Implications of Antibiotic and Bacteriophage Resistance in Environmentally Isolated *E. coli*

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Abstract

The increasing prevalence of antibiotic-resistant bacteria is an emerging problem for humans. Clinical misuse, overuse in agricultural and food settings, and limited numbers of new antibiotics have accelerated the proliferation of antibiotic-resistant bacteria. To confront this threat, scientists must develop new therapeutics that kill these antibiotic-resistant bacteria. In this study, we used *Escherichia coli* to analyze antibiotic and bacteriophage susceptibility. *E. coli* is a common, mostly benign, enteric, gram-negative bacteria. We isolated three *E. coli* strains from the Hans Groot Kill, a stream that runs through Union College's campus. We sought to assess various *E. coli* strains' antibiotic resistance, susceptibility to bacteriophages, and underlying resistance mechanisms. We compared the environmental *E. coli* isolates to known lab strains of *E. coli*. Using T4 bacteriophage, a well characterized viral pathogen that carries out a lytic cyle in *E. coli* bacteria, we conducted modified plaque assays with the three environmental isolates and lab strains "B" and "C." The environmental isolates were found to be highly resistant to T4 phage, while the lab strains were susceptible. We also conducted Kirby-Bauer antimicrobial disk susceptibility tests, using various gram-negative targeted drugs. We found the lab strains to be highly susceptible to all of the antibiotics, while the environmental isolates showed intermediate resistance to some of the antibiotics. Surprisingly, both environmental and lab strains were encapsulated. These patterns of resistance to phages and antimicrobial drugs suggest that environmental *E. coli* strains must have some mechanism for resistance that could be exploited as a novel means of treating drug-resistant infections.

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Introduction

Background

Since Alexander Fleming's accidental discovery of penicillin in 1928, there has been a rapid and explosive increase in the development of antimicrobial drugs (Aminov, 2010). Antimicrobial drugs comprise many chemicals that target a bacterial cell to kill it or arrest its growth. These new drugs have proved almost miraculous for many patients infected with previously fatal bacterial diseases.

The use of antimicrobial drugs has not been solely restricted to medical settings. Large scale industrial farming commonly uses large quantities of antimicrobial drugs in raising livestock (Manyi-Loh et al., 2018). Antimicrobial drugs are frequently overused in these large-scale farms, and eventually end up polluting the environment through animal waste (*ibid.*). This form of antimicrobial drug abuse is thought to lead to the natural selection for and the proliferation of resistant bacterial strains (*ibid.*). Since many of the bacteria that are present in livestock are pathogenic in humans, the emergence of resistant bacterial strains in livestock, and their increasing prevalence in the environment, have significant health ramifications for humans (*ibid.*).

In the twenty-first century, the proliferation of single and increasingly multi-drug resistant bacteria has challenged the healthcare system's reliance on antimicrobial drugs (Llor & Bjerrum, 2014). The erroneous prescription of antibiotics for viral infections and self-limiting bacterial infections, the failure to persist in taking a full course of antibiotics, and the excessive use of antimicrobial drugs in agriculture and aquaculture settings have led to the increase in number of antimicrobial drug-resistant bacteria (*ibid*.). The discovery and approval of new

antibiotic drugs have decreased by 90% over the last thirty years because of the time and cost-prohibitive nature of this process (Vera-Mansilla et al., 2022). These developments have reduced the effectiveness of antimicrobial drugs in treating infections.

Antimicrobial drug resistance can be mitigated, but not eliminated by responsible drug use. In many developing countries, antibiotics are available over the counter, and are thus prone to irresponsible use (Manyi-Loh et al., 2018). The diverse genome of bacteria means that every antimicrobial drug class ever developed has been met by resistance when tested on bacteria (Larsson & Flak, 2018). This indicates that, even with well-regulated antimicrobial drug schedules, these drugs have inherent limits, and alternate treatments must be explored in order to most effectively treat bacterial infections.

In 1917, Félix d'Hérelle developed a procedure to isolate particles that he named "bacteriophages" or simply "phages" from bacterial cultures. He was unaware of the exact nature of these particles, but their antimicrobial effect was apparent. Two years later, d'Hérelle successfully used phages to cure avian typhosis in a chicken. This was the first successful use of phage therapy (Summers, 1999). The principle behind phage therapy is simple. Like all living organisms, bacteria have viral parasites that selectively infect, replicate inside, and kill their hosts. Phage therapy uses viruses of bacteria, d'Hérelle's phages, to infect a bacterial infection in a human or animal host and thereby cure the disease.

As bacteria have become increasingly resistant to antimicrobial drugs, phage therapy is increasingly seen as a promising alternative treatment. Phages have been shown to infect and lyse clinically isolated bacteria strains, indicating the possibility for phage use in medical treatments of antibiotic-resistant bacterial strains (Sundar et al., 2009). Phages have high host specificity, meaning that a phage will not produce resistance in non-targeted bacterial strains,

unlike many broad-spectrum antibiotics (*ibid*.). While phage therapy is currently in medical use in several former Eastern Bloc countries: Poland, Russia, and the Republic of Georgia, it has only reached the clinical trial stage in the United States and other Western nations (Furfaro et al., 2018; Żaczek et al., 2020).

Escherichia coli and T4 Phage

One of the most studied bacterial species is *Escherichia coli*. *E. coli* is a Gram-negative, rod-shaped, facultative anaerobe that is commonly found in the gastrointestinal tract of humans and other mammals (Anderson et al., 2019). Its presence in water indicates fecal contamination (Sora et al., 2021). Some strains of *E. coli*, such as O157:H7, are extremely virulent, and can be spread rapidly through contaminated water (Rahal et al., 2012). *E. coli* is the main causative agent of urinary tract infections (UTI), the second most common bacterial infection. UTIs are so common that approximately 50% of women will have a UTI at some point in their lives. As in other bacteria species, recent decades have seen a marked increase in antibiotic resistance in *E. coli strains* (Vera-Mansilla et al., 2022). These factors make *E. coli* a relevant bacterium to study regarding antimicrobial drug and phage resistance.

*E. coli'*s role as an indicator organism for fecal contamination makes it an excellent candidate for an indicator of antimicrobial drug resistance in the environment (Anjum et al., 2021). Human and animal feces can enter streams and rivers through various processes including sewer overflow, agricultural and urban runoff, and direct animal defecation (Boehm & Sassoubre, 2014). Because *E. coli* is so common in humans and animals, it is frequently exposed to antimicrobial drugs, regardless of whether it is the intended target (Larsson & Flak, 2018). This exposure inevitably leads to the development of antimicrobial drug resistant bacteria strains

(*ibid*.). Study of antimicrobial resistance in *E. coli* strains isolated from the environment will thus provide insight into the presence of antimicrobial drug resistant *E. coli* in the environment, and may lead to the development of alternate treatments, such as phage therapy, *E. coli* infections in humans and animals.

