RSGC
Royal St. George's College

The Young Researcher

2020 Volume 4 | Issue 1

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Recommended Citation
Khanal, P. (2020). Cus efflux system and resistance to nanoparticles. *The Young Researcher*, 4 (1), 208-219. Retrieved from http://www.theyoungresearcher.com/papers/khanal.pdf

ISSN: 2560-9815 (Print) 2560-9823 (Online) Journal homepage: http://www.theyoungresearcher.com

The Cus Efflux System and *E. coli* Resistance to Green-Synthesized Silver Nanoparticles

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This study aimed to determine how the deletion of the *cusC* gene influences *E. coli* growth in a medium containing silver nanoparticles. Two strains of *E. coli* were ordered, one of which had a deletion of the *cusC* gene, and were placed into four culture tubes each, which all contained nanoparticles. A quantitative approach and a posttest-only control group design were executed to compare the growth of the two strains by measuring the tubes' optical densities (ODs) every hour while in school. A smooth line curve showed that the strain without the *cusC* gene ceased growth after some time while the parent strain continued to grow, and inferential statistics showed that a manipulated factor caused the difference in the final OD values of the strains. Thus, the deletion of the *cusC* gene appeared to reduce bacterial resistance when exposed to nanoparticles. This information can be used in the development of future therapeutics.

Keywords: Cus efflux system, silver nanoparticles, *E. coli*, *cusC*, bacterial resistance, optical density

Introduction

In the field of microbiology, bacterial diseases have become increasingly prevalent. While antibiotic treatments have been administered to those who fall ill, various bacterial strains have evolved and developed resistance to combat the toxic nature of these antibiotics. Such is the case when resistant bacteria survive and pass their genes to their offspring while the non-resistant bacteria die off and do not reproduce. The overall bacterial resistance to antibiotics has been significant enough for Alanis (2005, p. 704) to claim that "the era where acute or chronic bacterial infections used to be treated with 'antibiotics-only' appears to have come to an abrupt end." Therefore, with the seemingly ineffective nature of antibiotic treatments, an alternative antimicrobial agent and its impacts on bacterial resistance should be studied extensively.

Literature Review

Bacterial Antibiotic Resistance

There has been ample research regarding methods of bacterial resistance to antibiotics, one of which are through efflux pumps, which are mechanisms incorporated within the bacterial cell membrane that are responsible for expelling antimicrobial, or harmful,

agents, such as antibiotics. Tenover (2006) called attention to the various resistance mechanisms and described that these efflux pumps work quickly to ensure the antimicrobial agents do not collect within the cell before traveling to an active site to trigger their effects. Additionally, Marquez (2005) pointed out that MDR, or multi-drug resistant, efflux pumps are the most responsible for overall bacterial resistance to antibiotics and other antimicrobial agents due to their ability to expunge a wide variety of therapeutics. Delmar et al. (2013) gave a description of one such efflux pump, the Cus efflux system, which opens up to initiate the discarding process in the presence of heavy metals. More specifically, the CusC pump in this system is responsible for discarding copper and silver ions. Therefore, manipulating the gene expression of these pumps could be related to bacterial resistance. To add on, Blair (2015) presented a clear evaluation of how well efflux pumps function and established that they correspond to increased bacterial resistance when the genes coding for the pumps were overexpressed. In other words, influencing the genes that code for efflux pump structure will affect their efficiency in discarding antimicrobial agents. Overall, resistance mechanisms can be controlled; however, research regarding how methods of resistance can be manipulated has been lacking.

