-6}
Average of three trials (CFU/mL)	1.33×10^{9}	2.27×10^{8}	1.33×10^{9}	2.00×10^{9}	1.93×10^{9}	3.93×10^{9}

Table 9. Null percentage growth of mutated *E. coli* at Day 1, indicating all strains have reverted back to the original form.

1st Day	Parent *E. coli*			Mutant *E. coli*		
	Strain 1	Strain 2	Strain 3	Strain 1	Strain 2	Strain 3
Percentage of mutated growth (%)	0			0		

Table 10. Day 2 of reversion rate test showed absence of growth (CFU/mL) by parent and mutant *E. coli* strains previously exposed to gentamicin on LB agars with gentamicin concentrations.

2nd Day	Parent *E. coli* on LB agar with 750µg/			Mutant *E. coli* on LB agar with 512µg/mL of Gentamicin		
	Strain 1	Strain 2	Strain 3	Strain 1	Strain 2	Strain 3
Average of three trials (CFU/mL)	No growth for all dilutions (Neat, 10^{-1}, and 10^{-2}).			No growth for all dilutions (Neat, 10^{-1}, and 10^{-2}).		

Table 11. Day 2 of reversion rate test displayed growth (CFU/mL) by parent and mutant *E. coli* strains previously exposed to gentamicin on pure LB agars.

2nd Day	Parent *E. coli* on pure LB agar			Mutant *E. coli* on pure LB agar		
	Strain 1	Strain 2	Strain 3	Strain 1	Strain 2	Strain 3
	10^{-6}	10^{-4}	10^{-6}	10^{-4}	10^{-6}	10^{-6}
Average of three trials (CFU/mL)	1.73×10^{9}	2.00×10^{8}	1.00×10^{9}	2.00×10^{8}	8.67×10^{8}	9.33×10^{9}

Table 12. Null percentage growth of mutated *E. coli* in Day 2, indicating all strains have retained their condition.

2nd Day	Parent *E. coli*			Mutant *E. coli*		
	Strain 1	Strain 2	Strain 3	Strain 1	Strain 2	Strain 3
Percentage of mutated growth (%)	0			0		

Discussion

Rapid spread of antibiotic-resistant bacteria is increasing worldwide, presenting a problem to the efficacy of antibiotics (Lee Ventola, 2015). The mechanisms involved in developing resistance is regularly being researched and new ones are continuously identified in order to develop novel antibiotic treatments and analyze their effectiveness (Blair et al., 2014; Li et al., 2016), some of which are described in the results of this study. The growing number of worldwide cases of gentamicin-resistant *E. coli* strains, particularly involving children, is of great concern as it presents a severe risk in morbidity and mortality. Amongst the genes that induce its resistance, the *trkH* gene, which influences the bacterial metabolic activity, has been found to play a major role in resistance to aminoglycosides. Therefore, the gene, *trkH*, was investigated in this study—through its respective presence and absence in parent and mutant *E. coli* strains—to assess its ability to generate gentamicin-resistance in parent *E. coli* strains and also the extent of the induced resistance.

trkH gene presents a significant role in the metabolic activity of *E. coli*

Intracellular potassium ions are critical for the survival of bacteria and is essential in the maintenance of its homeostasis, adaptation to osmolarity, and activation of specific cellular enzymes (Su et al., 2009). *trkH* encodes the TrkH protein from the Trk system, which is responsible for the entry of potassium ions into the cell. In the mutant *E. coli* strains in this study, *trkH* is absent.

The growth curves of *E. coli* strains from original and post-gentamicin resistance induction LB broth cultures consistently showed the mutant-knockout *trkH* strains to have a lower growth (metabolic) activity than the parent strains (Figures 8 and 9), particularly in terms of their lower rates for doubling (Table 3), and their longer generation times, which suggest longer periods required for binary fission (Table 4).