To develop an assay that would explore the potential for phage therapy, we analyzed T4 phage's infectivity in *E. coli* strains isolated from the environment. T4 phage is a well-studied model lytic bacteriophage that is a pathogen of *E. coli*. Belonging to the family *Myoviridae*, T4 phage is physically and genetically similar to T2 phage and T6 phage and is thus classified as a T-even phage (Anderson et al., 2019). T4 phage's structure is composed of three main structures: a capsid, containing double-stranded DNA; a long cylindrical tail; and a series of six tail fibers (Yap et al., 2014; Anderson et al., 2019). T4 phage acts as a molecular syringe to infect a cell. T4 binds in a two-step process to E. coli's outer membrane; first, it reversibly binds to the outer membrane's lipopolysaccharide (LPS), then irreversibly binds to a phage receptor in the outer membrane; this secondary receptor varies by strain and can be outer membrane proteins, porins, flagella, or even the bacterium's capsule (Soundararajan et al., 2019; Gonzalez et al., 2014). After binding to the cell, a protein on T4's cell puncturing device bores a hole in the membrane and injects T4's DNA into the host cell (Yap et al., 2014). T4 phage propagates in a lytic cycle, wherein after adsorbing to the host cell and injecting its DNA, the host cell is hijacked to assemble new T4 phage virions and then lyse, expelling virion progeny that can initiate a new lytic cycle into the environment (Howard-Varona et al., 2017).

Our Study

In our study, we analyzed *E. coli* bacteria collected from the Hans Groot Kill, an urban stream in Schenectady, New York. We were drawn to this body of water on the basis of previous studies that had indicated that the Hans Groot Kill was highly contaminated with enteric bacteria. The source of this bacteria is unknown, but it is thought that Schenectady's aging sewer system contributes to this pollution. This means that humans are likely the ultimate source of the stream's contamination. After flowing through Schenectady, the Hans Groot Kill flows into the Mohawk River, thus contaminating the source of drinking water for some communities downriver (Willard-Bauer, 2021).

Our study aimed to analyze phage and antimicrobial resistance in *E. coli* using T4 phage and a panel of Gram-negative bacteria targeted antimicrobial drugs. The antimicrobial drug and phage susceptibility patterns of well-studied *E. coli* lab reference strains B and C were compared to those of *E. coli* environmental isolates from the Hans Groot Kill. Since these environmental isolates have a likely origin in the gut of humans or animals, this study has medical and industrial significance. *E. coli* environmental isolates come from a much harsher environment compared to lab strains. We reasoned that *E. coli* environmental isolates would be much more accustomed to competing with phages and other microbes for survival, and environmental isolates, through their human hosts, had been exposed to antimicrobial drugs and survived; thus, it was expected that environmental isolates would have strong resistance mechanisms to phages and/or antimicrobial drugs.

We analyzed T4 phage susceptibility in *E. coli* environmental isolates using a modified version of the small drop plaque assay described by Mazzocco et al. (Kropinski et al., 2009). This assay enabled us to serially dilute our T4 phage solution, and thereby assess bacterial

susceptibility to T4 phage at multiple concentrations. Antimicrobial drug susceptibility in our *E. coli* environmental isolates was assessed by performing a Kirby-Bauer susceptibility test, using a panel of Gram-negative specific drugs. Our aim was to assess whether environmentally isolated *E. coli* displayed resistance to antimicrobial drugs, and how this compared to their pattern of T4 phage susceptibility.

A well-studied resistance mechanism to both phages and antimicrobial drugs in bacteria is encapsulation. Encapsulated bacteria are surrounded by a polysaccharide layer that can act as an additional barrier to prevent phage adsorption and penetration of antimicrobial drugs into the cell. A study by Soundararajan et al. (2019) compared T4 susceptibility in a genetically modified capsule knockout *E. coli* strain Nissle 1917 to encapsulated *E. coli* strain Nissle 1917 and found that the unencapsulated bacteria were significantly more susceptible to T4 phage. We considered encapsulation as an easily testable mechanism that might explain differential phage and antimicrobial drug susceptibility in *E. coli* lab strains and environmental isolates.

Materials and Methods

Materials

Growth Media:

Growth media were purchased from VWR (Radnor, PA). *E. coli* lab strains and environmental isolates were maintained on BD DIFCO™ Tryptic Soy Agar (TSA) slants. In addition, BD DIFCO™ Tryptic Soy Agar (TSA) was supplemented with 1.5 mM calcium chloride and used as a growth medium in our small drop plaque assays. We used BD BBL™ Lactose Broth in our lactose fermentation tests, and BD BBL™ EMB Agar in our *E. coli* colony isolation tests. 1.5 mM Ca^{2+} BD BBL™ Tryptic Soy Broth (TSB) was used as a growth medium for our growth rate assays, small drop plaque assays, and Kirby-Bauer testing. We used BD BBL™ Mueller-Hinton Agar to perform our Kirby-Bauer testing.

Organisms and Phage

Laboratory strains of *Escherichia coli* B and C were obtained from Carolina Biological Supply (Burlington, NC) and grown on tryptic soy agar (TSA) slants supplemented with 1.5 mM calcium chloride. T4 bacteriophage suspension was obtained from Ward's Science (Rochester, NY) at a titer of 3×10^7 plaque forming units (PFU)/ml.

Antibiotic Testing

Kirby-Bauer testing was performed using six different types of antimicrobial drug-infused disks: tetracycline, kanamycin, sulfadimethoxine, chloramphenicol, nalidixic acid, and nitrofurantoin (Ward's Science, Rochester, NY).

Methods

Isolation of E. coli Environmental isolates

Through several tests conducted in a sequence, three *E. coli* strains were isolated from the Hans Groot Kill in Schenectady, NY. First, sterile glass bottles were filled with water samples collected from the Hans Groot Kill and this water was used to inoculate 5 ml lactose broth tubes each containing an inverted Durham tube. The lactose broth was incubated overnight at 37°C. A positive test (acid and gas production) indicated the presence of a lactose fermenting bacterium. EMB agar plates were subsequently quadrant-streaked with inocula from positive lactose broth tubes and incubated overnight at 37°C. Discrete colonies with a metallic sheen appearance were picked and transferred onto tryptic soy agar (TSA) slants and grown overnight at 37°C. These isolates were labeled "1," 2A," and "2B" (isolates 2A and 2B came from three separate colonies on the same EMB plate; strain 1 came from a second EMB plate). Gram stains were carried out on cultures of each isolate grown in TSB at 37°C for 24 hours. EnteroPluri-Tests (Becton, Dickinson & Company, Sparks, MD) were individually inoculated with inocula from each isolate to determine species identity.

Growth Rate determination

TSB tubes were inoculated with each *E. coli* strain and incubated for 24 hours at 30^{o}C. The following day, 50 µl of bacterial suspension was inoculated into sterile 5 ml TSB spectroscopy tubes and subsequently incubated in a shaking incubator at 37^{o}C. Absorbance readings for each culture tube was determined by spectrophotometry at a wavelength of 600 nm every 30 minutes for a maximum of 6 hours.

