Bacteria That Resist Centrifugal Force

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BACTERIA THAT RESIST CENTRIFUGAL FORCE

by

NICKOLAS GLEN KESSLER

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Biomedical Sciences in the College of Medicine and in the Burnett Honors College at the University of Central Florida Orlando, Florida

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Thesis Chair: Dr. Sean D. Moore
ABSTRACT

Our lab discovered that approximately 1 in 10,000 *Escherichia coli* cells in stationary phase remain in suspension after a high g-force centrifuge event. To establish the mechanism behind this curious phenotype, multiple mutant strains of *E. coli* were independently evolved such that the majority of their populations resisted migration when exposed to high centrifugal forces. Genomic DNA sequencing of the mutants’ revealed unique, isolated mutations in genes involved in capsule synthesis and exopolysaccharide (EPS) production. Each mutant exhibits a novel mechanism that allows them to remain in suspension. The mutants were further characterized by determining their growth rates, strengths of resistance to various centrifugal forces, the phenotype’s dependence on a carbon source, and timing of the phenotype’s presentation. The results revealed: comparable mutant generation times to the wild-type strain, variable resistance to centrifugal force, phenotype dependence on carbon source, and phenotype presentation during early stationary phase. To interrogate the mechanism by which these cells stay in suspension the production of EPS was quantified, and gene knock-outs were performed. Quantification of the EPS revealed approximately a seventeen-fold increase in EPS in the mutants’ compared to the wild-type strain. Gene knock-outs revealed the EPS produced can be attached to the outer-membrane or freely secreted into the media by different mechanisms. In addition, this mechanism was further confirmed as being responsible for the centrifuge resistant trait by attaching extracted EPS to polystyrene microspheres. Experimental results show that mutant extracted EPS treated beads caused increased bead retention in suspension compared to wild-type EPS treated beads. These results reveal that *E. coli* is using a novel mechanism to adapt to a new environmental factor introduced to remove the bacteria. With the discovery of this mechanism and the transferability to
inorganic objects industrial applications are now envisioned where particle sedimentation is controllable and mixtures remain homogenized by attaching optically transparent biomolecules.
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LIST OF ACRONYMS/ABBREVIATIONS

EPS – Exopolysaccharide
CA – Colanic Acid
LPS – Lipopolysaccharide

yea* – ΔyeaI, yeaJ’, yrfF A564P (mutant C06)

LB – Lysogeny Broth
RCF – Relative Centrifugal Force
OM – Outer-membrane

cps – capsule synthesis operon
I. INTRODUCTION

1.1 Escherichia coli

*E. coli* is a Gram-negative, rod-shaped prokaryote that can grow in aerobic or anaerobic conditions and has an optimal growth rate at 37 °C [1, 2]. *E. coli* is often used as a research model because it is a well understood organism that has a variety of metabolic capabilities, and can synthesize all cellular components from basic nutrients. Some of *E. coli*’s other notable qualities ideal for research studies are its quick generation time, gene uptake abilities, ease of isolation, hardiness, and low cost requirements [3].

1.2 Bacterial evolution

Environmental pressures can reduce the viability of phenotypically unfit bacteria, allowing cells with certain characteristics to outcompete and gain reproductive advantage over others. The evolution and adaptation of a species is a result of genetic variability, favoring an organism best suited for the environment. This is consistent with Darwin’s theory of natural selection. Small changes in an organism’s genome due to mutation can potentially lead to a better adapted species. It is believed that these genetic mutations are present before and occur independently to the exposure of an environmental factor [4]. This observation seems to be the case with the *E. coli* mutants that have been isolated for this study. The mutant strains were isolated by selecting for a small fraction of the population that expressed the trait that allowed them to remain in suspension after being subjected to a large centrifugal force [5]. If genetic variants were not present in the population before the exposure, all the bacterium would sediment in the same manner.
Furthermore, this could explain why each mutant isolated, regardless of what centrifugal force was used to generate the strain, is unique in its resistance to sedimentation.

1.3 Bacterial persistence

Bacterial persistence is a transient physiological response that is present in a small percentage of the population [6]. Persistence is characterized by a state of reduced growth that allows a small subset of the bacterial population to survive exposure to unexpected environmental elements [6]. An example of this characteristic is bacterial response to treatment with antibiotics. After exposure to antibiotics, most bacteria die off, however, due to the presence of cells in the “persistent” state at the time of exposure, some bacteria survive and remain in this state until reinstated into a preferable environment. Once these cells are moved to fresh medium, they can resume growth and form new colonies. Interestingly, these new colonies are not antibiotic resistant. If treated with another round of antibiotics, they are just as susceptible as before. This supports the notion that bacterial persistence is a temporary phenotypic state that is either on or off at any given time. On the other hand, antibiotic resistance is a trait that is always present in the genome or on a plasmid, allowing bacteria to have continuous growth in the presence of antibiotics [7]. When developing the mutants, the small percentage of the population that consistently resisted sedimentation in the presence of centrifugal force was selected for and isolated. However, if these cells are carried over to grow a new generation they do not exhibit the centrifuge resistant trait. In light of this, it is believed that there is a regulatory mechanism at the epigenetic level controlling this trait. Once the transition to a dominant centrifuge resistant mutant occurred, it seems that these cells had a permanent change in their genomic DNA to consistently present this trait.
1.4 Biofilms

Preliminary observations noted that when the mutants are grown on solid medium, they produce a colony morphology that is phenotypically distinct from the wild-type. This distinct morphology has increased biomass and a mucoid-like appearance characteristic of a biofilm. Biofilms consist of an aggregation of bacterial cells on a surface that produce a protective matrix [8]. They represent a form of protected growth that give bacteria the ability to maintain a relatively constant local environment in the presence of changing external conditions. This allows for rapid adaptation to various environmental factors [9]. Bacterial biofilms cause persistent microbial growth. The increased exterior bio-mass reduces the interior cells’ exposure to deleterious agents, making these colonies much harder to remove [8]. Persistent bacterial infections due to biofilm formation are responsible for chronic infections in the medical field. The increase in bio-mass could decrease the effectiveness of antibiotics by decreasing the drug’s penetrance, or decreasing the amount of extracellular material the cell uptakes. This is an area in need of further research because antibiotics are effective at treating planktonic bacterial infections, but not biofilm infections as seen in the disease Cystic Fibrosis [8].