Silver Nanoparticles

Silver nanoparticles (AgNPs) are small pieces of silver that have shown various cytotoxic effects on bacterial strains. Shrivastava et al. (2007, p. 8) identified and reported that "once inside the cell, nanoparticles would interfere with the bacterial growth signaling pathway by modulating tyrosine phosphorylation of putative peptide substrates critical for cell viability and division." By inhibiting cell division, nanoparticles jeopardize the survival of bacteria. Additionally, Morones et al. (2005, p. 2352) examined the effect of these nanoparticles, which "attach to the surface of the cell membrane and drastically disturb its proper function, like permeability and respiration". Disrupting the function of the cell membrane creates easy openings to allow other harmful substances to enter and affect bacterial cells. Salopek and Salopek-Sondi (2004) corroborated with Morones et al. as they gave cognizance to the charge of silver nanoparticles, which disrupts the structure of the cell membrane and eventually makes the cell vulnerable to additional antimicrobial agents. Moreover, nanoparticles can affect protein synthesis and inhibit the progression of the cell cycle. Liu et al. (2010) observed that nanoparticles arrested human cells in the S, or synthesis, phase of interphase in the cell cycle. DNA strands are replicated to prepare for cell division, or mitosis, during the S phase. Since silver nanoparticles have the effect of trapping bacteria in the S phase and not letting them progress, cell growth stagnates. Based on all of these studies, silver nanoparticles seem to be an effective alternative antimicrobial agent to antibiotics.

With the growing popularity of silver nanoparticles in the biology and medical fields, new methods of nanoparticle synthesis have emerged. Yakout and Mastoga (2015, p. 3538) performed and discussed one such method, green synthesis, in which they used nontoxic chemicals, such as starch, due to "simplicity, cost effectiveness, [and] compatibility for biomedical and pharmaceutical application". Research involving green synthesis of silver nanoparticles has been lacking since it is a fairly new concept, so an experiment focusing on their influence would establish a new understanding of them.

Research on Efflux Pumps

Current research on changes in efflux pump structure have shed light on mechanisms that affect the antimicrobial resistance of bacteria. Ni et al. (2016) presented evidence that colistin-resistant bacteria could be susceptible to colistin, an antibiotic, if efflux pump inhibitors (EPIs) were introduced to the bacterial strains, ultimately inhibiting bacterial growth within the medium. If efflux pump functions are disturbed by an outside force, bacterial resistance to certain antimicrobial agents will decrease, opening doors for studies on other inhibition methods. Furthermore, Anes et al. (2015) presented a clear evaluation of the EPIs when used in combination with antibiotics, which can undo the resistance that the bacteria have developed for ages. Anes et al. also claim that using EPIs with antibiotics would be an effective measure to treat bacterial diseases. Whereas inhibitors limit the function of efflux pumps, these efflux pumps can also be manipulated to increase their function. Sun et al. (2014, p. 263) focused on this overexpression, which "has

been frequently found in clinical isolates that have increased MICs to antibiotics." The MIC, or minimum inhibitory concentration, is the lowest concentration of antibiotics that will hinder bacterial growth. The increase in MIC means the bacteria are able to tolerate antimicrobial agents at higher concentrations. Thus, the efflux pump function has increased. All in all, changes to efflux pump functions have shown to influence bacterial resistance to antimicrobial agents, but testing alternative methods of efflux pump manipulation would provide a better picture of this cause and effect relationship.

Gap in Knowledge

With all the information surrounding silver nanoparticle cytotoxic effects and efflux pump function, the genes that code for these efflux pumps have not been touched upon often. Krishnamurthi et al. (2019) arrived at a conclusion in their study that population growth of *E. coli* staggered when grown in a medium containing silver ions. They hypothesized that mutations of the Cus efflux system conferring to overexpression causes increased bacterial resistance; however, they stressed that the Cus efflux system has not been directly mutated to test for *E. coli* resistance to silver nanoparticles. This gap in knowledge, along with the emerging use of green-synthesized silver nanoparticles, leads to the following research question: to what extent does the deletion of the *cusC* gene, which codes for the CusC efflux pump, influence *E. coli* resistance to green-synthesized silver nanoparticles within the growth medium? Based on previous research, overexpression of the *cusCFBA* operon, a section of bacterial DNA containing genes (including *cusC*) that code for Cus efflux pumps, should result in greater antimicrobial resistance and nanoparticle extrusion, and underexpression of this operon should produce the opposite effect. The purpose of this research is to explore another method of inhibiting efflux pumps and testing the effectiveness of this method by measuring bacterial resistance to silver nanoparticles. The execution of this study is significant in an age of medical research while also providing information useful in developing future therapeutics that can treat bacterial diseases.