This may be due to the absence of the *trkH* in these strains. Since these strains do not carry *trkH*, the influx of potassium ions into the cells is defective therefore their metabolic activity is impaired, highlighting the significance of *trkH* in *E. coli* metabolism. Also, a steep decline from Time 0 to Time 20 in the graph indicates reduction in the number of viable cells as the cells adapt to its new extracellular environment; this typically occurs before the commencing of the exponential phase (Bertrand, 2019).

Interestingly, several studies have described the role of the TrkH transporter in the increase of virulence in several bacteria (Stingl et al., 2007; Su et al., 2009; Alkhuder et al., 2010). In *Francisella tularensis*, a Gram-negative that occasionally causes bacteremia, an absence of *trkH* in knockout strains demonstrated the strains to be less virulent, which was examined from their rapid expulsion in the blood of infected mice. This was confirmed by a detected growth defect of the knockout via blood *ex vivo* (Haristoy et al., 2003; Alkhuder et al., 2010). A study by Su et. al, (2009) found that Trk system is essential for the virulence of another Gram-negative, *Salmonella enterica*. The Trk system was reported to influence the production of the effector proteins necessary for the bacterium's infection—allowing for cellular invasion. This was confirmed in mutants without the Trk system wherein *S. enterica* was observed to be less virulent and consequently, lower levels of effector proteins were produced. These findings signify the vital role of *trkH* in the biological processes in *E. coli*, such as the maintaining of its hemostasis and the activation of its enzymes that may potentially support and influence its capacity for virulence. Considering the increasing prevalence of *E. coli*-associated blood-stream_infections (BSI) and sepsis (Tumbarello et al., 2010; Shehab El-Din et al., 2015), specific inhibition of *trkH* may be exploited to develop novel therapeutic antibiotics that suppress the virulence of *E. coli*.

Furthermore, the reduction in the doubling rates and the increase in the generation times of the post-induction parent and the original and post-induction mutant *E. coli* strains resulted in the strains' high fitness costs—especially in the mutant strains from the post-gentamicin resistance induction culture which accumulated a loss of 43.4 (Table 6). The reduction also led to significantly decreased p-values in the statistical comparison of parent and mutant strains, which highlighted the essential role of *trkH* in the metabolic activity of *E. coli* (Table 5). The comparison between the parent and mutant *E. coli* strains from the original culture produced a p-value lower than 0.05: 7.77×10^{-3}; the parent from the original culture and the mutant *E. coli* from the post-induction culture also showed a markedly reduced p-value of 4.25×10^{-5}; and the parent and mutant *E. coli* from the post-induction culture presented an even lower p-value of 4.25×10^{-5} . Through the p-values, which illustrated existing consequential differences between the parent and mutant strains, and the fitness costs acquired by the mutant strains, the role of the *trkH* in the metabolism of *E. coli* is emphasized. As a result, the potential influence of *trkH* in the development of resistance to gentamicin is reinforced as resistance mutations are likely to confer fitness costs in bacteria; these mutations often occur in genes involved in cellular functions (Melnyk et. al, 2014). Therefore, mutation in *trkH* may confer gentamicin resistance in *E. coli* at the cost of its fitness.

Persistence of parent and mutant-knockout *E. coli* strains in increasing Gentamicin concentrations may be of heteroresistance

In the determination of MIC and in the induction of resistance in parent and mutant *E. coli*, the strains presented apparent resistance to gentamicin through adequate growth in the presence of the antibiotic. At maximum MIC of 512µg/mL, the parent strain produced an average growth (log CFU/mL) of 8.35 and the mutant strain, 8.47, which are only 0.998% and 11.1% lower than the respective averaged maximal growths of 8.36 and 8.58 (Figures 5 and 6)—far from the MIC cut-off of 90% lower growth than the maximum. Further, both the parent and mutant strains presented variation in their response to the different ranges of gentamicin concentration. In the first trial of MIC, the parent and mutant produced the highest growth at the maximum gentamicin concentration (512µg/mL), whilst in MIC 2 and 3, the highest growth values were produced in the middle range of concentrations. These varied results may indicate heteroresistance.