1.5 Colanic acid

The increase in colony size, morphological changes, and mucoid appearance seen in the mutants is characteristic of increased exterior cell biomass. Colanic acid (CA) has been noted to increase the volume of bacterial biofilms and produce a mucoid colony appearance [10, 11]. However, unlike biofilms that are composed of a community of cells with varying phenotypic expression, each cell in the mutant’s population seems to express an individual protective coat believed to be
composed of the EPS CA. CA is a repeating poly-anionic hetero-polysaccharide made up of: D-glucose, L-fucose, L-fucose. Branching off of this backbone chain is a three-unit polysaccharide consisting of: D-galactose, D-glucuronic acid, and D-galactose. In E. coli, the terminal D-galactose is thought to contain a pyruvate group attached to positions 4 and 6. The first L-fucose is believed to be acetylated at position 2 [12]. EPSs like CA have been noted to range in size from 0.5 to 2 mega-Daltons [13].

Figure 1. Structure of colanic acid. Schematic representation of colanic acid repeating polymer.

CA is believed to act as a protective capsule around bacteria that is produced in response to destabilization of the outer cell membrane, low temperatures, and solid surface growth [10, 14, 15]. It has been observed that production of CA makes bacteria resistant to bacteriophage infections by decreasing the phage’s ability to reach the cell membrane. In this context, CA production is a form of bacterial persistence and could be responsible for phage-resistant infections [10].
1.6 Preliminary studies

Our laboratory noted that a small fraction, approximately 1 in 10,000, *E. coli* cells stay in suspension after a hard centrifuge event (15,000 RCF, 10 min). It was discovered that using these bacteria to grow a new culture does not generate a centrifuge resistant strain initially. However, over multiple generations of reiterating this process this minority phenotype became the majority. A centrifuge resistant mutant was noted when an uncondensed pellet and an opaque medium was observed compared to the wild-type post centrifugation. Further investigation revealed that these cells are not less dense than the surrounding medium and they are not expending energy to swim against the centrifugal force.
II. MATERIALS AND METHODS

2.1 Research objectives and design

To identify the molecular mechanism *E. coli* is using to resist centrifugal force, multiple mutant strains were independently evolved such that the majority of the cell population exhibited the centrifuge resistant trait and mucoid phenotype. Three mutant strains and a wild-type parental strain were used for experiments. The generation time of each strain was identified to determine if the phenotype had an effect on growth physiology. To characterize this centrifuge resistant trait, the strength of each mutant was determined, the effect of different growth media on the phenotype, and the timing of phenotype’s presentation. Genomic DNA was sequenced to determine mutations responsible for the mechanism. A colorimetric assay was used to quantify EPS production in the mutants. Phage P1 transductions were used to knockout genes involved in CA linkage to determine how CA is associated with the membrane.

2.2 Evolving mutants that resist centrifugal force

*E. coli* strain BW30270 (CGSC #7925) was used for the evolution experiments. This strain is a prototrophic lineage of MG1655 and its genome has been sequenced by the Moore lab. *E. coli* cells were streaked-out for isolation of colonies on agar prepared with Lysogeny Broth medium with 5% sodium chloride (“Lennox”, LB) supplemented with 0.2% glycerol and incubated 18 hours at 30 °C. An isolated colony was chosen and inoculated in 10 mL of LB (0.2% glycerol) in a 125 mL baffled Erlenmeyer flask. For each evolution selection cycle, the culture was then grown to stationary phase at 30 °C in a shaking incubator at 220 rpm for 18 hours. A 1 mL aliquot of the culture was transferred to a 1.7 mL microcentrifuge tube and centrifuged at 3,250 RCF for 10
minutes. 750 µL of the supernatant was sampled and dispensed into a freezer stock tube with 250 µL of 50 % glycerol. The freezer stock was thoroughly mixed and 200 µL of the freezer stock were used to inoculate a new culture of 10 mL LB-glycerol in a 125 mL flask. Each consecutive inoculation, after following the protocol above, was noted as a new generation.

This procedure was repeated until the presence of an uncondensed pellet and cloudy supernatant of bacteria was observed post-centrifugation. At this generation, the sample was streaked on LB agar and grown at 30 °C overnight. A representative colony was then chosen that matched the dominant phenotype, which was consistently mucoid for each independent lineage. Stocks from the isolated colony were grown again in parallel to the wild-type parental strain to confirm the centrifugation phenotype and then given a reference name.

2.3 Growth rate studies
Mutant and the wild-type strains were grown overnight in 10 mL LB-glycerol, 125 mL flasks, 30 °C, in a shaking incubator at 220 rpm. After 18 hours of growth, the samples were diluted 1:100 using 990 µL of LB-glycerol and 10 µL of culture. The samples were vortexed and three aspirations of 75 µL 1:100 dilution and LB blank, was dispensed into a Corning Costar® polystyrene assay plate, 96-well, clear, clear flat bottom, non-treated, with a lid. The 96-well plate was then inserted into a spectrophotometer and data was obtained using BioTek Gen5 software. The spectrophotometer measurement settings were: 30 °C, slow continuous shake, absorbance measurement at 600 nm every 5 minutes, over 16 hours.
2.4 Strength of resistance

Mutant and wild-type strains were grown up overnight in 10 mL LB-glycerol, in 125 mL flasks, 30 °C, in a shaking incubator at 220 rpm for 18 hours. Eleven 1 mL samples of the cultures was dispensed into a 1.7 mL microcentrifuge tube. The samples were centrifuged at various speeds calculated by using this radial distance of the sample spot, 0.5 mL mark, from the axis of the centrifuge. This determined the centrifugal force experienced by the cells where sample measurements were taken from.

Ten centrifuge spins were performed in 1,000 RCF increments. Starting at 1,000 RCF and ending at 10,000 RCF. One final centrifuge spin was performed with an experienced force at the 0.5 mL mark of 15,000 RCF. After centrifugation, 100 µL of each sample were aspirated from the 0.5 mL mark and dispensed into a 96-well plate. The absorbance of the samples was measured at 600 nm and a LB-glycerol blank was subtracted from these values. The turbidity of each mutant sample at the 0.5 mL mark was compared to the wild-type at the same position and was used to determine the “strength” of each of the strains’ resistance to centrifugal force.

