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Article

Investigating facilitated biofilm formation in *Escherichia coli* exposed to sublethal levels of ampicillin

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SUMMARY

Communities of bacteria known as biofilms occupy surfaces to provide protection against environmental threats, including antibiotics. Biofilms formed by pathogenic bacteria *in vivo*, like on surfaces of medical implants, may cause serious infections. The reduced antibiotic penetration due to the presence of an extracellular polymeric substance matrix makes biofilm infections particularly difficult to treat. To investigate biofilm formation in the context of antibiotic treatments, we studied the effects of extended sublethal exposure to the antibiotic ampicillin on *Escherichia coli* biofilm formation and ampicillin resistance. We hypothesized that low ampicillin concentrations would facilitate biofilm formation by imposing selective pressure, while higher concentrations would eliminate biofilms. We exposed *E. coli* bacteria grown in LB medium to varying ampicillin concentrations throughout their growth. We quantified bacteria for biofilm formation using an optical density crystal violet assay. Acquired antibiotic resistance was measured after 24 hours of growth. Bacteria grown in low concentrations of ampicillin demonstrated increased biofilm formation over time, in comparison to bacteria selected under no ampicillin or high ampicillin. Measurements confirmed that biofilm-selected bacteria generally demonstrated higher antibiotic resistance than planktonic (free-floating) bacteria. Antibiotic exposure favors the more resistant biofilm bacteria, promoting instead of eradicating biofilm formation over time. The selection for biofilm-forming *E. coli* in ampicillin-present cultures *in vitro* provides crucial insight into the therapeutic efficacy of antibiotic treatments.

INTRODUCTION

Biofilms are communities of bacteria that form a structure to defend against external threats, such as antibiotics and host immune cells (1). Biofilm-associated cells display a different phenotype than planktonic, or free-floating cells; biofilms form an extracellular polymeric substance (EPS) matrix and regulate certain genes to allow for inter-cell communication, known as quorum sensing (1). Quorum sensing occurs via the secretion of autoinducer compounds (AI), which are then received by adjacent cells (1).

Biofilms defend bacteria from antibiotics, which are bactericidal substances commonly used to treat infections. Antibiotics work by disrupting vital cellular processes such as the function of the cell wall and/or the production of nucleic acids (DNA or RNA) and proteins, thus killing the bacteria or stopping cell division (2). Ampicillin is a broad-spectrum antibiotic due to its ability to work on multiple types of bacteria (3). Specific structures in ampicillin molecules called beta-lactam rings covalently bind to peptidoglycan synthesis proteins, inhibiting cell wall synthesis and thus killing the bacteria (4, 5).

However, an increasing concern accompanying the prevalent use of antibiotics is the development of antibiotic resistance. Resistance usually develops under evolutionary pressure, when random mutations contributing to an increased antibiotic tolerance are favored upon exposure to antibiotics and passed on to subsequent generations. This is a crucial concern in the medical field because many medical devices such as catheters and implants provide optimal surfaces for bacteria to attach and embed in an EPS matrix, forming highly resistant biofilm communities that put patients at risk for chronic infections (6). Failure to target biofilm-forming, pathogenic bacteria using standard antimicrobial techniques could lead to persistent infections by highly resistant or multi-drug resistant bacteria (6).

Bacteria in biofilm communities obtain antibiotic resistance mechanisms distinct from planktonic cells, which rely mostly on efflux pumps, drug-degrading enzymes, and target site mutations (6). The EPS matrix acts as a physical barrier that reduces the rate at which antibiotic molecules enter the cell (6). The lack of an EPS matrix barrier makes planktonic bacteria more susceptible to antibiotics due to a faster penetration rate of the antibiotic compounds (6). Thus, when low concentrations of ampicillin are present in the medium, a greater percentage of biofilm-associated bacteria survive and reproduce compared to planktonic bacteria. Previous studies suggest that the multicellularity of the biofilm structure allows individual bacteria within the biofilm to signal via quorum sensing, collectively up- or down-regulating genes related to surface attachment and pathways differentiation (6, 7, 8). This multicellular communication process is responsible for the increased antibiotic resistance in biofilm bacteria since deeper layers of the biofilm are not directly exposed to antibiotic stress.