Heteroresistance is a poorly characterized phenomenon commonly defined by a demonstrated resistance to certain antibiotics by a subset of bacterial population—through exhibiting numerous responses in the presence of varying concentrations (Falagas et al., 2008; El-Halfawy and Valvano, 2015). A possible heteroresistance mechanism is the ability of subpopulations of heteroresistant bacteria to "protect" sensitive subpopulations through chemical signals, such as indole in *E. coli*—a signal produced by growing cells to activate drug efflux pumps and oxidative stress protective mechanism (Lee et al., 2010; El-Halfawy and Valvano, 2015). These "protective" mechanisms may explain the persistent growth of *E. coli* strains in the presence of increasingly high gentamicin concentrations. This action in *E. coli* populations was investigated by Lee et. al (2010) through a culture subjected to increasing gentamicin concentration. In an environment of antibiotic stress, resistant isolates maintained high indole production of greater than 300µM; but in its absence, less amounts of indole were produced (<300µM).

Further, the co-culturing of resistance isolates with susceptible isolates generated optimal growth, unlike the culture consisting of individual isolates thus suggesting "protective" mechanisms being carried out. Through genome-wide transcription, various expressed genes were identified in indole-deficient mutants supplemented with suboptimal concentration of indole. The action of *mdtE* on multi-drug efflux pumps was found to be upregulated by indole, as well as the action of *astD*, which increased the production of succinate, a citric acid cycle intermediate. Likewise, the downregulation of certain genes that activate oxidative stress protective mechanisms were also detected. This includes *oxyS*, which is an RNA sensor for oxidative stress inside the cell, and *iscU*, which plays a role in bacteria's antibiotic-mediated death. These genes, present in both the parent and mutant-knockout *trkH* strains, may have been upregulated/downregulated by the production of indole, which explains their survival at high concentrations (Lee et. al, 2010; Förster and Gescher, 2014).

However, it is not conclusive whether the cells did exhibit heteroresistance. Considering that the MICs of the parent and mutant strains were not established, the actual starting MICs for the resistance induction were not adopted. It is possible that the strains retain high MICs therefore the usage of low concentrations in the resistance induction failed to encourage resistance in *E.* coli strains. As a result, for further work, increasing the MIC concentrations should be maintained as it is possible that the *E. coli* strains in this study possess high MIC for aminoglycosides as was observed in Apjok et al. (2019)'s study. The same *E. coli* BW25113, with the knockout *trkH* had MICs at 7,460µg/mL for Kanamycin and 9,585µg/mL for Streptomycin. The uncharted mechanisms of resistance exhibited by the strains during the MIC determination presents a crucial limitation. A way to overcome this and should be considered in further work is whole-genome sequencing using next-generation sequencing (NGS) technology (Qin, 2019). Through sequencing, mechanisms of genes encoding proteins essential in export and oxidative stress protection may be identified.

Extended lag phase by parent and mutant *E. coli* strains may indicate a post-antibiotic effect: tolerance

Referencing the studies mentioned that support the findings of this research, adaptation to the presence of antibiotics does not always result in full resistance. The results indicate that resistance is not the only mechanism in which bacteria offset the effects of antibiotic (Grant and Hung, 2013).

An anomaly was observed in the growth curve produced by parent and mutant *E. coli* previously exposed to gentamicin (Figure 7), which presented a linear-like curve—representing an extended lag phase. This is identified from averaged OD growth of the strains, which are greater than that of the control well. The averages of all control wells were calculated and compared to the averages of the strains to confirm that the curve does not represent cell death. Further, another deviance was observed during the induction of resistance in *E. coli* strains. Both parent and mutant strains persisted even at higher concentrations of gentamicin, wherein more than 50% of the tested wells for both strains presented growth (Figures 8-9).