2.5 Timing of phenotype expression

2 mL of the Mutants and wild-type cultures grown for 18 hrs in LB-glycerol (0.2%) were used to inoculate 18 mL of fresh LB-glycerol (0.2%) in a 125 mL flask. After the 1:10 inoculation, cultures were thoroughly mixed. A 1 mL sample was removed and the flask was placed in a 30 °C, shaking incubator at 220 rpm. 1 mL was subsequently removed every 30 min from the start of incubation.
The 1 mL samples were spun at 3,000 RCF for 3 min. 100 μL was removed from the 0.5 mL mark and dispensed into a 96-well plate. Using a spectrophotometer, the OD$_{600}$ was measured.

2.6 Growth medium’s effect on phenotype

Three different LB broth medias were prepared: LB no supplemental carbon source, LB 0.2% glycerol, and LB 0.2% glucose. *Wild-type* and the mutants were separately inoculated into 10 mL of the three medias mentioned above in a 125 mL flask and grown to stationary phase in a 30 °C shaking incubator at 220 rpm. Three, 1 mL aspirations of each sample removed and dispensed into a microcentrifuge tube. The samples were spun at 11,000 RCF for 3 min. 75 μL were removed from the 0.5 mL mark of the microcentrifuge tube and dispensed into a 96-well plate, and the absorbance at 600 nm was measured in spectrophotometer.

2.7 Genomic DNA

Due to the inability to pellet and harvest the mutant cells, a unique genomic DNA extraction method was adapted for this study. The mutant strains were grown at 30 °C, on agar LB-glycerol plates for 18 hours. Colonies were scraped from the plate using a pipette tip and inserted into a screw cap microcentrifuge tube with 0.1 mm zirconia/silica disruption beads added up to the 100 μL mark on the tube. Cells were re-suspended in 400 μL of P1 resuspension buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 μg/mL RNase A).

The cells were placed into a MP FastPrep-24 homogenizing instrument and lysed using the preset *E. coli* settings. Samples were spun at full speed for 1 min in a microcentrifuge. The lysing step
and full speed spin steps were repeated a second time to ensure thorough cell lysis. The aqueous phase was removed, avoiding cell debris and beads, then placed in a clean microcentrifuge tube. 500 µL of gel melting buffer (5.5 M guanidine thiocyanate, 100 mM acetic acid, potassium-acetate pH 5.0) were added to the aqueous phase. The sample was vortexed and lightly spun to remove liquid from the cap.

To the samples, 250 µL of isopropanol was added, vortexed, and spun at full speed. The supernatant was removed carefully, avoiding the cell pellet, and dispensed into a silica column spin tube and spun at 2,000 RCF for 30 seconds. The sample run-through was pipetted back on top of the column and spun through the column a second time to increase binding efficiency. The run-through was then discarded. 200 µL of gel melting buffer was added to the column and spun down. The column was washed three times with 200 µL of column wash buffer. 5X column wash buffer (CWB) was prepared by combining: 20 mM K-HEPES, 100 mM NaCl, 0.1 mM EDTA, pH 8.2. From the 5X CWB 10 mL was diluted in 40 mL of 95% ethanol, final pH above 7.5. The column was spun at full speed for 1 min for drying. 50 µL of DNA buffer (2.5 mM Tris-Cl pH 8.0, 1 mM EDTA) was added to the column and spun down. The run-through was added back to the column a second time for thorough elution of the DNA. Samples of DNA were quantified using a NanoDrop 3300 Fluorospectrometer and sent for Illumina DNA sequencing.

2.8 Exopolysaccharide release

Mutant and wild-type cultures were inoculated from isolated colonies on agar (LB-glycerol) plates and grown in 10 mL LB-glycerol in a 125 mL flask at 30 °C, in a shaking incubator at 220 rpm.
for 18 hours. The optical density at 600 nm (OD600) was measured in a 96-well plate to determine the density of the cultures. The Soosang Kang et al. (1966) protocol for the release of exopolysaccharides was followed with minor modifications [16]. 3 mL of overnight cultures were dispensed into large glass test tubes and placed into a boiling H2O bath for 15 min. Once the samples finished boiling, 1.5 mL of the supernatant was removed and placed into 1.7 mL microcentrifuge tubes and centrifuged for 10 minutes at 8,000 RCF using a Sorval SS-34 rotor at room temperature. After centrifugation, the supernatant of each sample was transferred into 3,500 MWCO dialysis tubing (Thermo Scientific) and clamped at the ends. The samples were then placed into 800 mL of deionized water in a 1 L beaker with a magnetic stir bar. Samples were dialyzed against deionized H2O (diH2O) for 2 hours and then exchanged for another 800 mL of diH2O. After another two hours, the diH2O was exchanged a final time and the samples were dialyzed overnight with stirring. Samples were carefully removed from dialysis tubing taking note of the volume removed from the bag. A final 50 mL sample of the dialysate was kept for the standard curve used to quantify CA. A separate methyl pentose quantification control procedure was performed on the final dialysate exchange and compared to diH2O to confirm thorough free fucose removal [16].

2.9 Exopolysaccharide quantification

The protocol from Zacharias Dische, (1947) for methyl pentose quantification was followed with minor modifications for quantification of exopolysaccharide production [17]. Using 10 mL of dialysate and 0.1 g of L-fucose, a 1% fucose solution was made. From this, a 1:10 dilution of the 1% fucose with dialysate was made, and then subsequent ½ dilutions were made from the 0.1 %
fucose solution. The percent fucose samples used for the standard curve were 0.025%, 0.0125%, 0.00625%, 0.003125%, and 0.00156%. The calculated μM concentration converted from % fucose used for the standard curve were: 1520 μM, 761 μM, 381 μM, 190 μM, and 95 μM respectively. L-fucose, a methyl pentose, is a constituent of CA [18]. 200 μL of the mutant samples and fucose concentrations were then dispensed into a large glass test tube and mixed with 2.25 mL of 3:1 sulfuric acid to diH₂O. The reaction was allowed to cool to room temperature in a diH₂O bath. The samples were then boiled for 10 minutes in a water bath. After boiling, the samples were placed in a room temperature water bath and cooled to 25 °C. Next, 50 μL of 3% cysteine-hydrochloride were added to the samples and allowed to react for 30 minutes at room temp with intermittent mixing. 130 μL of the samples were then transferred to a quartz cuvette and the absorbance was measured from 300 nm to 500 nm using a spectrophotometer using the BioTek Gen5 program. The absorbance values of the fucose solutions were used to make a standard curve [17].