While the vast majority of *Escherichia coli* strains are beneficial, some strains are responsible for intestinal infections and urinary tract infections (UTI) (7). Previous studies have concluded that uropathogenic *E. coli* has become extremely resistant to multiple antibiotics (6, 7, 8). In a previous study, 81% of uropathogenic *E. coli* isolates extracted from human samples were found to be multi-drug resistant (7). Additionally, *E. coli* isolates displayed high resistance to ampicillin, the most commonly prescribed antibiotic for UTIs (7, 8).

Despite the extensive research conducted on antibiotic

resistance, these studies focused mainly on biofilm formation as an antibiotic resistance mechanism itself, instead of how certain concentrations of antibiotics may exacerbate biofilm formation — an area of study crucial for evaluating antibiotics for therapeutic use on persistent biofilm infections. In this study, we aimed to examine the effect of prolonged exposure to sub-minimum inhibitory concentrations of ampicillin, or concentrations lower than the lethal threshold, on *E. coli* biofilm formation and antibiotic resistance. Since sublethal concentrations of ampicillin impose selective pressure favoring biofilm-forming bacteria, we hypothesized that low ampicillin concentrations would facilitate the evolution of biofilms, whereas near-lethal concentrations would have an adverse effect. We found that prolonged antibiotic use promotes biofilm formation, suggesting not only that biofilm cells are inherently more antibiotic-resistant, but also that biofilm communities strengthen in response to ampicillin over time. This study could provide a biofilm-related context on the extent of antibiotic resistance of *E. coli* in pharmaceutical manufacturing.

RESULTS

Biofilm Formation in Standard LB Environment

To determine the effect of different ampicillin concentrations on biofilm formation, we compared biofilm-forming capacities of bacteria grown in different ampicillin concentrations after four days of daily bead transfers by a crystal violet assay. Bead transfers imposed selective pressure on the bacteria since only the bacteria that produced biofilms would adhere strongly to its surface and could be separated from the non-biofilm-producing bacteria. OD_{595} measurements were taken to quantify biofilm formation, with higher absorbances indicating more biofilm-associated cells. *E. coli* bacteria selected for optimal biofilm formation over 4 days under low concentrations of ampicillin (1, 2, 5, and 10 µg/mL) displayed significantly higher biofilm-forming abilities compared to the control bacteria in Luria Broth (LB) with no ampicillin ($p < 0.05$) **(Figure 1A)**. Bacteria grown in the presence of 1 µg/mL ampicillin formed more biofilms than bacteria selected grown with 0 µg/mL or higher levels of ampicillin (2, 5, 10, 20 µg/mL) **(Figure 1A)**. However, when exposed to ampicillin concentrations near the minimum inhibitory level (>20 µg/mL), biofilm formation was completely eliminated, and the cells were unable to recover biofilm-forming abilities even in environments containing no ampicillin **(Figure 1A)**. Consistent exposure to 20 µg/mL ampicillin for 4 days produced no significant increase in biofilm formation by day 5 compared to the control ($p > 0.05$) **(Figure 1A)**. In a standard LB environment, biofilm selection at ampicillin concentrations closer to 1 µg/mL yields greater biofilm formation.

Biofilm Formation in Low Antibiotic Environment

Then, we investigated how selected cells form biofilms in an environment with persistent non-zero ampicillin concentrations, imitating how bacteria would respond to long-term antibiotic use. We exposed *E. coli* selected for optimal biofilm formation for 4 days under all experimental ampicillin concentrations to a low ampicillin environment of 2 µg/mL to compare biofilm formation against that of the non-ampicillin-treated and Day 1 bacteria, which are not biofilm-optimized nor ampicillin-treated ($p < 0.05$) **(Figure 1B)**. All experimental *E. coli* groups grown under non-zero ampicillin concentrations significantly increased biofilm formation compared to the Day 1 control. Bacteria grown in 5 µg/mL ampicillin yielded the highest crystal violet OD595 absorbance, indicating maximum biofilm formation **(Figure 1B)**. Ampicillin concentrations deviating from 5 µg/mL had an adverse effect on biofilm formation, but still yielded more biofilms compared to the Day 1 control culture ($p < 0.05$) **(Figure 1B)**. The Day 1 control formed no biofilms in 2 µg/mL ampicillin after 24 hours ($p > 0.05$) **(Figure 1B)**. These results suggest that selection in an ampicillin-containing medium overall increases biofilm formation, even when ampicillin continues to be present. The ANOVA data suggested that different ampicillin concentrations affected biofilm formation under both 0 µg/mL and 2 µg/mL conditions ($p < 0.05$). The interaction *p*-value