Methods

Research Approach

This study attempted to investigate how the deletion of the *cusC* gene, which codes for the Cus efflux system, affects *E. coli* resistance to silver nanoparticles via a quantitative approach. A quantitative approach best fit this experiment because the main measuring instrument used, a nanodrop, allowed for the collection of numerical data that would objectively describe the growth of *E. coli* in their respective culture tubes and would provide an explanation of the effect of Cus efflux pump mutations on bacterial survival. Previous studies have used the quantitative approach to explain cell behavior when exposed to silver nanoparticles. Krishnamurthi et al. (2019) utilized time-lapse microscopy and discovered that cell lengths of *E. coli* bacteria oscillated when exposed to silver ions by measuring their lengths over a period of time. They used this numerical data to determine how silver ions affect bacterial length. Thus, a quantitative approach would allow for the formulation of reasonable conclusions via the collection of numerical data.

Methodologies

A posttest only control-group experimental design, in which the control group and experimental group are only tested after a treatment is imposed on the experimental group, best fit this research study. The experimental group of *E. coli* was mutated before testing resistance to nanoparticles as they had to be ordered beforehand. Therefore, growth in both groups was observed at even intervals only after the experimental group was treated. The control group had the parent, or original, *E. coli* strain in culture tubes, while the experimental group had the mutant *E. coli* strain without the *cusC* gene in culture tubes. This experimental design allowed for the reduction of confounding variables from influencing the study. Changes in bacterial population were measured by recording the optical density (OD) of the culture tubes in a nanodrop at even intervals, and a growth curve was created based on the data collected. Various studies have conducted a similar experimental design for similar experiments. Salopek and Salopek-Sondi (2004) tested how silver nanoparticles affect bacterial growth. *E. coli* bacte-

ria were separated into two groups: one group was placed in media containing silver nanoparticles, and the other group was placed in media lacking nanoparticles. Then, the bacterial population was measured in even intervals. As for the method of measurement, the optical density of the culture tubes was recorded in a spectrophotometer every 30 minutes to measure bacterial population, as this is the most reliable and efficient way to quantify bacterial population.

Subject Selection

E. coli is one of the most common bacteria used in scientific research in the biology and biotechnology fields. Su et al. (2010) worked with *E. coli* to determine how different proteins in the cell membrane work together when exposed to copper or silver ions. Additionally, Delmar et al. (2013) determined that the Cus efflux pump opens up when *E. coli* are exposed to ionic copper or silver. Thus, *E. coli* is popular in the biology community because of its ease of accessibility and use. Furthermore, the rapid reproduction rate of *E. coli* allowed for data collection to take place within the limited given time frame. Therefore, *E. coli* was used to determine the relationship between *cusC* deletion and resistance to silver nanoparticles.

Data Collection

Before the experiment ran, the parent strain (designation number: BW25113) and the mutant strain (designation number: JW0561-1), which were both obtained from the Coli Genetic Stock Center at Yale University, were cultured onto an agar plate for two days. After culturing, data was obtained via frequent testing on the eight culture tubes used. These tubes were divided into two groups of four each; each tube in the control group received a colony of the parent strain, while each tube in the experimental group received a colony of the mutant strain. All tubes contained 5 mL of an LB (lysogeny broth) medium and 30 µL of a silver nanoparticle solution, which were synthesized via a redox reaction between soluble starch and silver nitrate ($AgNO_3$). The green-synthesis procedure by Yakout and Mostaga (2015) was mirrored in which 10 mL of 1% soluble starch was mixed with 50 mL of 1 mM $AgNO_3$ on a hot plate for 3 hours until the solution turned a brownish-yellow color. After the bacteria were placed in their respective tubes, they were placed in an incubator at a temperature of 37°. Their OD values were recorded by extracting 2 µL of each culture onto a nanodrop, with the wavelength set at 600 nm. In this experiment, an OD value of 1 is equivalent to approximately 10^8 bacterial cells. The nanodrop was blanked, or reset to an OD of 0, with the original LB each time. The OD of all eight tubes were measured every hour while in school over the span of two days before they were discarded.