Kirby-Bauer Antimicrobial drug sensitivity

TSB tubes were inoculated with *E. coli* lab strains and environmental isolates, and incubated for 24 hours at 30℃. New sterile 5 ml TSB tubes were inoculated with 50 µl of bacterial suspension from the 24-hour cultures and incubated in a shaking incubator at 37°C for two hours to achieve log phase of growth. Using sterile cotton swabs, 3+1 swabbing of Mueller-Hinton agar plates was carried out with each *E. coli* culture. Two plates were swabbed for each *E. coli* strain. Three antimicrobial disks were placed on each plate, with a blank disk in the middle of each plate to serve as control. This assay was performed using six different antibiotics: tetracycline, kanamycin, sulfadimethoxine, chloramphenicol, nalidixic acid, and nitrofurantoin. The plates were inverted and incubated for 24 hours at 37°C. Diameters for zones of inhibition were measured for each antibiotic.

Small drop Plaque Assay Protocol

5 ml TSB broth tubes were inoculated with *E. coli* B and C strains as well as environmental isolates and incubated for 24 hours at 30℃. New sterile 5 ml TSB tubes were inoculated with 50 µl of bacterial suspension from the 24-hour cultures and incubated in a shaking incubator at 37°C for two hours to achieve log phase of growth, as described above.

Serial dilutions were prepared in a series of ten microfuge tubes for each bacterial strain tested. 180 µl sterile TSB was added to the first dilution tube, whereas 100 µl of sterile TSB was added to tubes 2-10. 20 µl of T4 phage suspension was pipetted into tube 1 and mixed to ensure even resuspension. A 100 µl volume was transferred from the first tube into the second tube, mixed and serial, two-fold dilutions were carried out in tubes 3-8. Tubes 9 and 10 did not contain phage and served as controls. A volume of 11 µl from each two-hour *E. coli* culture was added to tubes 1-10. The solutions in each tube were gently mixed as described and incubated for 20

minutes at 37℃ to allow for phage adsorption onto bacterial cells. Individual TSA plates were divided into 10 sections each, labeled 1-10 and performed in duplicate for each strain. A 20 µl volume from each tube was pipetted onto their corresponding section on each TSA plate. Plates were allowed to dry and subsequently incubated overnight for 24 hours at 37℃. Presence or absence of plaques was visualized the following day; plaques appearing as clearance zones in the bacterial lawn.

Anthony's Method Capsule Stain

5 ml volumes of TSB tubes were inoculated with *E. coli* and incubated for 24 hours at 37℃. Microscope slides were first inoculated with a loopful of skim milk broth, then with a loopful of *E. coli* overnight culture. Slides were allowed to air dry without heat fixation. Slides were initially stained with a 1% crystal violet solution for two minutes, and then decolorized with a 20% copper sulfate solution. These slides were allowed to air-dry and subsequently viewed under oil immersion.

Results

Isolation of E. coli environmental isolates

Water collected from the Hans Groot Kill was shown to contain lactose fermenting bacteria through the lactose broth test (Figs. 1, 2). By quadrant streaking EMB agar plates with inoculum from positive lactose broth tubes, we successfully isolated several discrete *E. coli* colonies that displayed a characteristic greyish-green metallic sheen (Fig. 3). Each discrete colony on the EMB agar plates contained the progeny of one *E. coli* cell from the lactose broth. This meant that each discrete *E. coli* colony was a single *E. coli* strain, as all the cells in a discrete colony are genetically identical. All of the strains were Gram negative rods, consistent with the morphology and cell wall characteristics of *E. coli* (Fig. 4)

We used an Enterotube^{TM} to further analyze the metabolic properties of three different colonies isolated from the EMB agar. Even though each colony was confirmed to belong to the same species, *E. coli*, differences in lysine and ornithine decarboxylase activity as well as ability to ferment the sugar dulcitol among the three strains indicated that the three colonies represented different strains of *E. coli* (Table 1). This implied that genotypic differences existed among the three environmental isolates of *E. coli*.

Growth Rate Assay

Our growth rate assay provided insight into the timing of *E. coli*'s different growth phases. In a closed vessel system containing a defined concentration of nutrients within a set volume, the bacterial growth curve follows characteristic stages which include a tooling up phase (lag), an exponential growth phase (log), a stationary phase during which cell division ceases and an exponential decline or death phase (Fig. 5). Our concern was identifying the delineation

between the lag phase of *E. coli* growth and exponential or log phase of *E. coli* growth. The growth rates of *E. coli* environmental isolates and lab strains mirrored the relationship between a culture's increasing absorbance at 600 nm over time. It is worth noting that measuring the culture's absorbance gives an estimate of the total number of cells and does not distinguish between living and dead cells (Anderson et al., 2019). However, this technique was deemed adequate for the purposes of our experiment to determine when *E. coli*'s log phase of growth began.

The strains began their growths in lag phase as a plateaued slope (Fig. 6). After approximately 1.5 hours, absorbance values from every *E. coli* strain began to increase rapidly. This was determined to be the start of the log phase of growth. This growth phase continued for approximately 4.5 hours, until the *E. coli* entered the stationary phase of growth (Fig. 6). Based on this assay, all further experiments that tested antimicrobial or phage susceptibility on the *E. coli* environmental isolates and lab strains used two-hour cultures of *E. coli* to ensure that the *E. coli* were in their log phase of growth. It is well documented that bacterial sensitivity to antimicrobial drugs is optimal during the log phase of growth (Eng et al., 1991; Bolger-Munro et al., 2013; Anderson et al., 2019).

In addition, we observed differences in growth rates among the *E. coli* environmental isolates and lab strains. Strain C, and to a lesser extent B, displayed less steeper slopes compared to the *E. coli* environmental isolates, particularly environmental isolate 1 (Fig. 6). The faster growth rate of some *E. coli* isolates lends credence to the idea that the environmental strains are more robust than the *E. coli* lab strains.

Kirby-Bauer Antibiotic Testing Assay

Lawns of the various strains of *E. coli* were incubated overnight with disks containing six Gram negative-specific antimicrobial drugs (tetracycline, kanamycin, sulfadimethoxine, chloramphenicol, nalidixic acid and nitrofurantoin) on Mueller-Hinton agar plates, after which the diameters of the zones of inhibition were measured around each disk. A zone of inhibition is a circular clearance area around an antimicrobial disk on Mueller-Hinton agar, where the drug either kills or inhibits bacterial growth. Several observations were apparent from our initial measurements. First, each *E. coli* environmental isolate and lab strain had some degree of sensitivity to the antibiotics that we chose (Table 2). Therefore, our premise, that a two-hour *E. coli* culture would be in its log phase of growth and therefore susceptible to antibiotics, was correct. Second, apart from all three *E. coli* environmental isolates having intermediate resistance to two of the antibiotics: tetracycline and nitrofurantoin, all *E. coli* environmental isolates and lab strains were comparably susceptible to the four remaining drugs (Fig. 7, Table 2). Finally, we did not see substantial numbers of revertant *E. coli* colonies growing within the zones of inhibition, indicating these antibiotics were highly effective on our *E. coli* environmental isolates and lab strains (Fig. 7).