2.10 Coli genetic stock center

E. coli strains with select non-essential genes replaced by kanamycin resistance genes, referred to as knock-outs, were ordered from the Coli Genetic Stock Center (CGSC). The gene knock-outs ordered were chosen by proximity to mutated regions noted in the genomic DNA sequences, and whether the gene was involved in CA synthesis. Strains received from the CGSC were on filter disks. Agar LB-glycerol plates with approximately 20 μg/mL of kanamycin were prepared. The filter disks were placed on the agar plates with kanamycin selection and wet with 30 μL of LB-glycerol. Using a streaking stick, a streak was made from the disk downward on the plate. A new stick was used to streak in and out from this line in a zig-zag pattern. Agar plates were placed in
37 °C incubator overnight. An isolated colony was used to inoculate a 1 mL LB-glycerol with 20 μg/mL Kanamycin (kan) culture and placed in a 37 °C incubator shaking at 220 rpm. After 18 hours of growth, a freezer stock was made and labeled with the relevant knock-out gene and drug marker kan. The strain used for this study was rfaL734(del)::kan, also referred to as JW3597-1 [19].

2.11 Phage-P1 transduction

Knock-out strains were streaked for isolation on agar LB-glycerol, kan 20 μg/mL plates. These strains were referred to as donor strains. Mutant wild-type strains were also streaked on agar plates with drug selection as controls for antibiotic activity and are referred to as recipient strains. An isolated colony of the donor strain was used to inoculate 1 mL of LB-glycerol, 20 μg/mL kan and grown for 18 hours. 10 μL of the overnight cultures were used to inoculate 990 μL of P1-LB and placed in a 37 °C incubator shaking at 220 rpm. P1-LB was made by combining 40 mL LB-glycerol, 200 μL 1 M CaCl₂, 200 μL 2.5 M MgCl₂, and 200 μL of 20% glucose solution. A 1 mL mock-culture consisting of 40 μL of phage in P1-LB and a mock-infection with 1 mL of culture were included for comparison [20].

A noticeable turbidity began to appear in the cultures after approximately 1 hour. At this point 40 μL of a pre-existing lab stock of P1-phage was added to each sample and were placed back into the incubator. Once the culture turbidity cleared and the sample looked similar to the mock-culture control, the samples were removed from the incubator. 75 μL of chloroform was added to the
samples, vortexed, and let stand for 8 min. The sample was then moved to a microcentrifuge tube and spun in a centrifuge at max speed for 5 min. The clear supernatant was then aspirated and moved to a clean microcentrifuge tube. 50 μL of chloroform was added to this stock and stored in a 4 °C fridge. This sample was noted as the donor library for the desired knock-out gene and was labeled with the donor strain and kanamycin-resistance.

The recipient strains, C02, C05, C06, were streaked on agar LB-glycerol plates, without kanamycin selection. An isolated colony was used to grow a 1 mL LB-glycerol culture overnight in 37 °C incubator shaking at 220 rpm. The culture was transferred to a microcentrifuge tube and harvested at 3,000 RCF for 3 min. The growth medium was then aspirated and the cells were re-suspended in 400 μL of P1-LB. 100 μL of culture was used for each transduction. 100 μL of recipient cells and 100 μL of phage library stock were combined in a clean microcentrifuge tube and rapidly mixed. Samples in the microcentrifuge tubes were then placed into a large flask and put in a 37 °C incubator shaking at 220 rpm. At this point the infection had started and after 30 min of incubation the samples were pulled out of the incubator. Once the samples were removed, a light spin was used to collect liquid from the top of tube.

Next, 200 μL of 1 M Na-citrate and 1 mL of fresh LB-glycerol was added to the samples. The samples were then placed back into the incubator for 1 hour. After incubation, the tubes were moved to a microcentrifuge and the cells were harvested at 5,000 RCF for 5 min. The supernatant was carefully removed using a pipette, without disturbing the cell debris, and discarded. To the concentrated cells, 100 μL of LB-citrate (LB supplemented with 100 mM Na-Citrate pH 5.5) were
added. The samples were capped and vortexed to re-suspend the cells. The contents were then transferred to an agar LB-glycerol plate with 20 µg/mL kan and evenly distributed with a spreader. The plates were placed in a 30 °C incubator for 18 hours.

From this plate, 4 colonies were chosen and re-streaked again on a selective agar-glycerol plate containing 1-5 mM Na-citrate, 20 µg/mL kan. One colony was streaked for isolation in each quadrant. These plates were grown for 18 hours at 30 °C. From these plates, an isolated colony was chosen from each quadrant and suspended in 5 µL of diH2O. 1 µL of this was used as the template DNA for a PCR reaction with relevant primers. 100 µL of LB-glycerol was then added to the remaining 4 µL and half of this was used to grow an overnight culture under kanamycin selection. Using the overnight culture, a freezer stock was made [20].

2.12 Polymerase chain reaction

Using the re-streaked transduced cells, grown on agar LB-glycerol with 20 µg/mL kan, an isolated colony was touched with a pipet tip and re-suspended in 5 µL of diH2O. A master-mix was prepared with: 2X OneTaq® Hot Start Master Mix with Standard Buffer (New England BioLabs®), diH2O, 200 nM forward primer, 200 nM reverse primer, and 1 µL of re-suspended colony. 25 µL was used for each reaction. NEB’s 2X OneTaq® Hot Start Master Mix with Standard Buffer protocol was followed exactly. Primer annealing temperature was calculated using template nucleotides and NEB’s online calculator. A BIO RAD thermocycler was used for PCR reaction.
Thermocycler settings: Step 1 - 94 °C 30 sec, Step 2 - 94 °C 30 sec, Step 3 - 53 °C 45 sec, Step 4 - 68 °C 1 min 40 sec, Step 2, 3, 4 repeated 29 times, Step 5 - 68 °C 5 min, Step 6 - 18 °C 10 min.