Analysis

After two weeks of testing and obtaining results, the data for all eight tubes were used to construct a smooth line graph in which the x-axis was time elapsed and the y-axis was the OD of the test tubes. The graphs between the two groups were then compared to each other and to an ideal bacteria growth curve (see Appendix A). Abnormalities in the graphs, such as premature stagnation or dips, were taken into account and recorded. In addition, inferential statistics was used to determine whether the difference between the mean bacterial populations of the two groups was a chance variation or a variation due to the deletion of the *cusC* gene in the mutant strain of *E. coli*.

Validity

Construct validity was established by only using the optical density of the culture tubes for its intended purpose of measuring bacteria population and then analyzing these results. In addition, internal validity was ensured by conducting this experiment in a classroom laboratory setting in order to reduce the effects of confounding variables. The protocol was performed in a laminar flow hood to prevent contamination of the culture tubes from bacteria in the air. Also, the culture tubes were placed in an incubator at a constant temperature of 37°, and there was minimal outside interference with the incubator. Therefore, differences between resistance to nanoparticles between the parent strain and the mutant strain could be confidently attributed to the deletion of the *cusC* gene. Furthermore, external validity was ensured by having a representative sample of *E. coli* and a culture environment, LB, that resembles natural *E. coli* habitats.

E. coli are identical in nature, so any sample obtained would represent the population of *E. coli* well, and the results obtained in this study can be attributed to the entire *E. coli* population; also, they were grown in a representative environment, so these results can be attributed to any *E. coli* culture, not just those grown in a laboratory.

Results

Overview

The purpose of this experimental study was to determine the extent to which the *cusC* deletion influences *E. coli* resistance to green-synthesized silver nanoparticles. Data were collected from February 10 to February 12, 2020, in which the ODs of each of the eight culture tubes incubated at 37°C were recorded on a nanodrop every hour while in school. Then, two tests were executed to determine the extent to which the deletion of the *cusC* gene influenced growth. The first test was to compare the line graphs of the parent strain group and the mutant strain group with each other and the ideal bacterial growth curve (see Appendix A). The second test used inferential statistics to confirm the extent to which the differences in the final OD values were due to chance or due to the deletion of the *cusC* gene.

Visual Representation of Growth

Each hour, 2 µl of each culture were placed on the nanodrop to measure the respective tube's OD value. These obtained values (see Appendix B) were used to construct a smooth line graph showing the change in OD over time in the tubes containing the parent strain and the tubes containing the mutant strain. Figure 1a shows the line curve for the change in OD over time for the parent strain tubes, and Figure 1b shows the line curve for the change in OD over time for the mutant strain tubes.

As shown in the two figures, silver nanoparticles may have influenced both the parent strain and the mutant strain. In Figure 1a, the parent strain seemed to have entered the exponential phase between 21 and 22 hours, but its growth stagnated at around 23 hours before dropping from 24 to 26 hours. Eventually, the strains grew again at a steady rate. Compared to the ideal bacterial growth curve, the parent strain seemed to have reached the stationary and death phases fairly quickly before recovering and re-entering the lag and exponential phases. In Figure 1b, the mutant strain seemed to have entered the exponential phase at around the same time as the parent strain, but its growth was more rapid. Its growth then declined rapidly between 23 and 24 hours before growth appeared to have ceased. Compared to the ideal bacterial growth curve, the mutant strain seemed to have also reached the stationary and death phases fairly quickly, but it did not seem to re-enter the exponential phase since growth stagnated altogether. This trend is somewhat similar to the study conducted by Salopek and Salopek-Sondi (2004) in which they let *E. coli* grow on agar plates in different concentrations of silver nanoparticles. In their case, growth of the bacteria was delayed when exposed to nanoparticles, similar to how growth stagnated in this experiment.