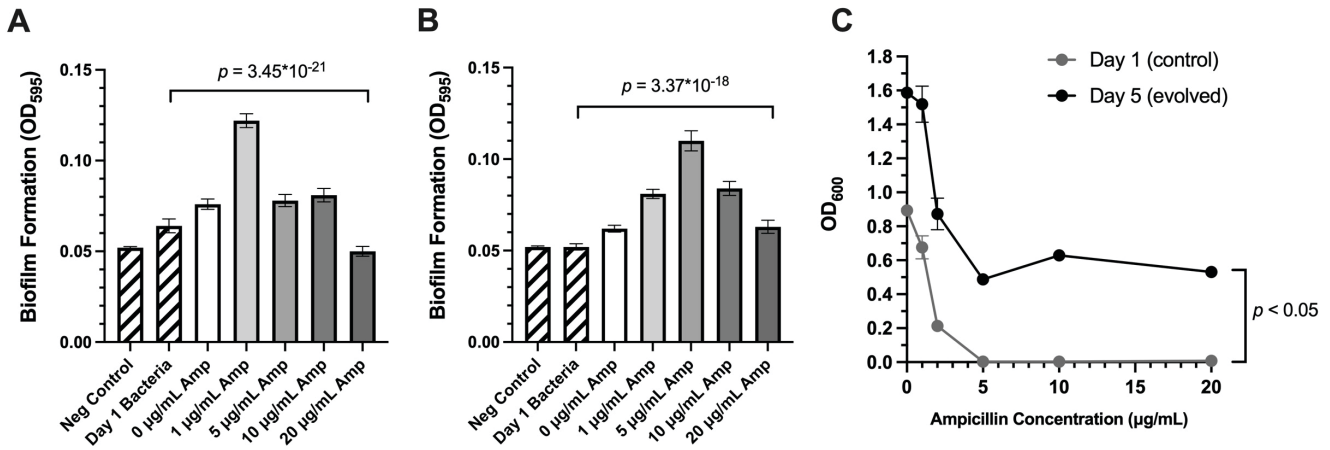

Figure 1: Final biofilm formation and antibiotic resistance of *E. coli* cells selected for optimal biofilm formation after 4 generations of bead transfer in growth media containing varying concentrations of ampicillin. (A, B) Quantification of biofilm formation via absorbance at 595 nm following a crystal violet assay on bacterial samples grown for 4 days under 0-20 µg/mL ampicillin, after transferring to LB with no ampicillin **(A)**, and LB with 2 µg/mL ampicillin **(B)**. **(C)** Antibiotic resistance of bacteria selected for biofilm formation on Day 5 (black) in comparison to planktonic bacteria on Day 1 (gray) was measured through OD_{600} assays (n = 3). Day 1 and Day 5 optical densities of all ampicillin concentrations were found statistically significant via one-way ANOVA tests ($p < 0.001$) **(C)**. Error bars represent ± mean SD.

had an order of magnitude of -27, strongly indicating that the final concentration played a role in the relationship between initial concentration and final biofilm formation.