The extended lag phases and persistent growth of parent and mutant *E. coli* may be attributed to a post-antibiotic effect (PAE). PAE is the persistent suppression of bacterial growth after subsequent temporary exposure to antibiotic (Stubbings et al., 2006). Fridman et al. (2014) discovered that shifts in the length of lag phase during sub-inhibitory antibiotic exposure allowed bacteria to develop tolerance and was its first strategy in response to antibiotic stress. PAE was reproduced in Li et al. (2016)'s study where *Pseudomonas putida* produced an extended lag phase and decreased growth rate in the presence of ciprofloxacin and tetracycline. After exposure to antibiotics, *P. putida* exposed to tetracycline regrew after 10 hours of lag phase following subsequent culture in broth; but bacterial cells exposed to ciprofloxacin did not regrow within 24 hours of it. In addition, antimicrobials inhibiting synthesis of proteins and nucleic acids, which include aminoglycosides and fluoroquinolones, have been found to induce prolonged PAE in *E. coli* (Stubbings et al., 2006); this corresponds to the growth demonstrated in the curve by the parent and mutant strains after exposure to sub-optimal gentamicin concentrations. To further examine this effect, the growth curve of *E. coli* may be analyzed for a longer period to detect the endpoint of the lag phase and the extent of PAE.

These findings are vital as these could suggest a type of PAE: tolerance. Tolerance is the adapting of bacteria in the presence of inadequate antibiotic treatment by transiently delaying their growth and persisting in the environment—and is illustrated by an extended lag phase. In the case of this research, inadequate treatment is the inconsistent short periods of exposure to a gentamicin concentration before its doubling. Tolerance is an area less understood than resistance as it is less universal and genes behind it are not fully identified; therefore, research into it is crucial as it may explain the failure of some antibiotic treatments (Fridman et al., 2014; Levin-Reisman et al., 2019). This effect protects cells from the action of most bactericidal antibiotics, like gentamicin, since these require actively dividing cells (Mascio et. al, 2007).

Furthermore, tolerant bacteria may be more problematic than resistant ones, since tolerant strains enhance their resilience to the antibiotic even in high concentrations—unlike resistant strains that deteriorate when concentrations increase past its MIC (Levin-Reisman et al., 2019). Therefore, an in-depth analysis using whole genome sequencing could be beneficial in detecting mutated genes that could induce tolerance through the comparison of tolerant and resistant strains.

However, the *trkH* gene may not be involved in the strains' development to tolerance as the gene is absent in gentamicin-exposed mutant strains. Mutations progressing to tolerance and resistance can interact interdependently therefore it is possible that while *trkH* induces resistance, another gene induces tolerance. A combination of these phenomena may explain the varied results of this study. This further highlights the danger of antibiotics losing its potency (Levin-Reisman et al., 2019).

Meanwhile, in the disk diffusion test of the same strains, no growth was identified on their cultured MH agars; likewise, in the reversion rate test, there was no growth on agars with gentamicin, whilst optimal growth on the ones without (Tables 7-8 and 10-11). These outcomes coincide with the growth curve of the mutant strains (Figure 7). The graph revealed the strains to be dormant therefore not replicating. Premature culture of the strains on the agars may have occurred, wherein inoculation of fewer nonreplicating cells were added onto the presence of antibiotics; because there were fewer cells in the population, there were not enough healthy cells to multiply on the agars thus strains were unable to produce growth. To overcome this, the culture may be left to incubate longer to ensure cells have adapted to their new environment and have come out of the lag phase.

A method that may be done in future work to thoroughly examine tolerance is through the antimicrobial gradient method, Etest—an *in vitro* method to determine MICs on agars using a labelled reagent strip consisting of a continuous gradient of dried antibiotic concentrations for the quantification of MICs. MIC is marked by the intersection of the ellipse and the strip—elliptical inhibition zone is formed from the interaction between the concentration gradient and the inoculated bacterial culture (Sader and Pignatari, 1994; Jorgensen and Ferraro, 1998; Fridman et al., 2014). This test allows easier comparison of MICs between the parent and a tolerant strain as the production of similar zones of inhibition may imply tolerance but will have different results on the growth curve.