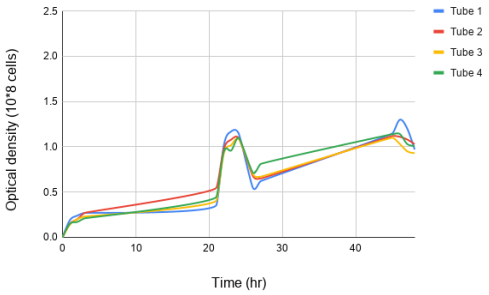

Figure 1a. Change in OD of parent strain tubes over time (left)

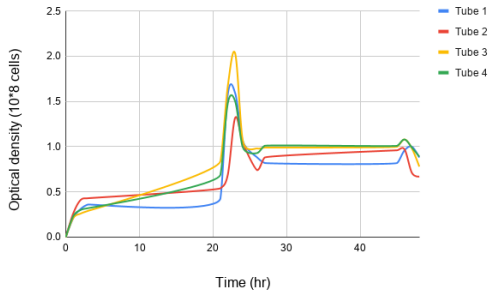

Figure 1b. Change in OD of mutant strain tubes over time (right)

Chance Variation Calculation

To determine whether the deletion of the *cusC* gene truly had an effect on bacterial growth, the OD values at 48 hours were taken. The sample mean of the OD values of the parent strain tubes, or $\bar{x}_1$, was 0.985, with a standard deviation of approximately 0.04435. The sample mean OD values of the mutant strain tubes, or $\bar{x}_2$, was 0.805, with a standard deviation of approximately 0.10279. Then, using inferential statistics, a null hypothesis was formed, which was that the difference between the means of the OD values of the two groups is zero, or $\mu_1 - \mu_2 = 0$, in which μ_1 is the true mean OD value of all parent strain tubes, and μ_2 is the true mean OD value of all mutant strain tubes. Since the original hypothesis stated that the mutant strain growth would stagnate, and the sample data showed that $\bar{x}_1 - \bar{x}_2 = 0.18 > 0$, the alternative hypothesis formed was $\mu_1 - \mu_2 > 0$. An alpha (α) significance level of 0.05 was used to make sure the null hypothesis was not prematurely rejected. After running a two sample t distribution test with approximately 4.08 degrees of freedom, the obtained P-value was 0.016. In context, this P-value means that assuming $\mu_1 - \mu_2 = 0$, there is only a 1.6% chance that a sample mean difference would be at least as extreme as 0.18. Therefore, the difference in means most likely did not occur due to chance.

Summary of Results

Overall, the results suggest a relationship between the deletion of the *cusC* gene and resistance to nanoparticles. First off, mutant strain growth seemed to stagnate after a large decline in growth while parent strain growth steadily increased under the same conditions. In addition, the difference in final OD values between the parent strain and the mutant strain was likely not a coincidence.

Discussion

Overview

To reiterate, the purpose of this study was to determine the extent to which the deletion of the *cusC* gene influences *E. coli* growth in medium containing green-synthesized silver nanoparticles, which attempted to fill a gap in research relating to how mutations of the *cusCFBA* operon affect bacterial growth when exposed to nanoparticles. This relationship was examined using a quantitative approach and more specifically a true experimental method with a post-test-only control group design. A parent strain with the *cusC* gene was separated into four culture tubes from a mutant strain without the *cusC* gene, which were also placed in four culture tubes. Both strains were incubated at 37°C, and their ODs were measured on a nanodrop every hour during the school day for 48 hours. After data collection, the obtained OD values were plotted on a smooth line graph to examine differences in growth, and inferential statistics was used to determine whether chance variation was a reasonable conclusion for the differences in data. The smooth line graphs show that growth of the two groups were similar in the beginning until the exponential growth and subsequent decline in growth of the mutant strain was sharper, and the parent strain resumed its growth after the decline, whereas growth ceased for the mutant strain. Inferential statistics showed that there is a low probability of obtaining two sample mean OD values in which their difference is as extreme as the calculated difference of 0.18.