Increased Antibiotic Resistance in Biofilm-Selected *E. coli*

We quantified growth under antibiotic exposure through OD absorbances of 24-hour cell cultures as a proxy for antibiotic resistance. We compared antibiotic resistance in biofilm-selected *E. coli* to the control samples through OD_{600} measurements in an ampicillin-containing medium. Cell densities (OD_{600}) correspond to growth rate and cell viability, which was used to approximate antibiotic tolerance. For all experimental ampicillin concentrations, *E. coli* selected for optimal biofilm formation after 4 days of bead transfers, displayed an increase in antibiotic resistance, reaching a significantly higher OD_{600} value after 24 hours of growth compared to Day 1 bacteria grown for 24 hours in their respective ampicillin concentrations ($p < 0.05$). For Day 1 bacteria, the OD was measured to be 0.003 for ampicillin concentrations of 5 µg/mL and over, indicating a near absence of growth, and thus, antibiotic sensitivity **(Figure 1C)**. Bacteria selected for biofilm formation on Day 5 plateaued at an OD of 0.487 for ampicillin concentrations of 5 µg/mL and greater, a significant increase in antibiotic tolerance compared to Day 1 bacteria, even for near-lethal concentrations ($p < 0.05$) **(Figure 1C)**. These results indicate increased antibiotic resistance in biofilm-selected cells.

Morphological Differences Arise under Selective Pressure of Ampicillin

We plated ampicillin-resistant and sensitive bacteria on LB agar plates to observe changes in colony morphology. When ampicillin was introduced to the growth medium, colonies plated on Day 5 showed changes in morphology compared to colonies on the starter plate and the 0 µg/mL ampicillin Day 5 culture. Colonies from the 0 µg/mL culture were uniformly spaced, generally consistently sized, and were all white, indicating colony homogeneity **(Figure 2A)**. Colonies from the 5 µg/mL culture, however, developed an increased size, an opaque yellow color, and a tendency to form clusters compared to colonies on the 0 µg/mL plate **(Figure 2B)**. The varied colony morphologies in the plates exposed to ampicillin may indicate a more diverse gene pool in populations under selective pressure. Overall, we found that the presence of ampicillin in lower concentrations accelerates the selection of biofilm bacteria, causing high amounts of biofilm to form in fewer generations and that these phenotypic changes correspond to differences in colony morphology.

DISCUSSION

Our data support our hypothesis that low concentrations of ampicillin induce additional selective pressure, causing the selection for biofilm-forming bacteria to proceed at a faster rate than bead transfers alone. We found that Day 5 bacteria produced a higher cell density in 24 hours under varying ampicillin concentrations compared to Day 1 bacteria, suggesting that the improved biofilm-forming ability observed in *E. coli* cultures exposed to low concentrations of ampicillin is attributable to the difference in antibiotic tolerance between biofilm and planktonic cells. At near-lethal concentrations of ampicillin, however, biofilm formation was almost eliminated to zero. Since EPS matrices are not completely invulnerable to penetration by antibiotics, ampicillin concentrations approaching the near-lethal level of 20 µg/mL were able to effectively suppress biofilm formation by significantly reducing cell density.

Furthermore, biofilm-associated bacteria reached higher cell densities compared to planktonic bacteria when grown in environments without ampicillin, implying an overall increase in fitness due to the selection for mutations unrelated to antibiotic resistance. A potential explanation for this phenomenon is the selection for genes involved in flagella synthesis, a structure responsible for motility in some bacterial cells. Biofilm-associated bacteria are selected for the ability to advance through stages of the biofilm cycle

