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Aaron Li 4/11/17

Restricted Evolution of Gph to Rescue *ΔserB E. coli* through Motility Selection

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Abstract

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Under selective conditions, the promiscuous catalytic functions by enzymes can often be targets for evolution. Enzymes with beneficial mutations that affect its catalytic ability with these side-reactions can evolve to become more efficient at its new function. This phenomenon has been demonstrated in auxotrophic *ΔserB Escherichia coli* where the phosphoglycolate phosphatase, or Gph, protein was found to be one of three proteins that rescued the phosphoserine phosphatase ability of the *E. coli*. However, mutation suppression is often achieved through multiple, different mechanisms and so the evolutionary extent of any particular mechanism cannot be easily elucidated. Using plates with semisolid media and chemoattractants, the auxotrophic cells with the fittest mutations were selected for through their extent of motility. Alternating between using this motility selection to evolve the Gph protein in *E.coli* with mutations in serine catalysis, *ΔserB,* and DNA repair mechanisms, *ΔmutS,* and then chemically transforming its *gph* plasmid, we demonstrate a method to specifically evolve the *gph* gene without accumulating mutations in the genome, thereby restricting evolution to only Gph. A custom plasmid containing the *gph* gene and other inducer and antibiotic genes was first constructed and transformed into *ΔserB ΔmutS E. coli* before evolution on the semisolid media. We also use a similar method to show the possibility of semi-continuous evolution of Gph. Instead of selecting for and transforming the plasmids of the fittest cells into new ones, we aimed to show that conjugation can be an alternative mechanism of gene transfer to evolve the *gph* plasmid.

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I am thankful for Dr. Matsumura's assistance in discussing my experiments and helping me troubleshoot problems in my experimental design or technique. In addition to my adviser's mentorship, I am also grateful for the help of my fellow lab members Hussein Salameh and Brindar Sandhu for their support and advice throughout my project.

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Introduction:

Evolution underpins the ability of a species to adapt to its environment. While evolutionary changes confer fitness advantages to a species in its niche, these advantages can often be attained from multiple, different molecular changes. Antibiotic resistance in bacteria, for instance, can be achieved through different mechanisms such as modified efflux pumps or the presence of enzymes that degrade antibiotics like β-lactamases (Soto, 2013). Often these macroscopic evolutionary consequences begin from molecular changes in enzymes. In addition to their primary reactions, enzymes are able to catalyze secondary reactions though with much lower affinities. This substrate ambiguity of enzymes characterizes their ability to catalyze the same reactions towards multiple substrates (Miller, 2004). In addition, their catalytic promiscuity refers to their potential for catalyzing reactions outside of their function (Bornscheuer et al, 2005). In spite of the low catalytic efficiencies associated with substrate ambiguity and catalytic promiscuity, sufficiently strong selective pressures have been shown to favor the evolution of these secondary catalysis reactions to suppress mutations (Patrick et al, 2007). Often, the suppression of a mutation is achieved through multiple compensatory mechanisms, similar to antibiotic resistance, as demonstrated in auxotrophic *ΔserB E. coli* strains where the evolved colonies were found to be rescued by one of Gph, HisB, or YtjC with evolved phosphoserine phosphatase-like activity (Yip and Matsumura, 2013). While mutation suppression through multiple mechanisms is common and beneficial, we seek to develop a method to strictly evolve a specific protein, in this case the protein phosphoglycolate phosphatase (or Gph), in order to focus on the effects and extent of evolution on this protein and how these changes impact the organism's fitness. We intend to construct a custom plasmid containing Gph and other inducer or antibiotic genes and insert it into a Δ*serB* Δ*mutS* double mutant *E. coli* strain to observe how

mutations accumulating in the plasmid's *gph* gene may compensate for the mutant's lack of phosphoserine phosphatase ability. Selection for evolved mutants will be performed on semisolid agar plates with chemoattractants, where cells are inserted onto the center of the plate and the fittest ones are those that manage to migrate outward the farthest (Topp and Gallivan, 2007). To prevent chromosomal mutations from contributing to the fitness of the host strain, chemical transformations will be performed to transfer the evolved *gph* plasmids into fresh Δ*serB* Δ*mutS E. coli* strains after every round of motility selection. Crystallographic and kinetic assays will then be performed and compared to that of native Gph to observe what evolutionary changes allowed Gph to better catalyze serine. In particular, we posited that the accumulation of mutations will cause the Gph's active site to more closely resemble that of phosphoserine phosphatase.