Small drop Plaque Assay

When tested for their susceptibility to the bacteriophage T4 lytic cycle, we observed noteworthy differences between the three *E. coli* environmental isolates and the two lab strains. Our *E. coli* environmental isolates 1, 2A, and 2B demonstrated complete resistance to T4 phage, with no discernible difference in *E. coli* growth from the highest phage concentration to the lowest (Fig. 8). This was contrasted with the results from our *E. coli* lab strains B and C, which

showed high levels of susceptibility to T4 phage at the highest phage concentrations (Fig. 8). Interestingly, the small drop plaque assays with *E. coli* lab strains showed substantial numbers of revertant colonies, or colonies that grew in the areas where the phage infections had taken place (Fig. 8). This indicated that phage resistance was present in a small number of cells in the *E. coli* lab strain populations.

Capsule Stain

The bacterial capsule is composed of a polysaccharide matrix that surrounds the cell wall and serves as a protective barrier against biocides, including antimicrobial drugs and phages (Fig. 9). We carried out capsule stains on lab and environmental strains to determine whether the observed phage resistance could be explained by the selective expression of a capsule in the environmental strains. Our findings revealed that individual, rod-shaped cells from both lab strain C and isolate 2A were surrounded by a capsule (Fig. 10). The capsules appeared as clear areas, resembling halos, around *E. coli* cells of both strains. Capsule stains carried on the other two environmental isolates similarly revealed the presence of capsules surrounding individual cells (data not shown). These findings indicate that, not only were the *E. coli* environmental isolates encapsulated as we had predicted, but the *E. coli* lab strains were encapsulated as well (Fig. 10).

Discussion

Our experiments resulted in several important conclusions about antimicrobial and phage resistance in our *E. coli* environmental isolates and created some new questions for future studies to address. First, our *E. coli* environmental isolates did not possess widespread antimicrobial drug resistance as we had predicted. Second, our *E. coli* environmental isolates were totally resistant to T4 phage. Most significantly, we developed a series of procedures that enable rapid screening for phage and antimicrobial resistance in *E. coli* environmental isolates.

It is important to note that our experiment was a pilot study, and was thus limited in its scope and depth of analysis. One major limitation of our study was the small number of environmental isolates of *E. coli*. Even though many studies have shown that bacterial strains isolated from the environment frequently possess and express antimicrobial resistance genes, our three *E. coli* environmental isolates' phage and antimicrobial drug resistance patterns simply may not reflect those of the thousands of *E. coli* strains that may be present in the Hans Groot Kill or other environmental sources (Colomer-Lluch et al., 2014; Larsson & Flach, 2021; Haberecht et al., 2019). To better analyze the drug and phage resistance patterns in the Hans Groot Kill, further research is required. A redesign of this study to allow for a high throughput analysis of many *E. coli* environmental isolates may provide greater insight into the antimicrobial and phage resistance characteristics of the typical *E. coli* strain in the Hans Groot Kill.

A second limitation is that our study only analyzed *E. coli* from one environmental source. The Hans Groot Kill's *E. coli* may not represent the *E. coli* of a rural stream, or even another urban stream. Whether a stream is in a rural or urban setting has been shown to have

significant effects on the stream's chemistry, pollution levels, and microbiome (Hosen et al., 2017). To make well supported conclusions and analyze the threat of antimicrobial drug and phage resistance in bacteria, from a study such as ours, it would be necessary to analyze *E. coli* from more streams, in areas with different levels of human development. In short, our study's effectiveness could be markedly optimized if its scope were larger.

Environmental phage isolation is another important area for further research. Our small drop plaque assay proved to be an effective means for visualization of phage infectivity in *E. coli* that was significantly less time consuming compared to the more common double agar layer method of plaque visualization described by Kropinski et al. (2009). Previous research has shown that phages isolated in hospital settings from patients infected with multi drug resistant *E. coli* are able to effectively eradicate clinically isolated *E. coli* strains in vivo and in vitro (Rahmani et al., 2015). Even in partially antimicrobial drug-resistant bacterial strains, environmentally isolated phages can be used synergistically with antimicrobial drugs to control a phage infection (Valério et al., 2017). This illustrates the importance of phages in effectively treating drug resistant bacterial infections.

Growth Rate

Our growth rate assay was successful in delineating the point when our *E. coli* environmental isolates and lab strains entered their log phase of growth. In all experiments where phage and antimicrobial susceptibility was tested, we needed to use metabolically active, actively dividing *E. coli*, so knowing the start of the log phase was essential to this study's success. One important observation was that the *E. coli* lab strains and environmental isolates were all observed to enter log phase at about the same time, at 1.5 hours of incubation. Since this time is common to our *E. coli* lab strains and environmental isolates, it is reasonable to infer that growth

rate is conserved across most *E. coli* strains; thus this 1.5-hour incubation time frame can be used in future studies for any environmentally isolated *E. coli* strains.

The difference in growth rates between *E. coli* environmental isolates and lab strains is not easily explained from a fitness perspective. We saw that the *E. coli* environmental isolates generally had faster growth rates and greater bacteriophage resistance compared to lab strains. Growth rate is a measure of bacterial fitness; a bacterium with a higher growth rate, all else being equal, is more fit than a bacterium with a lower growth rate (Pope et al., 2010). Previous research has indicated that when a bacterium acquires phage resistance, its growth rate actually decreases (Avrani & Lindell, 2015). Therefore, it is interesting to observe that our *E. coli* environmental isolates have acquired phage resistance and yet, display a higher growth rate than our *E. coli* lab strains.

Kirby-Bauer Antibiotic Testing

We thought that since the *E. coli* environmental isolates likely originated from human feces, they would have been exposed to and acquired resistance to several antimicrobial drugs. It is intriguing to observe that the *E. coli* environmental isolates were highly susceptible to four out of the six antibiotics that were tested. Indeed, tetracycline and nitrofurantoin showed only intermediate resistance with the *E. coli* environmental isolates, not full resistance. This indicates that conventional antibiotics would still be sufficient to treat most infections with *E. coli* strains (Anderson et al., 2019).

In recent years, previous studies have shown an increase in drug resistant *E. coli* in both clinical and community environments (Ibrahim et al., 2012; Ansari et al., 2015). This increase has posed tremendous threat to public health because of the ubiquity of *E. coli*; for example, pathogenic *E. coli* causes 80% of urinary tract infections (Croxall et al., 2011). The emergence of

intermediate resistance towards tetracycline and nitrofurantoin seen in our *E. coli* environmental isolates is a troubling indication that these *E. coli* strains may further develop intermediate and/or complete resistance to other antimicrobial drugs. Such developments would be of grave concern to physicians and other medical professionals seeking to treat *E. coli* infections with common drugs.