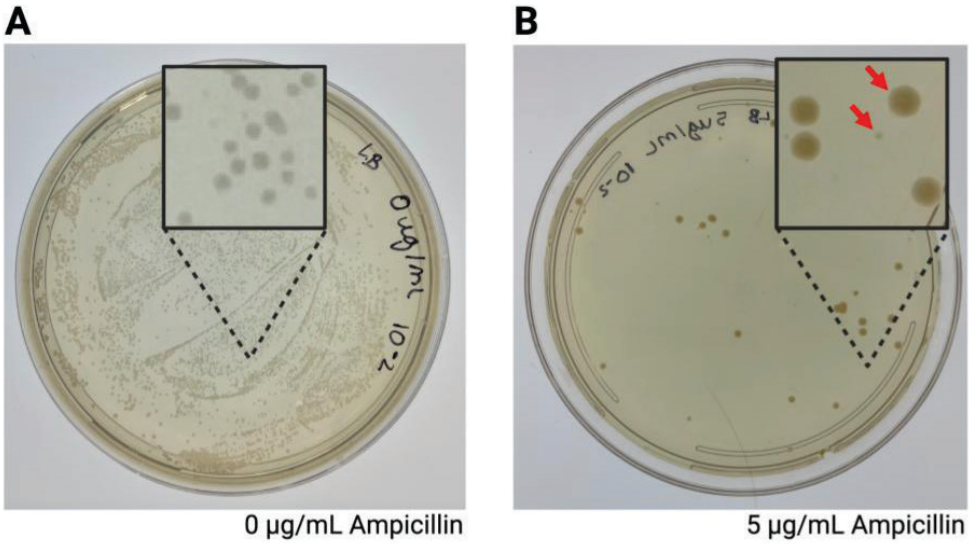

Figure 2: Morphological differences arise from colonies grown in ampicillin-containing media. (A) Colonies of uniform size formed after plating the 0 µg/mL ampicillin control culture indicate genetic uniformity. **(B)** Bacteria grown in a 5 µg/mL ampicillin medium displayed varying colony sizes. Red arrows indicate ampicillin-resistant and surrounding satellite colonies that differ in size and morphology.

(attachment, maturation, detachment, and recolonization), which requires high motility and thus, effective synthesis of the flagella (9). A previous study suggested that flagella abundance in *E. coli* cultures directly correlates to growth rate due to changes in the regulation of the flagellar master regulator gene FlhD4C2, which supports our observation of increased growth rates in biofilm-selected bacteria (10). Another point that remains elusive is the cause of the different colony morphologies observed in the 5 µg/mL ampicillin plate. We suspect that the increase in colony size corresponds with the increased OD_{600} values on Day 5 since both phenotypes imply an increased growth rate. However, this change was not seen in the 0 µg/mL Day 5 culture with bead transfers, demonstrating that morphological changes are induced by the presence of ampicillin, not the selection for biofilm-forming bacteria. In populations exposed to antibiotics, small satellite colonies formed around the bigger, resistant colonies. The smaller colonies are likely antibiotic-sensitive but were able to survive due to their proximity to a resistant colony, indicating the presence of chemical signaling or collaborative behavior (11). Ampicillin-resistant bacteria are known to secrete β-lactamases enzymes into their close surroundings, which then hydrolyze to break the β-lactam ring present in a wide range of antibiotic compounds (11). This detoxifies the environment surrounding resistant cells, allowing the survival of certain susceptible populations.

Although our findings generally align with current theories on antibiotic resistance and biofilms, we have yet to pinpoint mutations in specific genes that allow for these phenotypic changes. Genome sequencing of morphologically distinct colonies will provide us with a more accurate understanding of the selection for biofilm-assisting traits on a genetic basis.

A potential source of uncertainty in our data that could limit our interpretations is the transfer of beads. When beads were transferred using forceps, some biofilm could have rubbed off or residual planktonic bacteria could have stuck to the bead and transferred to the new tube. We minimized this uncertainty by only picking up the bead once and making the transfer as fast as possible to minimize the amount of biofilm removed in the process of bead transfers. Another source of uncertainty is the sterility of the experiment. Since we conducted the experiment over an extended period of time with multiple instances of exposure to air, any contamination that may have occurred in the *E. coli* cultures over the 5 days could impact the OD_{600} and crystal violet absorbencies.

Overall, exposure to low concentrations of ampicillin yielded a significant increase in biofilm formation in both environments, indicating that selection for optimized biofilm formation occurs at a faster rate in the presence of ampicillin, while concentrations over 10 µg/mL have an adverse effect. The results of our study have implications for the efficacy of ampicillin in the *in vivo* treatment of *E. coli* biofilm infections, which can be more broadly applied to antibiotic therapy. The tendency for bacteria to resist antibiotics through biofilm formation remains a current medical issue with the emergence of multidrug-resistant strains. Our findings show that more appropriate antibiotic doses need to be determined before designing ampicillin-based pharmaceuticals to be administered in order to minimize antibiotic resistance and biofilm formation. Additionally, alternative approaches could better eradicate resistant biofilms. Further studies with quorum sensing inhibitors, matrix-degrading enzymes, and photodynamic therapy are needed to provide alternative solutions.