2.13 Restriction enzyme digest
Targeted gene replacement by kanamycin resistance was confirmed using restriction enzyme digestion. The restriction enzyme Eag 1 (NEB®) was used because of its specificity for a cut site in the kanamycin resistance gene in the transduced mutants. 2 μL of the amplified colony PCR product was mixed with 0.6 μL of Eag 1 and placed into a 37 °C incubator or 1 hour with periodic mixing. Samples were then loaded into a 1% agarose gel.

2.14 Ethidium bromide gel electrophoresis
1 g of agarose was dissolved in 100 mL of TBE buffer: 50mM Tris, 50 mM Borate, 0.5 mM EDTA. The solution was heated in a microwave and mixed until the agarose was completely dissolved. 7 μL of Ethidium Bromide were added to the warm solution and mixed. The solution was then poured into a casting tray with a twelve well lane comb. The solution was allowed to cool until it solidified and the comb was removed.

The gel was removed from the casting tray and placed into a BIO-RAD gel electrophoresis tank. TBE buffer was added to the tank until the gel was submerged and 14 μL of Ethidium Bromide was added to the buffer and mixed. 2 μL of Bromophenol Blue load dye and 2 μL of each DNA
sample were mixed. Bromophenol Blue load dye was prepared by combining: 1 mL 50% glycerol, 4 µL 250 mM EDTA, 1 µL 10% SDS, 20 µL Bromophenol Blue. The total 4 µL mix was aspirated and dispensed into the corresponding well of the agarose gel. Leads from the power supply were connected to the tank and power setting was set to 120V. Electrophoresis was allowed to proceed for 30 min, and the gel was visualized using a proteinsimple® imager.

2.15 Bio-conjugation of CA

CA extracted from the mutants and wild-type used for the colorimetric assay were buffer exchanged using 3500 MWCO dialysis tubing. Samples were dialyzed against 50 mM Na-MES, and 100 mM NaCl for 2 hrs. 1 mL of CA samples was combined with 100 µL of 1:10 dilution of blue, amine coated, 1.0 µm, polystyrene microspheres from Polysciences, Inc. in a 5 mL snap cap microcentrifuge tube. 1 mL of 50 mM Na-MES, and 100 mM NaCl was added to the CA-bead reaction. Samples were allowed to react overnight at room temp. Samples were centrifuged in a swinging bucket centrifuge at 3,200 RCF for 8 min. 100 µL were removed from the 1 mL marked and dispensed into a 96-well plate. The absorbance spectrum was measured from 500 nm to 700 nm, to quantify the amount of beads in suspension. A photo was taken of tubes post-centrifugation to visually confirm blue beads stayed in suspension.
III. RESULTS

3.1 Mutants that resist centrifugal force

Opaque supernatant and an uncondensed pellet, post-centrifugation, marked the presence of a mutant. The mutant phenotype was further confirmed by the presence of mucoid like colonies on agar LB-glycerol plates (Figure 1).
Figure 2. Mutants that resist sedimentation by centrifugal force. *Wild-type* and evolved mutants centrifuged at 15,000 RCF for 3 minutes (A). Mutant mucoid phenotype on agar LB-glycerol (0.2%) (B). Post-centrifugation the mutants’ revealed an opaque supernatant and uncondensed pellet. The mutant strains were streaked on solid medium plates and revealed a large mucoid colony phenotype compared to *wild-type*.
3.2 Effect of mucoid phenotype on growth rate

A growth study performed on the mutants and wild-type strain to compare the doubling time and general cell growth physiologies. The mutants’ generation times were comparable to the wild-type’s.
Figure 3. Growth study $A_{600}$ data. $A_{600}$ absorbance measured every 5 min for 18 hours at 30 °C. Error bars represent standard deviation from the mean.
Figure 4. Generation time equation. $A_{600}$ values were Log$_2$ transformed and derived to determine the most linear region of growth. This region was plotted and fit with the linear equation line. The inverse of the slope from the equation was used to determine the generation times of the mutants and wild-type.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>36.9 min</td>
<td></td>
</tr>
<tr>
<td>C02</td>
<td>36.2 min</td>
<td></td>
</tr>
<tr>
<td>C05</td>
<td>36.9 min</td>
<td></td>
</tr>
<tr>
<td>C06</td>
<td>36.1 min</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Generation times at 30 °C
3.3 Strength of resistance to centrifugal force

Pre-spin culture samples (0 RCF) of the mutant have the same amount of colony forming units (CFU) as the wild-type. Wild-type cells pellet out of suspension immediately at low g-force spins. C02 progressively sediments out of suspension as the spin speed is increased. C05 and C06 retard their sedimentation more than C02 at higher spin speeds. However, noting the higher variability at 15,000 RCF indicates that C05 and C06 can be forced to migrate at higher g-force spins, albeit at a slower rate than C02.
Figure 5. Strength of resistance to centrifugal force. Strength is represented by cell density post-centrifugation. Samples centrifuged at various g-force for 3 min. Sample taken from 0.5 mL mark. A_600 measured to determine cell density. Error bars represent standard deviation from the mean.

Figure 6. Forced sedimentation of mutant C02. Higher g-force spins reveal a clearer supernatant and larger pellet mass, showing cells can be forced to sediment. C02 samples above were from experiment performed in Figure 4.
3.4 Effect of growth media on phenotype

The availability of a supplemental carbon source in the medium affects the mutants’ ability to resist centrifugal force. The mutants are the most resistant when grown in LB-glycerol (0.2%). LB-glucose (0.2%) media significantly diminished the OD$_{600}$ post-centrifugation in mutant C02 and decreased C05 to a lesser degree. C06 grown in LB-glucose (0.2%) had a negligible effect on its resistance to centrifugal force. In LB only media, all the mutants’ resistance to centrifugal force decreased significantly.
Figure 7. Growth media’s effect on mutants’ resistance to centrifugal force. Culture grown for 18 hrs. at 30 °C supplemented with different carbon sources. 1 mL samples were centrifuged for 3 min at 11,000 RCF. The OD$_{600}$ of samples from the 0.5 mL was measured and used to determine the mutants’ strength of resistance to centrifugal force.
3.5 Timing of phenotype presentation

Mutants C05 and C06 present the centrifuge resistant phenotype during early stationary phase.