Significance of Results

The results show that both strains grew in a similar nature for the first 21 hours. Growth was slow but progressing until the 22nd hour when growth exponentially increased for both strains. The mutant strain had a higher growth peak at 23 hours, but its growth ultimately dropped before stagnating. The parent strain grew at a steady rate after 26 hours after a small drop at 24 hours. Both curves had another small drop in growth after 46 hours. Afterwards, a two sample t distribution test was executed to determine how likely the difference in the mean OD values at 48 hours between both strains was as extreme as was observed. The difference in sample means was 0.18, which was only 1.6% probable assuming that the true difference in mean OD values is 0.

In terms of the graphs, there seems to be enough evidence to conclude that the deletion of the *cusC* gene influenced bacterial growth. First, both strains experienced the lag phase for about 21 hours. This

extended lag phase corresponds to the study conducted by Krishnamurthi et al. (2019) which concluded that the lag phase of *E. coli* was significantly elongated when exposed to nanoparticles. After 26 hours, growth of the parent strain increased, and growth of the mutant strain stopped. Additionally, the drop of growth was sharper for the mutant strain from 24 to 26 hours. All of these factors seem to indicate that the silver nanoparticles affected growth of the mutant strain more than the growth of the parent strain. Franke et al. (2003) gave cognizance to the fact that the CusC efflux pump is responsible for full resistance to heavy metals. Therefore, it would be logical that the deletion of the *cusC* gene, which codes for the CusC efflux pump, decreases bacterial resistance to silver nanoparticles. Overall, this result is significant in the field of biology and biotechnology because the above trends show that green-synthesized silver nanoparticles, which have not been explored in the field often, are effective in inhibiting *E. coli* growth. Since green synthesis is a simple and cheap method of obtaining silver nanoparticles, as it only requires soluble starch and silver nitrate, future medicine and pharmaceuticals that treat bacterial diseases could be made more cost efficient.

However, the larger peak growth for the mutant strain deviated from its expectation that it would not grow well in the presence of silver nanoparticles. This phenomenon could be explained by the mutant strain initially growing better in the presence of silver nanoparticles than the parent strain. Krishnamurthi et al. (2019, p. 8) commented that approximately 7% of bacterium were able to "win the battle" against nanoparticles and eventually reproduce, which may explain the initial large growth for the mutant strain before quickly dying off. Also, imperfections in the laboratory setting or equipment could have contributed to this high peak. Overall, this experiment should be conducted in a better setting with better equipment and timing to determine whether this higher peak is of any significance.

The results are also statistically significant because the obtained P-value, 0.016, is less than the α significance level of 0.05. Again, this P-value means that there is only a 1.6% probability of obtaining two samples of culture tubes in which their mean OD values have a difference at least as extreme as 0.18. Therefore, the null hypothesis is rejected because there is convincing evidence of a difference in the true mean OD values between the parent strain and the mutant strain. Thus, the extent of the difference in mean OD values between the parent strain and the mutant strain was probably caused by an outside factor. Since this experiment was carefully controlled in a sterile environment with little outside interference, and the independent variable was the presence of the *cusC* gene, this factor most likely contributed to this difference, which corresponds with previous studies; Delmar et al. (2013) asserted that the CusC efflux pump serves as the last step in heavy metal extrusion within *E. coli* and essentially opens the outer membrane channel. Therefore, it would be logical to conclude that when the CusC pump is ineffective via a deletion of its gene, silver nanoparticles cannot be extruded, and these nanoparticles can carry out their cytotoxic effects within bacteria. Overall, this result is significant because it rules out chance variation as a factor influencing the growth of the parent strain and the mutant strain.