Small Drop Plaque Assay

All three environmental isolates of *E. coli* displayed complete resistance to T4 phage. These findings raise several interesting implications. Since our control *E. coli* lab strains B and C were highly susceptible to T4 phage, these observations demonstrate that the T4 phage preparation was infectious as expected. This in turn strongly suggests that the three *E. coli* environmental isolates must have acquired a resistance mechanism that was absent in the lab strains. T4 is an extremely common phage in the environment (Desplats & Krisch, 2003). Therefore, it is unsurprising that the *E. coli* environmental isolates expressed high levels of resistance to T4 phage. Fortunately, there are many different bacteriophage species and strains in the environment (Rohwer, 2003). It is likely that there is another type of phage that could successfully infect our *E. coli* environmental isolates. In addition, there may be temperate phages that have already infected our environmental isolates and exist as prophages in the *E. coli* chromosome (Cieślik et al., 2021; Kasatiya, 1970). These phages would still be capable of being induced into a full lytic cycle under certain conditions (i.e.: high nutrient levels or following genotoxic damage) (Anderson et al., 2019).

Studies have shown that prophages are difficult to identify because they are so well integrated into the bacterial genome (Tang et al., 2021). One method of prophage identification

uses genome sequencing and comparison to known phage genomes. The tremendous genetic diversity of bacteriophages and insufficient sequencing data for cross referencing means that many prophages are missed (*ibid.*). To solve this problem, Tang et al., (2021) developed "Prophage Tracer," an algorithm that uses sequencing data to identify prophages in bacterial genomes through overlapping split-read alignment in the genome. These recent advances in prophage identification may fuel future studies in rapidly identifying and inducing lysis in bacteria infected with temperate phages, expanding our ability to use these phages in phage therapy.

This study was effective in developing and optimizing the use of a small drop plaque assay to analyze phage susceptibility in *E. coli*. This assay can be repeated in the future with lytic phages purified from the environment from which *E. coli* strains are isolated, or with environmentally sourced temperate phages induced into a lytic cycle. Environmental phages will likely have coevolved with the *E. coli* environmental isolates. Consequently, environmental phages will likely be more successful, compared to T4 phage, in inducing lysis in *E. coli* strains isolated from environmental sources.

Phage and Antimicrobial Resistance Mechanisms

Encapsulation was a simple and easily testable hypothetical resistance mechanism for us to analyze in this study. A bacterium's capsule acts as a barrier that prevents phage and antibiotic adsorption or entrance into the cell (Soundararajan et al., 2019). Our results clearly demonstrated that *E. coli* environmental isolates and lab strains were both encapsulated. These findings strongly suggest that encapsulation was not a likely phage or antimicrobial resistance mechanism within our *E. coli* environmental isolates.

Phage and antimicrobial resistance can be explained by a number of other subcellular mechanisms in *E. coli*. A study by Soundararajan et al. (2019), proposed five mechanisms for phage resistance: self-protection, genome modification, CRISPR-Cas9, a restriction modification system, and phage inactivation. Encapsulation is a form of self-protection, but any sort of barrier around a cell, or a phage receptor modification could also be considered self-protection. Genome modification describes mechanisms such as superinfection exclusion, wherein an established virus prevents a secondary infection by the same or a closely related virus (Folimonova et al., 2012). CRISPR-Cas9 is a mechanism by which a cell genetically "remembers" a previous phage infection and can thereby target and cleave the intruding phage genome before it can infect the cell. A restriction modification system is a cellular defense mechanism whereby a cell produces restriction enzymes that target foreign DNA sequences for degradation. Phage inactivation can take many forms; one possible mechanism occurs when a phage is inactivated by binding to a vesicle or outer membrane structure (Soundararajan et al., 2019).

Clearly, bacteria may acquire phage and antimicrobial resistance through many different mechanisms. Future research to determine specific subcellular phage and antimicrobial resistance mechanisms may require knockout gene experiments, genome sequencing, or other genetic analysis (McCloskey et al., 2018). Restriction Enzymes can be identified through DNA cleavage experiments and methylation site identification (Ryu & Rowsell, 2008). Lipopolysaccharide (LPS) composition from resistant bacteria and membrane protein mutations can be identified through SDS page analysis (Filippov et al., 2011). CRISPR is a fairly recently discovered defense mechanism in bacteria, with considerable ongoing research. One promising means of determining if CRISPR is active in a bacterium is through the presence of Cas proteins.

Cas proteins can be identified by comparing a protein's amino acid sequence to Cas protein sequences that have been sequenced from other bacteria (Makarova & Koonin, 2015).

Future Research

Our study demonstrated the need for future research on antimicrobial drugs and phage resistance in *E. coli*. Future studies should focus on a high throughput analysis of phage and antimicrobial drug resistance in many *E. coli* environmental isolates from different environmental sources. This, combined with an analysis and identification of resistance mechanisms in *E. coli*, will enable researchers to make well-founded conclusions about *E. coli* phage and antibiotic resistance, and its medical and industrial implications.

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Appendix

Figures & Tables

Figure 1. The Hans Groot Kill. This urban stream is highly contaminated with enteric bacteria. Shown are our two water sampling sites from which the *E. coli* environmental isolates were obtained.

Figure 2. Lactose broth fermentation test. Left panel, sterile lactose broth tubes containing Durham tubes. Right panel, in the presence of a lactose fermenting organism, acid is produced and the pH indicator phenol red turns yellow. Gas is also produced, visible as a bubble in the inverted Durham tube.

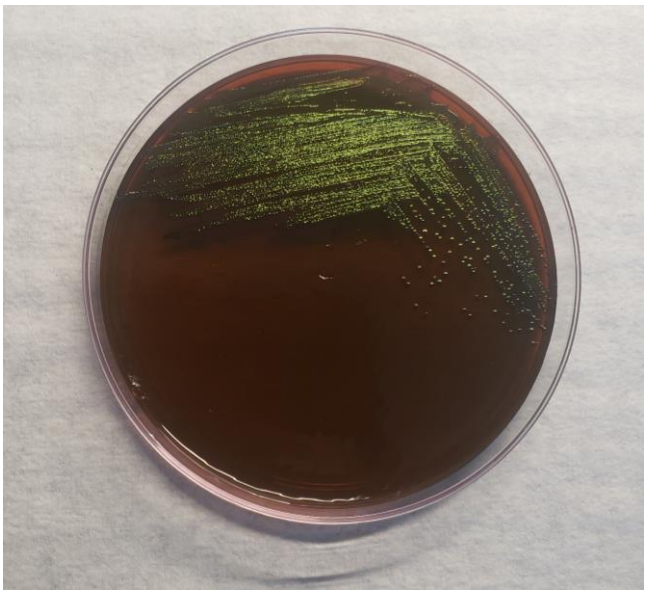


Figure 3. Quadrant streak of *E. coli* grown on Eosin Methylene Blue (EMB) agar. EMB agar is selective for gram-negative enteric bacteria and differential for lactose fermenting bacteria. *E. coli* colonies produce a greyish-green metallic sheen on EMB agar.