C02 presents the phenotype farther along in stationary phase.
Figure 8. Timing of centrifuge resistant phenotype presentation. Stationary phase cultures diluted 1:10. Immediately after dilution (0 min) mutants still show phenotype by remaining in suspension post-centrifugation. The centrifuge resistant phenotype increases in strength for mutants C05 and C06 at 210 min. This corresponds to early stationary phase in whole culture OD_{600}. C02 presents the phenotype further along in stationary phase.
3.6 DNA sequencing

Genomic DNA sequencing of the mutants revealed mutations in regions involved in the regulation of capsule synthesis, membrane structural integrity, biofilm formations, and swarming motility. These regions have been noted in their association with capsule synthesis and exopolysaccharide, CA in particular, in *E. coli* strain K12. Of the nine mutants that the genomic DNA was extracted, seven were sent for Illumina sequencing, including a *wild-type* strain. Of the six mutants, three were used for this study. Two strains, C02 and C05, were chosen because of their isolated gene mutations. This mitigated the chances of a confounding gene mutation altering experimental results. C06 was chosen because of the robustness of its phenotype. The mutations were further confirmed using primers designed to flank each mutated gene region and PCR amplifying the product. Samples were sent for Sanger Sequencing and the results were consistent with genomic Illumina sequence reads (results not shown).

Whole genome sequencing of the mutant strain C02 revealed two mutations: a silent point mutation in *narH* (GCC to GCA, alanine) and a missense mutation in *rcsC* (CTG to CGG, leucine to arginine). Mutant C05 contains a 42 nucleotides deletion in *lpp* that removes amino acids Lys26 through Ala39. Strain C06 revealed a total deletion of *yeal*, a partial deletion of the front portion of *yeaJ*, and a missense mutation in *yrfF* (GCG to CCG, alanine to proline). The mutants C02 and C05 from this point will be referred to by their genomic mutations *rcsC* L840R, *lpp* ΔK26-A39 respectively. Because of C06’s impractical name of Δ*yeal*, *yeaJ’, yrfF* A564P it will from now on be referred to as *yea*°.
Figure 9. Genomic DNA gel. DNA was extracted using an adapted protocol and visualized using ethidium bromide on a 1% agarose gel. Strains C02, C05, C06 and wild-type were used for this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutated Gene</th>
<th>Gene Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>C02</td>
<td>rcsC L840R</td>
<td>Transmembrane sensor kinase protein involved in signal transduction. Conveys signals of envelope conditions to the cell and regulates the cps (capsule synthesis) operon [14].</td>
</tr>
<tr>
<td>C05</td>
<td>lpp ΔK26-A39</td>
<td>Meurin lipoprotein. 1/3 tethers the outer-membrane to peptidoglycan layer, providing the membrane structural integrity. The other portion is attached to the outer-membrane and exposed to the cell surface [21].</td>
</tr>
<tr>
<td>C06</td>
<td>ΔyeaI</td>
<td>A diguanylate cyclase that produces diguanylic acid. Diguanylic acid is a secondary messenger noted to reduce early biofilm formation [22].</td>
</tr>
<tr>
<td>yrfF A564P</td>
<td></td>
<td>Inner membrane protein that has been associated with the down regulation of Rcs pathway [23].</td>
</tr>
<tr>
<td>yeaJ’ – partial deletion</td>
<td>Putative diguanylic cyclase that produces diguanylic acid. Noted for its larger involvement in swarming motility at 37 °C [24].</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Mutated genes and their putative actions. Mutations revealed by genomic DNA sequencing of the mutants and the genes’ putative actions.
3.7 Exopolysaccharide quantification

The mutants produce approximately 17 times more methyl-pentose, a constituent of CA, than the wild-type strain. Treated methyl pentose has a distinct peak absorbance at 400 nm. A standard curve was generated using pure L-fucose, a methyl pentose. The standard curve was used to quantify the amount of methyl pentose polymer in the mutants and wild-type. Each mutant produces approximately the same amount of methyl-pentose pentose in whole culture preps.
Figure 10. L-fucose absorbance spectrum and standard curve. Various L-fucose solutions were made from a 1:1 dilution of a 0.1% L-fucose solution. Absorbance spectrum of the various L-fucose solutions measured using a spectrophotometer (A). L-fucose solution $A_{400}$ values were used to generate a standard curve and standard curve equation (B). The standard equation was used to determine the quantity of methyl pentose in the mutant and wild-type samples.
Figure 11. Total methyl pentose polymer absorbance spectrum. Mutant and wild type samples grown for 18 hours at 30 °C post treatment with colorimetric assay. Each mutant produces approximately the same amount of methyl pentose polymer. The mutants produce substantially more methyl pentose polymer than the wild-type. Error bars represent the standard deviation from the mean.

<table>
<thead>
<tr>
<th>Strain</th>
<th>A_{400}</th>
<th>[Methyl Pentose]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.119</td>
<td>85 μM ± 9</td>
</tr>
<tr>
<td>C02 (rcsC L840R)</td>
<td>1.714</td>
<td>1414 μM ± 64</td>
</tr>
<tr>
<td>C05 (lpp ΔK26-A39)</td>
<td>1.701</td>
<td>1404 μM ± 56</td>
</tr>
<tr>
<td>C06 (yea*)</td>
<td>1.672</td>
<td>1379 μM ± 55</td>
</tr>
</tbody>
</table>

Table 3. Total methyl pentose polymer concentrations. Mutant and wild-type methyl pentose concentrations were calculated using the L-fucose standard curve (figure 10) and the A_{400} values from the colorimetric assay.
Figure 12. Supernatant methyl pentose polymer absorbance spectrum. Samples were centrifuged for 30 min, at 15,000 RCF. After centrifugation the supernatant was sampled and the quantity of methyl pentose was determined by performing the colorimetric assay and measuring in a spectrophotometer.