Lack of nutrients in LB broths may have affected cell composition of strains

The results of the growth curve, disk diffusion test, and reversion rate test were ambiguous and inconclusive therefore it is possible that other factors are causing the extended lag phase and absence of growth on the agars.

Due to restricted volumes of broth used in the propagation of parent and mutant cultures in the 96-microwell plates during resistance induction, it is probable for there to have been scarce nutrients available in the broth culture. The restricted environment of a batch culture, defined by the transfer of a part of a bacterial culture into a new medium, provides limited nutrients for bacterial strains which can only support rapid and optimal growth for a limited period (Vonshak, 1985; Brown et. al, 1990)—hence, cultures during the resistance induction may be considered as batch cultures. The depleted nutrients in the culture can influence cells to adapt through the conservation of consumed nutrients within the cell, modification of cell composition which can lead to cellular disruptions, alteration of cell composition or surface to increase the affinity for nutrients, and/or reduction of cellular growth rate (Brown et. al, 1990). Consequently, the continuous exposure of the parent and mutant populations to less nutrients (through the lower volumes of broth in the 96-microwell plates) may have caused disruptions in the strains' cell composition—and in mutant strains, continuous exposure to gentamicin (in the broth) also occurred therefore being exposed to even lesser nutrients. Thus, it is possible for the strains to have evolved through mutations in their cell composition and/or surface in order to increase their affinity for more nutrients in a competitive environment (Brown et. al, 1990). In addition, because of the decreased nutrients for the strains, cells may have shifted to dormancy to conserve remaining energy for survival. Considering the lengthened period of resistance induction, it is probable that the gentamicin-exposed cells required more time to adapt to nutrient-rich conditions in higher volumes of LB broth. Additionally, this event may be linked—theoretically—with tolerance, in which through the presence of reduced nutrients, cells adapted through alterations causing an extended lag phase.

To redress this, longer incubation time in a nutrient-sufficient broth could be done in which the cultures are resuspended daily in fresh broth before the analysis of growth curves, allowing them to adapt and modify cellular structure in optimal conditions.

Moreover, the possible manifestation of mutations in cell composition may explain the broth-cultured mutant strains' significant increase in susceptibility to gentamicin and streptomycin, despite lacking *trkH*, which encodes the TrkH transporter that facilitates in the cell entry of aminoglycosides, and its shift in its sensitivity pattern towards cotrimoxazole and cephalothin (Figure 10). It may also explain the absolute inability of cells to grow in the presence of the antibiotic disks. Changes in the outer and cytoplasmic membranes in gram-negatives were found to be associated with increased sensitivity to antibiotics including gentamicin. It was found that *E. coli* in a carbon-limited batch culture produced more lipopolysaccharide (LPS). The exhibition of a heightened production of LPS by *E. coli* may then be used to confirm for the existence of scarce nutrients through the Limulus amebocyte lysate (LAL) Gel-Clot assay, a quantitative assay used to measure LPS over a concentration range (Mehmood, 2019).

In addition, during the reversion rate test, gentamicin-exposed parent and mutant strains cultured on pure LB agar produced sufficient growth on Day 1 and optimal growth on Day 2 (Tables 8 and 11), but absence of growth on agars with gentamicin. This may suggest that the nutrient-deficient broth did drive *E. coli* cells to adapt through mutations in cell composition and/or surface; hence, when grown in nutrient-rich optimal conditions, the metabolic activity of the strains recovered. Although the purpose of the test was to investigate the stability of the gentamicin-resistance mutation in gentamicin-exposed parent *E. coli*, this was not completed due to the cells remaining in lag phase and not producing profiles during disk diffusion. Therefore, whether *trkH* was mutated is unknown. The indefinite results amplify the importance of gene sequencing that may be done through PCR. For future work, it can be performed on a group of *E. coli* cells exhibiting reduced metabolic activity and another group exhibiting maximal metabolic activity for comparison and confirmation.