The next experimental step to determine the underlying genetic changes responsible for the observed phenotypes can be achieved by sequencing the genomes of biofilm-selected and planktonic bacteria. Since colony morphology may be indicative of biofilm-forming ability and antibiotic resistance, as observed on the 5 µg/mL ampicillin plate, sequencing the genomes of bacteria that formed different-sized colonies can more definitively identify possible mutations induced by the selective pressure of ampicillin. Further studies include exposing bacteria selected for biofilm formation in ampicillin to media containing other types of antibiotics (e.g., streptomycin, tetracycline, gentamicin, etc.) to test whether acquired antibiotic resistance applies to a broader spectrum of antibiotic types.

MATERIALS AND METHODS

Control and Preliminary Testing

Before beginning the 5-day sequence of biofilm selection, the growth of *E. coli* (HB101 K12 strain, Bio-Rad Laboratories) was measured in its planktonic form under varying concentrations of ampicillin, to set a baseline value for antibiotic tolerance without the selective pressure induced by the beads. *E. coli* K12 bacteria were streaked on an LB agar plate for single colonies and incubated at 37˚C overnight. Then, single colonies were picked from the starter plate and inoculated into culture tubes containing 5 mL of liquid LB medium with 0, 1, 2, 5, 10, and 20 µg/mL of ampicillin. Concentrations were determined by preliminary testing as the optimal range, after testing a wider range of concentrations up to 2 mg/mL of ampicillin. The preliminary optical densities measured at 600 nm (OD_{600}) after 24 hours of growth revealed that ampicillin concentrations higher than 20 µg/mL yielded no growth. After 24 hours of growth in 0, 1, 2, 5, 10, and 20 µg/mL ampicillin, OD_{600} measurements were conducted to quantify cell growth. Then, a crystal violet assay was performed to measure the extent of biofilm formation without selective pressure from the bead. *E. coli* cells were cultured in identical conditions as for the OD_{600} measurements. After 24 hours of growth, the liquid was pipetted out of the culture tubes, leaving only the biofilm that had formed on the walls of the tubes. Five mL of crystal violet solution was added to each tube and incubated for 15 minutes to stain the biofilm. Ten mL of deionized water was added to each tube, incubated for 1 minute, and removed to rinse excess crystal violet solution. One mL of 95% ethanol was added into each tube and vortexed to dissolve the stained biofilm. From the resulting sample, 200 µL were pipetted into each well of a 96-well microplate 3 times for each concentration. Absorbance at 595 nm was measured using a microplate reader.

Ampicillin-Assisted Biofilm Selection

Bacteria were selected according to the following modified procedure of the Evolving STEM *Pseudomonas fluorescens* Experimental Evolution Protocol (12). The 5-day sequence of biofilm selection was started by performing polystyrene bead transfers daily in *E. coli* grown in LB containing the same ampicillin concentrations used in the control cultures. The single colonies of *E. coli* were inoculated into culture tubes containing 5 mL of liquid LB with ampicillin concentrations of 0, 1, 2, 5, 10, and 20 µg/mL, in addition to a negative control

with no bacteria to ensure no contamination **(Figure 3)**. A sterile black polystyrene bead was placed in the media for each of the experimental groups to provide a surface for cell attachment and biofilm formation. The tubes were grown for 24 hours in a 37 °C water bath while shaking at 60 rpm. For each ampicillin concentration, three biological replicates were cultured independently. Additionally, three tubes of pure LB with no antibiotics were inoculated without a bead as a negative control.

After 24 hours of growth, the black beads from the previous day in each ampicillin environment were transferred to 1.5 mL microcentrifuge tubes filled with 950 µL of PBS buffer using sterile forceps. Each centrifuge tube was vortexed for 1 minute to transfer the bacteria from the surface of the bead to the surrounding PBS. A 500 µL of cell suspension from each 1.5 mL centrifuge tube was pipetted into culture tubes containing 5 mL of LB with the corresponding ampicillin concentration. Additionally, 50 µL from the previous day's negative control culture with no bead was pipetted into a new tube containing 5 mL of LB. A white bead was added into each of the culture tubes except the negative control. All culture tubes were incubated for 24 hours in a 37 °C water bath **(Figure 3)**.