<table>
<thead>
<tr>
<th>Strain</th>
<th>A_{400}</th>
<th>[Methyl Pentose]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.023</td>
<td>5.2 ± 13 µM</td>
</tr>
<tr>
<td>rcsC L840R</td>
<td>1.191</td>
<td>978 ± 58 µM</td>
</tr>
<tr>
<td>lpp ΔK26-A39</td>
<td>0.657</td>
<td>534 ± 50 µM</td>
</tr>
<tr>
<td>yea*</td>
<td>1.026</td>
<td>844 ± 55 µM</td>
</tr>
</tbody>
</table>

Table 4. Supernatant methyl pentose polymer concentration. Values calculated using the standard curve equation in figure 8.
3.8 \textit{waaL} knock-out

Deletion of \textit{waaL} was confirmed by growth on selective media containing kanamycin medium and a cut PCR product shown in figure 11. \(\Delta waaL\), \textit{rcsC} L840R had no effect on total methyl pentose production in mutant \textit{rcsC} L840R. In mutant \textit{lpp} K26-A39, \textit{waaL} deletion substantially reduced total methyl pentose production from 1404 \(\mu\text{M} \pm 56\) (\textit{lpp} K26-A39) to 1144 \(\mu\text{M} \pm 59\) (\(\Delta waaL\), \textit{lpp} K26-A39), an approximate 19\% decrease. \(\Delta waaL\) substantially reduced total methyl pentose production in mutant \textit{yea*} from 1379 \(\mu\text{M} \pm 55\) (\textit{yea*}) to 953 \(\mu\text{M} \pm 59\) (\(\Delta waaL\), \textit{yea*}), an approximate 31\% decrease.

Supernatant methyl pentose production in \(\Delta waaL\), \textit{rcsC} L840R was not significantly altered. \(\Delta waaL\), \textit{lpp} K26-A39 changed the supernatant methyl pentose production from 534 \(\mu\text{M} \pm 50\) (\textit{lpp} K26-A39) to 419 \(\mu\text{M} \pm 59\) (\(\Delta waaL\), \textit{lpp} K26-A39) an approximate 22\% decrease. \(\Delta waaL\), \textit{yea*} had a decrease in supernatant methyl pentose production from 844 \(\mu\text{M} \pm 55\) (\textit{yea*}) to 359 \(\mu\text{M} \pm 57\) (\(\Delta waaL\), \textit{yea*}) a decrease of approximately 57\%. 
Figure 13. Agarose DNA gel of ΔwaaL mutant transduction recipients. *Wild-type, and rcsC L840R control. Mutant transduction recipients were PCR amplified using flanking waaL primers. Successful P1 transduction of waaL::kan was confirmed by growth on selective media and specific restriction digest of the kanamycin resistance gene using Eag1. Labeled ΔwaaL lanes were used for experiments.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total [Methyl Pentose]</th>
<th>Supernatant [Methyl Pentose]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔwaaL, rcsC L840R</td>
<td>1519 µM ± 59</td>
<td>969 µM ± 55</td>
</tr>
<tr>
<td>ΔwaaL, Δlpp K26-A39</td>
<td>1144 µM ± 59</td>
<td>419 µM ± 59</td>
</tr>
<tr>
<td>ΔwaaL, Δyea*</td>
<td>953 µM ± 59</td>
<td>359 µM ± 57</td>
</tr>
</tbody>
</table>

Table 5. ΔwaaL total culture and supernatant methyl pentose polymer concentration.
3.9 Bio-conjugation

Blue, amine functionalized, 1.0 μm, polystyrene micro-spheres were treated with extracted CA. Mutant CA extract was able to keep beads in suspension to varying degrees. Mutant yea* kept the most beads in suspension, followed by rcsC L840R, and then lpp ΔK26-A39. Wild-type beads treated with CA did not keep beads in suspension. Beads quantities in suspension were determined by light scattering 500 nm to 700 nm.
Figure 14. 1.0 μm polystyrene micro-spheres treated with CA extract. Extracted CA samples from the colorimetric assay were coupled to blue, 1.0 μm polystyrene micro-spheres. Photo taken after centrifuging samples for 8 min at 3,200 RCF.

Figure 15. Absorbance spectrum of 1.0 μm polystyrene micro-spheres treated with CA. Samples spun for 8 min at 3,200 RCF. 100 μL of sample was measured in a spectrophotometer.
IV. DISCUSSION

It is curious that a fraction of bacteria remains in the supernatant after a high centrifugal force event. However, the mechanism by which these cells remain in the supernatant after exposure to thousands of centrifugal g-force has never been investigated. In this study the centrifuge resistant trait was characterized by defining growth conditions, genomic DNA sequencing, quantifying EPS production, and using gene deletions. The results reveal that these cells are using a novel mechanism stimulated by independent gene variations to adapt to this new environmental variable.

Preliminary studies revealed that the mutants are not less dense than the medium and are not actively swimming against the force. The mutants can be forced to sediment by increasing the centrifugal force and duration of spin, and wild-type cells have been shown to pellet through the suspension when mixed with the mutants. It seems that the cells are using an energy efficient method to retard their sedimentation when exposed to centrifugal force. Knowing that altering the medium with different carbon sources affects the phenotype’s strength, it is believed that there could be a feedback mechanism regulating this trait dependent on the availability of a carbon source.

Whole genome sequencing of the centrifuge resistant mutants revealed isolated gene mutations in the C02 and C05 strains, whereas the presence of three separate gene mutations in the C06 strain suggests that multiple pathways may be contributing to the production of CA in synergistic manner.
RcsC is a transmembrane hybrid histidine kinase located in the inner membrane of *E. coli*. RcsC has been noted to modulate capsule synthesis by transmitting signals of envelope conditions to the cell [25]. Genomic DNA sequencing of the C02 mutant revealed a missense mutation in *rcsC* L840R. The change from a non-polar leucine to a charged arginine could result in a conformational shift in the protein. This conformational change could be causing an upregulation of the *Rcs* pathway, thus stimulating capsule synthesis.