However, the difference in final OD values appears to be small, which raises the question of the practical significance of this study. If this difference is as minute as 0.18, then chance variation may truly have had an effect instead of the deletion of the *cusC* gene. Thus, this study should be replicated to determine the extremity of the influence of the *cusC* gene on bacterial growth. Nevertheless, the data have statistical significance.

Limitations

Despite its straightforwardness, many obstacles were encountered concerning the developed procedure. First, the OD of the culture tubes could not be recorded between school days because the building was closed, which led to gaps in data collection. Thus, the line graphs were not continuous, and making comparisons between the line graphs became difficult because the OD of the culture tubes during these times were unknown. In addition, the cost of silver nanoparticles was well outside the budget for this experiment, so these nanoparticles were synthesized from scratch using common laboratory substances. The color change of the reaction was the only indication that nanoparticles were present, so it was un-

certain whether these nanoparticles were abundant enough for the experiment due to the lack of resources for a screening process.

Due to limited resources, there was no indicator that determined how both strains grew in the culture tubes. In fact, the experiment failed the first time it was executed. An inoculating loop was used to transfer the bacteria directly into the culture tubes from the disks they were delivered on. However, the strains did not grow in the culture tubes overnight. The next week, the disks were placed on agar plates, and the bacteria were allowed to grow. Then, using an inoculating loop, one colony was selected to place into each culture tube. This time, there was growth in all eight tubes overnight. In addition, gaps in data collection result in uncertainty over the true growth trends of the two strains because they could not be monitored every hour. All in all, the lack of resources extended the duration of the experiment, and the gaps in data collection led to unanswered questions about *E. coli* growth. The conclusions made in this study should be confirmed by future studies with better resources.

Future Studies

Future studies can focus on the specific pathways that green-synthesized silver nanoparticles take to inhibit cell growth, especially bacteria lacking the *cusC* gene. This study only determined the extent of the relationship between the deletion of the *cusC* gene and bacterial growth and predicted the nature of the bacterial strains while being grown. Hence, this study opens up many more questions about how the deletion affects growth when exposed to other metals and how deletions of genes that code for other efflux pumps affect growth. Ultimately, future studies can use this experiment as a jumpstart to answer more questions about *E. coli*, efflux pumps, and silver nanoparticles.

Conclusion

The original hypothesis of this study, which stated that mutations in the *cusCFBA* operon causing the underexpression of the CusC efflux pump should result in reduced antimicrobial resistance and nanoparticle extrusion, seems to be supported through the data collected. The mutant strain was not able to grow in the presence of silver nanoparticles after some time, which was significant when compared to the parent strain that continued to grow in the same environment. Additionally, inferential statistics showed that a factor outside of chance variation must have had an effect on the differences in growth between the two strains. Overall, the hypothesis along with the obtained data worked together to develop the following argument: the deletion of the *cusC* gene appears to inhibit the growth of *E. coli* in medium containing green-synthesized silver nanoparticles. With this assertion, this study attempted to address a gap in knowledge concerning how manipulating efflux pump function through a novel method influences bacterial resistance to an emerging antimicrobial agent. By manipulating efflux pump function through the deletion of a key gene that codes for an important efflux pump in *E. coli*, this study effectively fills this gap by visualizing and calculating the major differences caused by this manipulation.

The silver nanoparticles in this study were of utmost importance because of their method of synthesis. In a process that has not been replicated often, the silver nanoparticles were synthesized through the reaction of silver nitrate and soluble starch, both of which are easily accessible and fairly inexpensive. Thus, the observable effect of these green-synthesized silver nanoparticles on both the parent strain and mutant strain of *E. coli* opens more doors to investigate their true nature and severity so that future therapeutics are able to incorporate them and thus reduce the cost of production. Also, this study affirms that efflux pumps play a key role in bacterial resistance to antimicrobial agents, thus opening more doors to investigate practical methods to inhibit their function in the human body when people are infected. Ultimately, this study has taken a step towards answering how to combat the trend of increased bacterial resistance to antimicrobial agents.