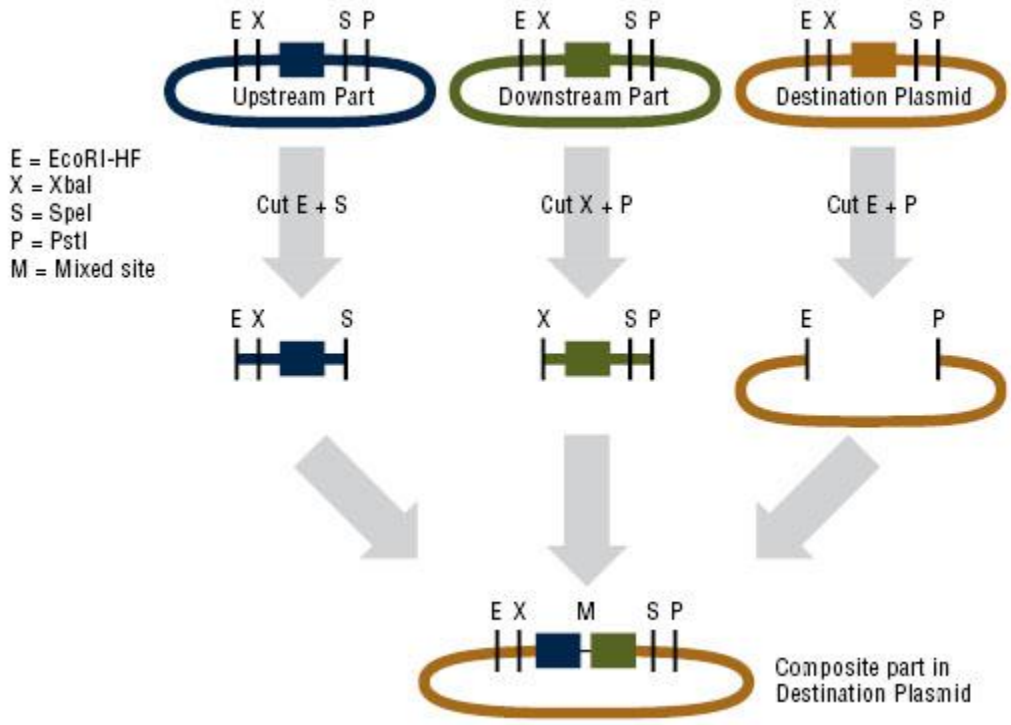
In addition to motility selection, we also attempt to develop a semi-continuous method to evolve the *gph* plasmid using bacterial conjugation. Whereas our motility selection followed by transformation requires us to isolate and transform plasmids into new strains frequently, we seek to use bacterial conjugation instead of transformation as a means of delivering the evolved plasmid into new strains to mimic a more natural process of evolution. We hypothesized that in both motility selections, Gph will be the only protein that will rescue the Δ*serB* mutation but the semi-continuous method would facilitate evolution with less manual labor. However, we acknowledge that using conjugation may not necessarily evolve Gph faster than using transformation because cells may fail to conjugate under too stringent conditions.

Material and Methods:

Plasmid Preparation Protocol:

Liquid cultures were centrifuged at 13,000 rpm for 2 minutes to form pellets. After centrifugation, supernatant was disposed and pellets were resuspended in 250 μL of P1 buffer (Tris-EDTA) using a vortex. After pellets were resuspended, 250 μL P2 buffer (SDS and NaOH) was added to lyse the cells (Lezin et al, 2011). To neutralize the lysation process and allow for plasmid binding, 350 μL of N3 buffer (Guanidinium hydrochloride) was added after one minute. Tubes were then centrifuged at 13,000 rpm for 10 minutes at 4°C. After centrifugation at 4°C, the supernatant was extracted and inserted onto Qiagen filter tubes along with 750 μL of PB buffer, a binding buffer. Tubes were once again centrifuged for 1 minute at 13,000 rpm at room temperature to filter out all non-plasmid cellular components. After each centrifugation, the spin columns, containing the plasmid, were transferred to new 1 mL tubes. For the last centrifugation, the spin columns were transferred to new 1.5 mL tubes and 50 μL of EB, an elution buffer containing Tris-Cl, was added to elute the plasmid DNA onto the new tubes.

BioBrick Assembly Method:



Our plasmids were designed to contain four restriction sites in a particular sequence-EcoRI-HF, XbaI, SpeI, and PstI- collectively called a BioBrick (Sleight and Sauro, 2013). The BioBrick model is unique in that the restriction sites XbaI and SpeI can be ligated with one another to form a non-restriction site. This allows a gene of interest to be cut and inserted into another plasmid with a BioBrick region while preserving all four BioBrick restriction sites. In general, a gene segment that is to be inserted upstream of a set of genes in a pre-existing BioBrick will be cut at the restriction sites EcoRI and SpeI while the insertion vector that contains the pre-existing BioBrick will be cut at sites XbaI and PstI (Shetty et al, 2008). On the other hand, gene segments to be inserted downstream will be cut at XbaI and PstI while the insertion vector will be cut at EcoRI and XbaI.