Results of the fitness costs reinforce the influence of the *trkH* gene and of the nutrient-deficient environment in the bacterial metabolic activity

Interestingly, both the parent and mutant *E. coli* strains from the broth culture of the resistance induction displayed significantly increased fitness costs when compared to the mutant strain from the original culture (Table 6). The cost of fitness of the mutant strain from the original culture is due to the absence of the *trkH* in its genome; but interestingly, the parent strain post-resistance induction demonstrated a greater loss of 29.6%, 75.3% higher than the original mutant strain. Theoretically, the post-induction parent strain will have the same level of fitness as the original parent strain, however its results state otherwise. Since environmental factors can also influence the PMF thus affecting the growth activity of bacteria, it may be that the lack of nutrients, which stimulate cell modification, affected the TrkH transporter of the parent strains (Brown et. al, 1990). Meanwhile, the post-induction mutant strains expectedly exhibited a fitness cost owing to the absence of *trkH*. However, compared to the original mutant strain, it garnered an even greater fitness cost of 43.4%, 83.2% greater than the original mutant and 31.8% higher than its parent counterpart. This outcome suggests that its metabolic activity was not only affected by the loss of *trkH* but may also have been due to the scarcity of nutrients in the broth cultures during the resistance induction, stimulating alterations in cell composition to adapt.

In addition, since the gentamicin-exposed strains were still in lag phase, its doubling and generation times were not calculated and statistically analyzed; the hypothesis cannot be investigated on strains unexposed to the antibiotic. Therefore, the analysis of the hypothesis of whether the loss of *trkH* has can influence the development of gentamicin resistance is ineffective. Still, the loss of *trkH* in the mutant strains was observed to significantly affect the metabolic growth activity of the unexposed strains as was seen through their doubling and generation times and the subsequent fitness costs (Tables 3-4, and 6). Consequently, the p-values exemplify significant difference in the metabolic activity between the mutant strains and the parent strains (Table 5). Additionally, the p-value from the comparison between the parent strains from the original culture and the post-resistance induction culture is greater than 0.05, implying no vast difference in their metabolic activity (Table 5). These results indicate the loss of *trkH* has an impact in the metabolism of *E. coli* and highlights the outcomes of the growth curves in which the mutants consistently demonstrated lower growth activity (Figures 8-9).

Intriguingly, the p-value of the comparison between the parent and mutant strains from the post-resistance induction was 4.77×10^{-5}, which is profoundly lower than that of strains from the original culture, 7.77×10^{-3}. It could allude to the nutrient-deficient broth that influenced the expansive difference between the strains from the two cultures' growth activity. Also, in the comparison between the original parent and the post-resistance induction mutant, the p-value presented was conspicuously lower than 0.05: 4.25×10^{-5}. The value is broadly disparate from that of the comparison between the original strains, despite the post-induction mutant being similar to the original mutant—not subjected to gentamicin and carrying a deficiency of *trkH* in its genome. This further illustrates the lack of nutrients that may have affected its metabolic activity.

Therefore, in line with the produced p-values, there was no major change in the metabolic activity in the parent strains, whereas in the mutant strains, their metabolic activity significantly shifted, especially in the post-induction strain. This strengthens the role and function of the *trkH* in the growth activity of *E. coli* through the PMF and may also insinuate the important role of nutrients in the stability of its metabolism.

Conclusions

According to the experimental results and the statistical analysis of the data, the *trkH* gene has a role in the metabolism of *E. coli*. The hypothesis was not further analyzed due to the gentamicin-exposed cultures of mutant and parent *E. coli* strains from the resistance induction still being in lag phase—therefore, its doubling and generation times were absent. Still, the function of *trkH* gene was investigated through its absence in the mutant strains. Because of its absence in the mutant-knockout *trkH E. coli*, there may have been insufficient intracellular levels of potassium ions thus the mutant strains were unable to grow optimally as was observed from their growth curve profiles (Figures 8-9), their doubling and generation times, and their subsequent fitness costs (Tables 3-4 and 6).