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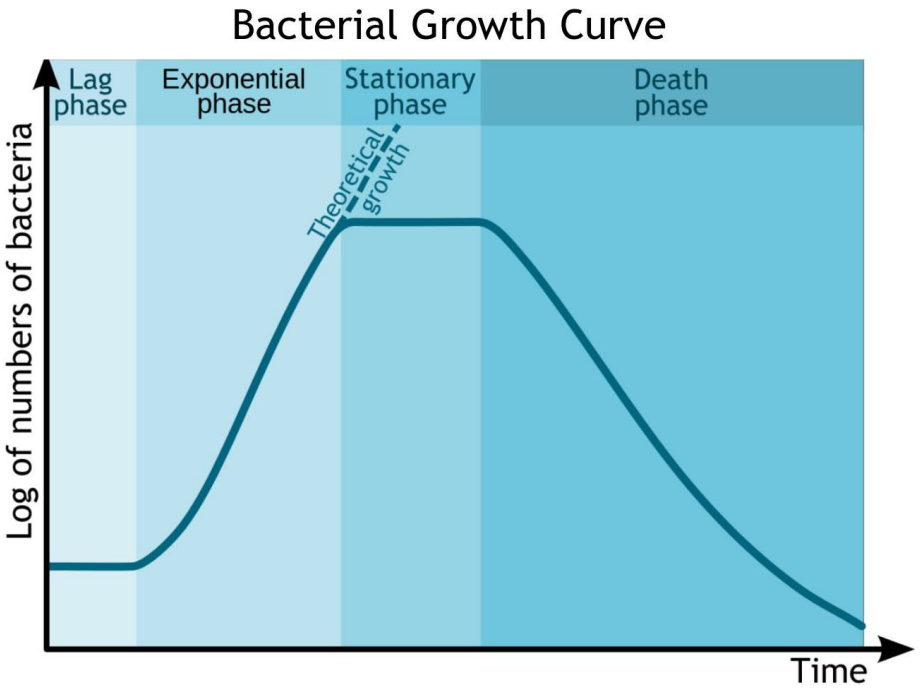
Appendix A

Ideal Bacterial Growth Curve

Figure A1. Growth curve showing the stages of bacterial growth (Komorniczak 2009)



Appendix B

Obtained OD Values

Table B1. Recorded optical density values for each culture tube

Time (hr)	Optical density (10^8 cells)							
	cusC+ (parent strain)				*cusC*- (mutant strain)			
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 1	Tube 2	Tube 3	Tube 4
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.19	0.15	0.14	0.15	0.21	0.26	0.21	0.23
2	0.24	0.20	0.20	0.17	0.31	0.41	0.26	0.30
3	0.27	0.27	0.23	0.21	0.36	0.43	0.29	0.32
21	0.36	0.55	0.41	0.45	0.43	0.54	0.84	0.69
22	1.02	0.98	0.93	0.95	1.58	0.69	1.67	1.48
23	1.17	1.08	1.02	0.96	1.59	1.32	2.03	1.50
24	1.15	1.10	1.09	1.10	1.07	1.05	1.08	1.00
26	0.54	0.67	0.69	0.71	0.88	0.74	0.98	0.93
27	0.62	0.65	0.67	0.81	0.82	0.88	0.99	1.01
45	1.15	1.12	1.10	1.14	0.82	0.96	1.00	1.01
46	1.30	1.11	1.03	1.14	0.96	0.97	1.08	1.08
47	1.20	1.08	0.95	1.03	1.00	0.71	0.98	0.99
48	0.97	1.03	0.93	1.01	0.88	0.67	0.78	0.89