After another 24 hours of growth, the next round of bead transfers was conducted by transferring the biofilm-colonized white bead from the previous day into a new culture tube filled with LB and its respective ampicillin concentration **(Figure 3)**. A new black bead was added into each culture tube to select for bacteria that detaches and re-colonizes the new bead. The culture tubes were incubated at 37 °C for 24 hours. The bead transfer process was repeated after 24 hours by transferring the black beads into new culture tubes with new white beads. At this point, the bacteria have been selected for optimal biofilm formation after four days of selection with daily bead transfers.

Final Quantification of Biofilm Formation and Antibiotic Resistance

The biofilm-selected bacteria from the Day 4 tubes were plated on LB agar plates to observe changes in colony morphology and density. White beads from the Day 4 culture tubes were transferred to 1.5 mL microcentrifuge tubes filled with 950 mL PBS buffer and vortexed until all bacteria were removed from the bead, resulting in the undiluted sample (10^0). A serial dilution was performed by pipetting 100 µL of the 10^0 cell suspension into 900 µL of PBS to make a 10^{-1} suspension, and subsequently, a 10^{-2} dilution by diluting 100 µL of the 10^{-1} cell suspension into 900 µL of PBS. A 100 µL of the 10^{-2} dilution was pipetted onto a LB agar plate and spread evenly using a plate spreader. The 6 resulting plates were incubated overnight at 37 ˚C.

Furthermore, bacteria selected for biofilm formation under ampicillin concentrations of 0, 1, 2, 5, 10, and 20 µg/mL for 4 days were transferred to new culture tubes containing no ampicillin (0 µg/mL) and low ampicillin (2 µg/mL) to the assay for their respective ability to form biofilms under controlled growth conditions. High ampicillin concentrations were omitted from the final assay to ensure a sublethal dose. The white bead from one of each Day 4 culture tubes was transferred to a 1.5 mL centrifuge tube with 950 µL of LB and vortexed to remove all bacteria from the bead. Then, 50 µL of cell suspension was pipetted into each corresponding Day 5 culture tube. There were a 0 µg/mL and a 2 µg/mL tube for each sample, with 12 tubes total. Fifty µL from the negative control tube of Day 4, with no bead pipetted directly into the Day 5 tube. The Day 5 tubes contained no bead to allow the bacteria to form biofilms on the inner surface of the tube. The bacteria were incubated overnight in a 60 rpm orbital shaker within a 37 ˚C water bath to prepare for growth quantification and a crystal violet assay the following day.