However, there presents a limitation in this research as the intracellular potassium ion-level in the strains was not confirmed. An assay that may be employed to confirm this is the inductively coupled mass spectrometry (ICP-MS), which can determine intracellular concentrations of potassium ions as was used in a study by Jensen et al. (2014). The researchers evaluated the response of *Haloarcula marismortui* in the presence of external potassium stress—the halophilic archaeon possesses an ATP-regulated potassium transporter similar to the Trk system in *E. coli*. Using ICP-MS, intracellular potassium ions in lysed and sonicated centrifuged cells can be detected; the measured concentrations can then be used to determine the average potassium ion concentration (mol) in a single cell, whilst referencing a standard curve of cell density against optical density (Jensen et al., 2014).

Nevertheless, the findings in this research provide strong evidence for the significant role of the *trkH* in *E. coli* and emphasize the importance of the TrkH transporter in its growth. In addition, it revealed some interesting insight into the complex evolution of bacteria, in terms of its adaptation to the presence of antibiotic which in this case is gentamicin. The parent and mutant strains previously exposed to gentamicin in the resistance induction were unable to produce antibiotic sensitivity profiles, but when grown in pure LB agars under standard conditions in the disk diffusion and the reversion rate tests, were able to produce optimal growth. Further, the parent and mutant strains from post-induction LB broth cultures exhibited differing sensitivity profiles to the original culture. These results may indicate genes responsible for cell composition and/or surface have transiently altered to adapt to changes in the extracellular environment of the *E. coli* cells. Through this process, bacterial cells were able to survive albeit decreased metabolic activity, and when introduced to ideal conditions, reverted back and continued growing, optimally, as seen in the agars without gentamicin in the reversion rate test (Tables 8 and 11).

Furthermore, the results in the MIC and in the growth curve profiles appear to be in conflict. In the MIC, results indicate heteroresistance in both the parent and mutant strains, but regarding the growth-curve profiles and the data from the resistance induction, results imply tolerance. These outcomes illustrate that resistance is not a “black or white” event. The presence of sub-inhibitory antibiotic concentrations may not induce resistance in bacteria, but instead, may cause heteroresistance, tolerance, and/or transient alteration of cell composition or surface in response to antibiotic stress. Whether these events will progress to mutations is uncertain and should be investigated in further researches. None theless, these events pose danger to the potency of antibiotics, particularly gentamicin. Considering the significance of gentamicin as a first-line treatment for infections, such as those of neonates, research into these phenomena must be executed as these may provide more understanding to the bacterial evolution in the presence of antibiotics. Gaining insight into these events may serve as backbone for the research of novel therapy.

Thorough investigation of the *trkH* gene may also be useful as this gene is involved in atypical resistance, and thus, only confers minor resistance. It is unknown whether this gene did contribute to the events described in this study, since its detection using straightforward methods is difficult. Methods from Wistrand-Yuen et. al (2019)’s study, namely laboratory selection and reconstitution experiment, followed by comparative analysis of susceptible and resistant clinical isolates for identification of possible presence in resistant isolate could be adapted to discover atypical resistance genes.

Lastly, it is imperative that whole-genome sequencing must be done in future work when investigating the actions of *trkH* and/or the mentioned phenomena in this research.

Using dye-based quantitative reverse transcription PCR (RT-qPCR), mutated genes will be detected at real time, and its function and effects on bacterial evolution after the presence of antibiotics can be analyzed (Bustin and Nolan, 2004). Transcriptome sequencing may also be used to investigate the functional parts of the entire genome, consequently revealing any changes in the molecular components of the cells and tissues (Wang et. al, 2009).

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Conflicts of Interest

The author has no conflicts of interest related to this study.

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