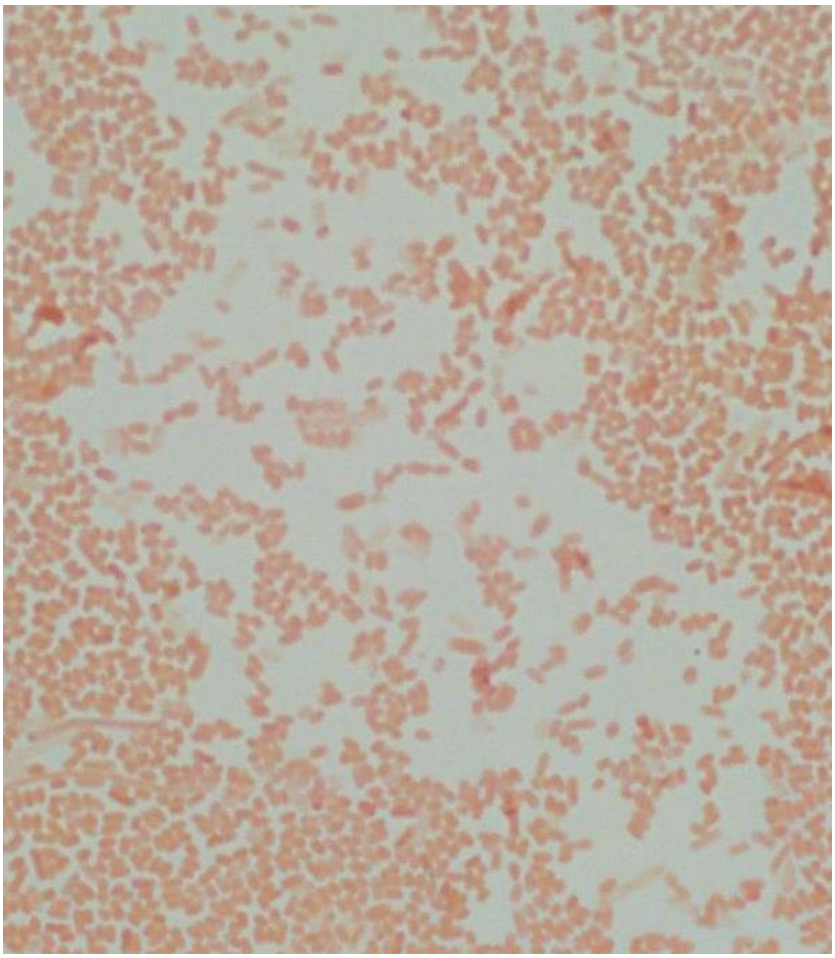


Figure 4. Gram stain of *E. coli* strain C. Pink rods can be observed in this smear, typical of a Gram-negative cell wall.

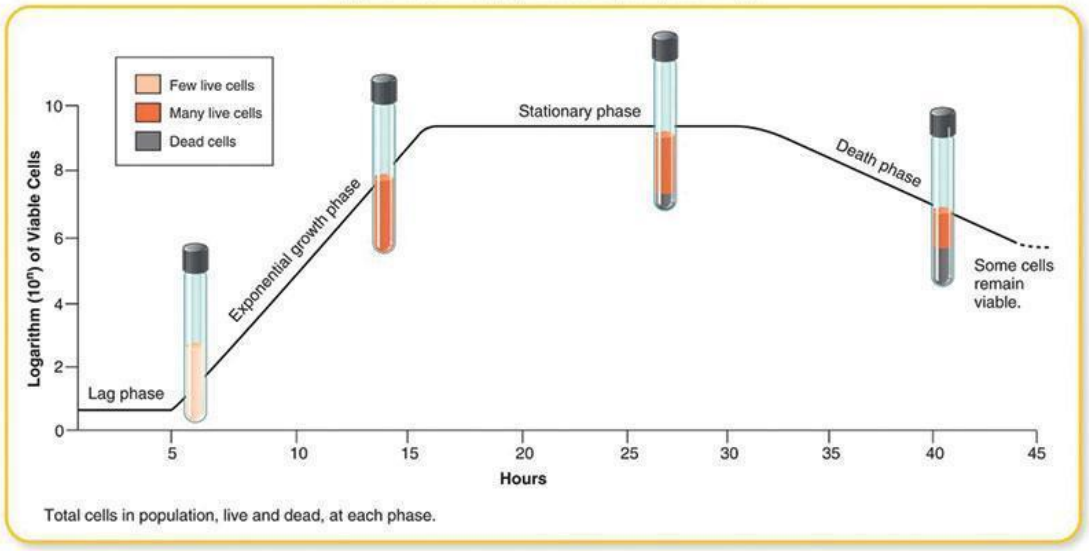


Figure 5. The bacterial growth curve in a closed system depicting lag, log (exponential growth), stationary and decline (death) phases.

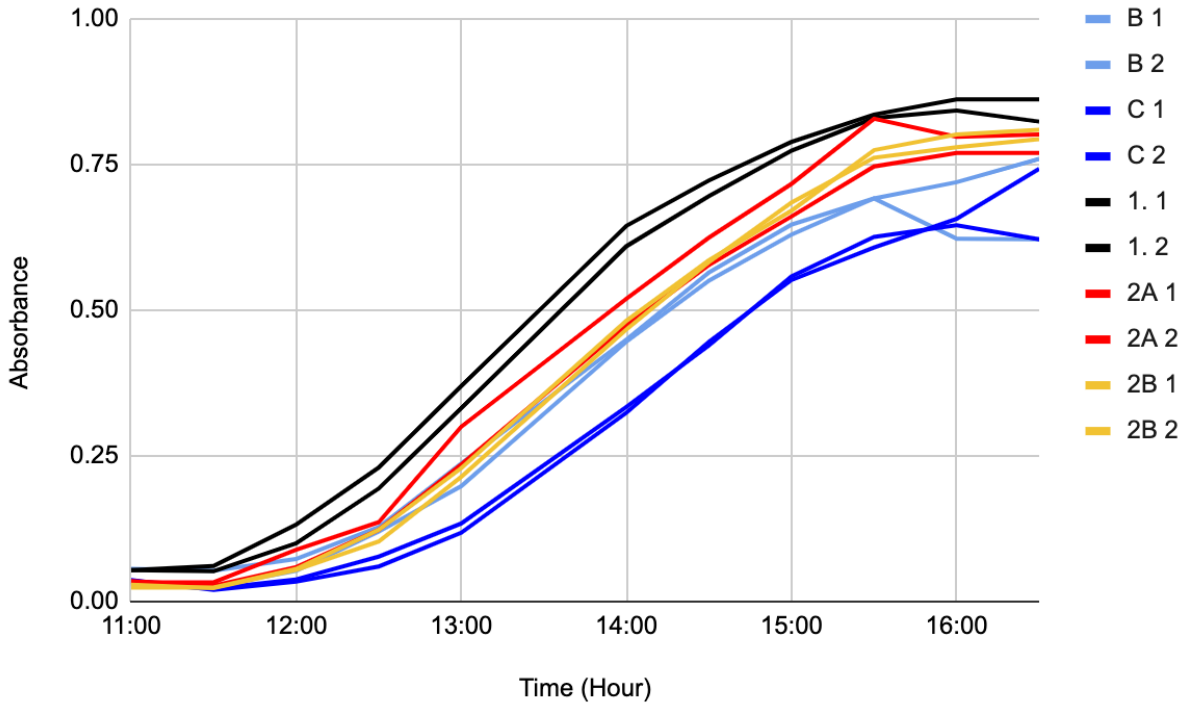


Figure 6. Growth rates of *E. coli* lab strains and environmental isolates. Each *E. coli* strain was grown in 5 ml 1.5 Ca^{2+} TSB in a 37℃-shaker incubator. Absorbance was measured by spectrophotometry at a wavelength of 600 nm every 30 minutes. Log phase begins at 1.5 hours of incubation.