To determine if bacteria selected for optimal biofilm

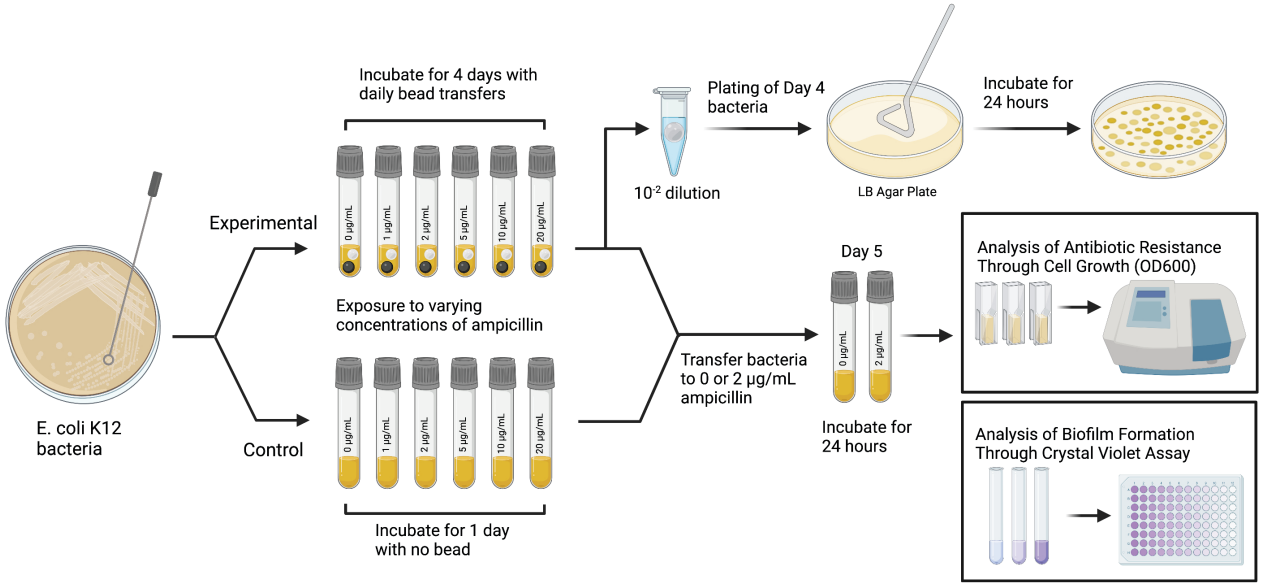

Figure 3: Schematic illustration of the experimental setup and procedure. Single *E. coli* colonies were inoculated into culture tubes containing LB with ampicillin concentrations ranging from 0 to 20 µg/mL. Bacterial cultures selected for 4 days with daily bead transfers were compared with those grown for 1 day without beads to test for changes in biofilm formation and antibiotic resistance. Biofilm formation was quantified using a crystal violet assay, and antibiotic resistance was measured using OD_{600} readings after a 24 hour incubation period.

formation (Day 5) evolved antibiotic resistance compared to the control (Day 1) bacteria, we compared OD_{600} values of the Day 5 cultures under the 6 ampicillin concentrations with the control OD_{600} values taken on Day 0, each after 24 hours of growth in 37 °C. For each ampicillin concentration, 1 mL of cell suspension was distributed into a 1.5 mL cuvette, and the optical density at 600 nm was measured using a spectrophotometer. For each concentration, three technical replicates were conducted by using three separate cuvettes and averaged.

Finally, the biofilm-forming ability of biofilm-selected bacteria transferred to 0 µg/mL and 2 µg/mL of ampicillin from antibiotic concentrations of 0, 1, 2, 5, 10, and 20 µg/mL ampicillin were quantified through a crystal violet assay followed by OD_{595} measurements. The cell suspension was carefully pipetted out of each culture tube to preserve the biofilm. Then, 5 mL of crystal violet solution was added to each tube and incubated for 15 minutes. A 10 mL of DI water was added to each tube, incubated for 1 minute, and removed to rinse away excess crystal violet solution. One mL of 95% ethanol was added into each tube and vortexed to dissolve the stained biofilm. Two hundred µL of the resulting ethanol solution was pipetted into each of 5 wells of a 96-well microplate for each concentration. Absorbance at 595 nm (OD_{595}) was measured using a microplate reader.

Statistical Analysis and Control Variables

The data collected from three trials of the OD_{600} growth assays and five trials of the crystal violet assay were averaged and plotted as a function of ampicillin concentration. Each data set was analyzed for statistical significance by a one-way ANOVA ($\alpha = 0.05$) for both final ampicillin concentrations of 0 µg/mL and 2 µg/mL, and p-values were indicated in **Figure 1**. There were several control variables used in this experiment to minimize the interference of data by factors unrelated to ampicillin concentration. First, the incubation time between bead transfers and before the final crystal violet assay was strictly controlled at 24 hours ± 30 minutes to ensure that an increase in biofilm formation was not attributed to longer incubation times. Second, all growth conditions except for ampicillin concentration (media, temperature, rpm, etc.) were controlled to ensure that the only differing condition between runs was ampicillin concentration. Third, the cell count of the initial inoculums for the Day 5 OD_{600} and crystal violet assays were controlled by pipetting the same volume (50 µL) of cell suspension into 5 mL of LB medium so that OD_{600} values and crystal violet absorbances were not skewed by changes in volumes of the initial inoculums.

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