The lipoprotein *lpp* is noted as one of the most numerous proteins in *E. coli*. It was discovered by Cowels et al. that two different forms of Lpp, a free and a bound form, occupy different regions in the cell. The paper reveals that the free form is exposed to the cell surface and the bound form links the peptidoglycan layer to the outer-membrane. This protein plays a major role in the membranes structural integrity. In the absence of Lpp, the cell has increased membrane permeability and periplasmic leakage [26]. Mutant C05 revealed a deletion in *lpp* K26-A39. Knowing the role of Lpp in membrane structural integrity, it is possible that this deletion is triggering the cell’s envelope stress response system and therefore causing the cell to be in a constant capsule production state [27].

Previous research performed by Sanchez et al. noted the involvement of *yeaI* in the regulation of swarming motility and pre-biofilm formation in *E. coli*. YeaI and YeaJ are diguanylic cyclase proteins that synthesizes the secondary messenger diguanylic acid [22]. The paper shows that in ΔyeaI cells, the production of diguanylic acid is decreased, resulting in an increase in early biofilm formation. YeaJ is thought to be involved in decreasing swarming motility when *E. coli* is grown
at 37°C on solid medium. In mutant C06, yeai is fully deleted and yeaj is partially deleted. Based on the results from Sanchez et al.’s paper, it is possible that the deletion of yeai is diminishing the production of diguanylic acid, thus signaling a transition into a biofilm growth phase. Due to YeaJ’s high activity at 37°C and decreased activity at lower temperatures, it is believed to have little involvement in C06’s phenotype because these cells were selected at 30°C and experiments were carried out at 30°C [24].

Is has been noted by Dominguez-Bernal et al. that yrfF down-regulates the Rcs pathway in Salmonella. Rcs regulates a multitude of pathways, one of particular note is the capsule synthesis operon (cps). Interestingly, the deletion of yrfF is lethal [23]. For a cell to survive a yrfF deletion, the Rcs pathway must also be inactivated. Genomic DNA sequencing of C06 revealed a missense mutation in yrfF A564P. Due to proline’s unique R-group structure and bulkiness compared to alanine, it is possible that this caused YrfF’s conformation to shift. If YrfF’s conformation is altered to a less active or inactive state it could diminish the regulation of Rcs. This would secondarily cause the upregulation of the cps operon resulting in EPS capsule production.

Because of the distinct mucoid phenotype as well as the mutated genes associated with capsule synthesis and biofilm formation, it was hypothesized that the mutants are increasing CA production [28]. Quantification of the total methyl pentose polymer confirmed a hyper-production of methyl-pentose, a unique sugar in CA. All the mutants produce approximately the same amount of CA, however, they each differ in their ability to resist centrifugal force. By quantifying the amount of unattached CA it was discovered that the weakest mutant, rcsC L840R, seems to be
freely excreting more CA into the medium than the stronger mutants *lpp* K26-A39 and ΔyeaI, yeaJ, yrfF A564P. Based on this result it seems that the mutants that are more robust at resisting centrifugal force have a higher proportion of CA attached to their cell membranes.

It was discovered by Meredith et al. that *waaL* can transfer CA to the LPS-core molecule in the periplasm. This is subsequently flipped to the outer-membrane in a less understood process [29].

![Figure 16. WaaL mechanism of EPS attachment to LPS.](image)

To determine if CA is covalently attached to the membrane or secreted into the extracellular space, *waaL* was deleted in the mutants. The ΔwaaL, rcsC L840R mutant had a negligible effect on total CA production, however, the total production of CA in the ΔwaaL, *lpp* ΔK26-A39 and ΔwaaL, ΔyeaI, yeaJ, yrfF A564P did decrease. Quantifying the proportions of attached and unattached CA, the results of the *waaL* deletion are inconclusive with regards to *waaL*’s effect on CA attachment. However, these results reveal that there must be other mechanisms independent of *waaL*, involved in the majority of CA export and presentation to the cell’s surface. It is now
hypothesized that the Wza translocon might play a major role in the simultaneous linkage and export of CA in a continuous fashion, possibly altering between attachment of long CA polymers to the inner-membrane via undecaprenyl-diphosphate and secretion of CA polymers into the extracellular space [31]. Based on these results it is now thought that these large EPS polymers are covalently linked to the inner-membrane and jutting out into the extracellular space, potentially revamping the conventional thought process that EPSs are linked to the outer-membrane and exposed to the extracellular environment.

Figure 17. New predicted EPS covalent attachment model via Wza translocon. EPS polymers are simultaneously built on undecaprenyl-diphosphate and exported out of the cell by Wza. The polymer remains covalently linked to the undecaprenyl-diphosphate molecule in the inner-membrane. This pathway has the ability to shed or retain the linkage of this polymer [31].

The extracted CA samples coupled to the polystyrene microspheres revealed that the amount of beads retained in suspension was not dependent on the concentration of CA. Each strain produced approximately the same amount of CA, however the CA extracts varied with their ability to keep
the microspheres in suspension. This suggests that the mechanism by which the cell produces CA and presents CA to the cell membrane might have an effect on its reactivity with other objects.

**Future studies and applications**

Now that the basis of the mechanism for centrifuge resistance is known, the next step would be to completely delete the genes responsible for CA production. This would confirm whether CA is solely responsible for the centrifuge resistant mechanism and could potentially reveal another mechanism that could compensate for its absence. Another important study is to determine if the genes involved in centrifuge resistance are dominant or recessive. This could be done by transforming a plasmid containing wild-type genes into the mutants and seeing which phenotype results. Determining the inheritance pattern would allow better understanding of how this process is regulated, and reveal potential ways to control it. Controlling this mechanism could optimize processes in industrial settings where microbes are utilized.

In light of the variability of the mutants’ CA reactions with the microspheres, the bio-conjugation reaction needs to be optimized by determining the most reactive functional groups and suitable reaction conditions. Once the transferability of these optically transparent biomolecules to other particles is achieved, controlling phase separation of heterogeneous mixtures can be envisioned.

EPS production by this mechanism appears to have the ability to attach EPS to the OM or secrete EPS into the extracellular medium. Investigating the signals involved in the decision to attachment EPS or secrete EPS may have implications in the medical field where EPSs are known virulence factors and cells secreting EPS can have an added advantage of evading a host’s immune system.
V. REFERENCES