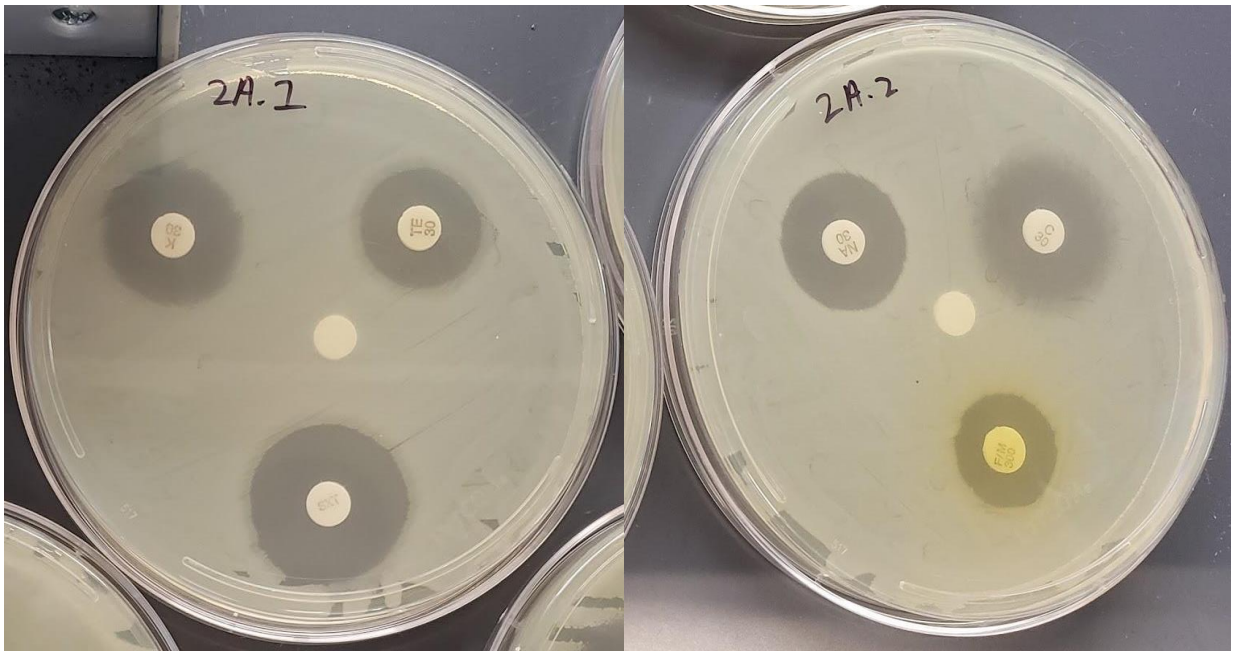


Figure 7. Kirby Bauer antimicrobial drug sensitivity profile of *E. coli* strain “2A”. A bacterial lawn was seeded on Mueller-Hinton agar plates, tested against six different antimicrobial drugs: Tetracycline, Kanamycin, Sulfadimethoxine, Chloramphenicol, Nalidixic Acid, and Nitrofurantoin; and incubated overnight at 37ºC. Each plate contains three antibiotic disks and a central control disk. Note the clear zones of inhibition around each antibiotic disk. These plates are representative of the methods used for all *E. coli* strains.

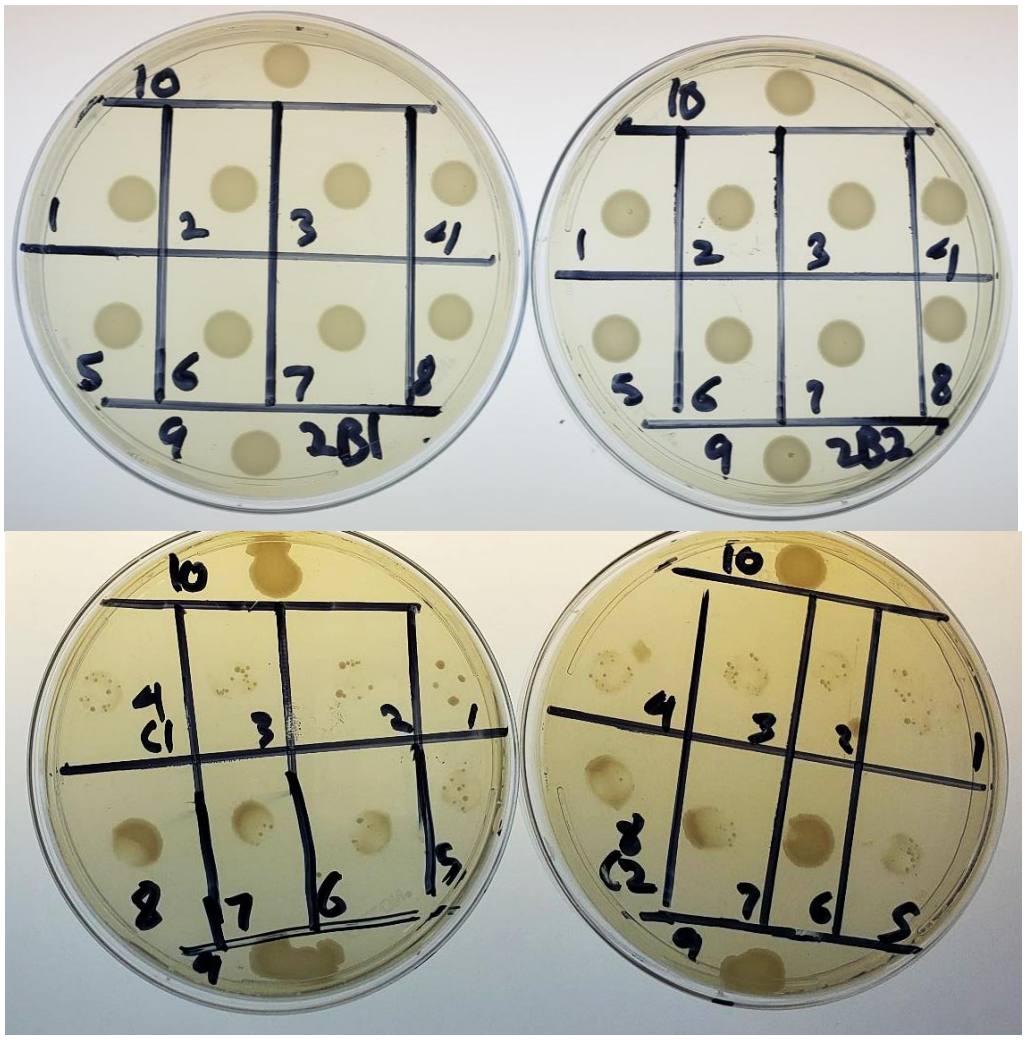


Figure 8. Environmental isolates are resistant to T4 lytic phage. A modified small drop plaque assay was carried out to determine phage susceptibility of *E. coli* laboratory strains and environmental isolates, and is shown in duplicate. Sections 1-8 are serial T4 phage dilutions mixed with *E. coli*; the sections marked "1" have the highest phage concentration, $1.5*10^{4}$ pfu/µl "8" have the lowest phage concentration, $1.5*10^{-4}$ pfu/µl and "9" and "10" have no phage. Top panel: *E. coli* strain 2A, depicted as representative of our environmental isolates, exhibited complete resistance to T4 phage as noted by the absence of plaques. Bottom panel: with *E. coli* lab strain C, plaques are visible at the highest phage concentrations, which demonstrates high susceptibility to T4 phage lytic cycle.

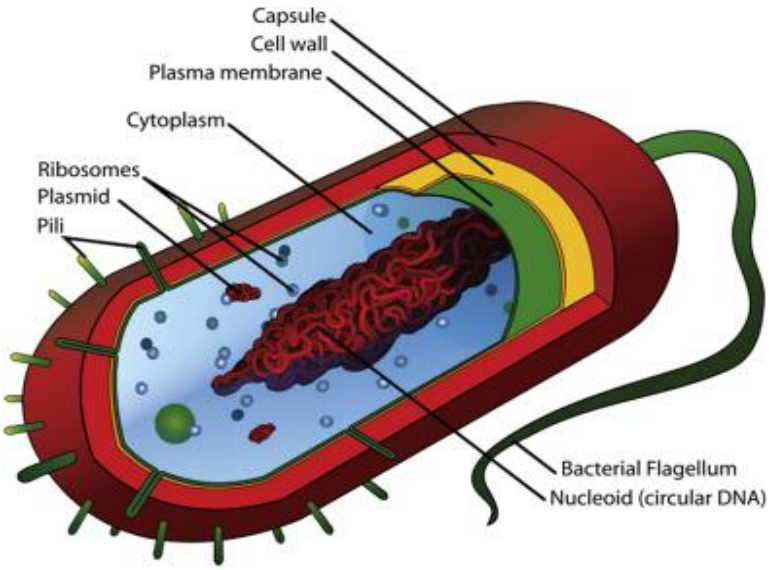
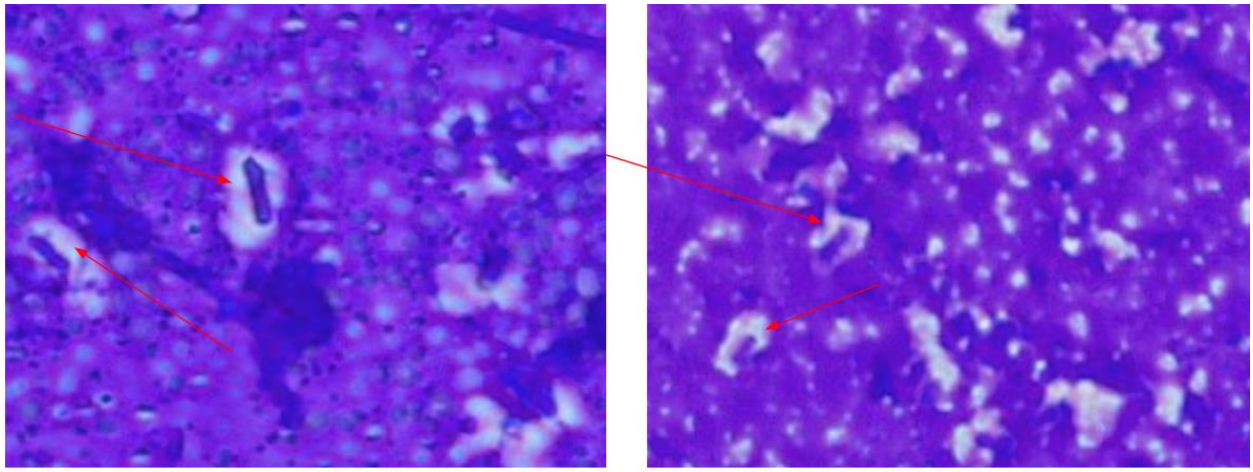


Figure 9. Diagram depicting an encapsulated bacterium. The bacterial capsule is external to the cell wall and consists of a polysaccharide matrix (Nazir et al., 2019).



C **2A**

Figure 10. Capsule stains of representative *E. coli* strains. Bacterial smears were stained according to Anthony's method. Left panel, *E. coli* strain C. Right panel, *E. coli* strain 2A. The clear halo surrounding the rod-shaped cells indicates the presence of a capsule (red arrows in both panels). Both environmental isolates of *E. coli* and lab strains were found to be encapsulated.

E. coli Environmental Isolates

	1	2A	2B
Glucose	+	+	+
Gas	+	+	+
Lysine decarboxylase	-	+	+
Ornithine decarboxylase	-	+	+
H_2S/ Indole	-/-	-/-	-/-
Adonitol	-	-	-
Sorbitol	+	+	+
VP	-	-	-
Dulcitol	-	+	+
Urea	-	-	-
Citrate	-	-	-
Lactose	+	+	+
Arabinose	+	+	+

Table 1. Enterotube identification for the three environmental isolates of *E. coli*. "+" indicates a positive result for a test while "-" indicates a negative result. Variation in lysine and ornithine decarboxylase activity as well as dulcitol fermentation was observed among the isolates.

Antimicrobial Drug	Target	B	C	1	2A	2B
Tetracycline	Ribosomes	S	S	I	I	I
Kanamycin	Ribosomes	S	S	S	S	S
Sulfadimethoxine	Folate synthesis	S	S	S	S	S
Chloramphenicol	Ribosomes	S	S	S	S	S
Nalidixic Acid	DNA replication	S	S	S	S	S
Nitrofurantoin	rRNA, DNA	S	S	I	I	I

Table 2. Kirby-Bauer antimicrobial drug sensitivity assay in laboratory strains and environmental isolates of *E. coli*. “I” indicates intermediate resistance whereas “S” indicates susceptibility to the antimicrobial drug. Except for intermediate resistance to the drugs Tetracycline and Nitrofurantoin observed in the environmental isolates, all strains showed high susceptibility to the other antimicrobial drugs. No strain exhibited complete resistance to any of the drugs tested.