Restriction Digest Protocol Using BioBrick sites:

We performed restriction digests for the inserts and vector plasmids separately in 100 μL solutions. Our plasmids that served as the vector needed to be cut only by two restriction enzymes since the region of interest was much larger than the unwanted segment (Schuler and Zielinski, 1989). Due to this size difference, the unwanted segment was later filtered out. However, those that served as inserts, with the region of interest being smaller than the unwanted segment, was cut with a total of 4 restriction enzymes because filtration could not be used to eliminate the unwanted segments (Matsumura, 2016). Two were used to cut out the region of interest, leaving the unwanted segment, and another two were used to cut out small segments slightly before and after the ends on the unwanted segments, leaving short DNA segments called neutramers (Matsumura, 2016). Since the unwanted segment could not be filtered out, cutting out these neutramer ends rendered the unwanted segment unable to interfere in the ligation step (Matsumura, 2016). Each solution contained 2 μg of insert or vector plasmid, 10uL of New England Biolabs CutSmart Buffer, 2μL of each required restriction enzyme and filled with deionized H_2O until volume reached 100μL. Prefix or suffix neutramers were added to their respective mixtures and incubated at 37°C overnight. To confirm proper cuts, 1 μL of each of the digests along with its corresponding uncut counterpart was inserted into separate agarose wells with loading dye for electrophoresis and ran at 160V for about 25 minutes. Gel red was used to stain the gel for 20 minutes and sizes were estimated using the Clone Manager diagrams of the original plasmids along with visual comparison to the Lambda HindIII ladder.

GeneRead Size Selection:

Since GeneRead size selection filters out DNA fragments less than 150 bps, we performed the procedure to filter out the small unwanted segment that remained when a region of interest that was larger was cut out. 400 μL of buffer SB1 was added to 100 μL of our restriction digests. The solution was then inserted onto MinElute spin column and centrifuged for 1 minute at 13,000 rpm in order to discard the flow-through. 700 μL of 80% ethanol was then added to the spin column and centrifuged again for 1 minute at 13,000 rpm. Afterwards, the spin column was centrifuged dry at the same speed and time. The column was transferred over to a 1.5 mL centrifuge tube where 30 μL of buffer EB was finally added to the column and spun at 13,000 rpm for 1 minute to elute the DNA.

Ligation:

20 fmoles of both vector and insert were mixed with 6 μL of EcoRI unlinker and 6 μL SacI unlinker along with 1 μL of 1/20 diluted NEB T4 DNA ligase. 4 μL of 5x Invitrogen T4 DNA ligase buffer was then added and deionized H_2O was added until the total solution was 20 μL. Five ligation tubes were made in this manner with four serving as controls: 1) vector but no insert and ligase, 2) vector and ligase but no insert, 3) vector and insert with ligase, 4) insert but no vector or enzyme, and 5) insert and ligase but no vector. All five mixtures were placed in a thermocycler cycling between 10°C for 30 seconds and 30°C for 30 seconds overnight.

Transformation:

200 μL of competent cells frozen at -80°C were thawed on ice. Once thawed, 3.4 μL of 1.4M 2-Mercaptoethanol was added to the competent cells in order to improve transformation efficiency (Ito et al, 1983). 25 μL of the competent cell mixture was then inserted into each PCR tube at

4°C (up to 8 tubes) on a thermocycler. 1.25 ng of DNA to be transformed was added to each tube and the thermocycler setting was changed to “Heat Shock” which kept the cells at 4°C for 30 minutes and raised the temperature quickly to 42°C for 40 seconds and then back down to 4°C (Froger and Hall, 2007). After the heat shock, 100 µL of SOC broth was added to each PCR tube and the thermocycler setting was changed to 37°C for 60 minutes. Once the hour was up, 100 µL of the cells in each tube were placed onto plates containing a mix of antibiotics that select for the transformant. For every plasmid to be transformed, a negative control was also made, in which the same transformation protocol was performed but with no plasmid added, to ensure transformation results were verified.

Synthesis of T1-lacI-PT5-sfBFP-gph-IMBB2-pUC57-mini:

Our goal was to isolate genes of interest from a plasmid, cut and insert them upstream or downstream into another vector possessing the BioBrick sites, and transform the new plasmid into new *E. coli*. Liquid cultures of *E. coli* strains containing T1-lacI-PT5-rpmH-sfBFP-IMBB2-pUC57-mini and gph-IMBB2.4-pUC57-mini were each inoculated in 2 mL LB broth and 2µL(*antibiotic) overnight at 37°C on a rotator. Plasmids from both were simultaneously isolated using the aforementioned protocol. Restriction digests were then performed using T1-lacI-PT5-rpmH-sfBFP-IMBB2-pUC57-mini as the vector (70 µL of plasmid, 10 µL of 10x NEB CutSmart buffer, 2 µL SpeI-HF, 2 µL PstI-HF, and 16 µL deionized H_2O) and gph-IMBB2.4-pUC57-mini as the insert (86 µL of plasmid, 10 µL of 10x NEB CutSmart buffer, 1 µL XbaI, 1 µL PstI-HF, 1 µL EcoRI-HF, and 1 µL SacI-HF) and both digests were placed at 37°C overnight. After 16 hours, 10 µL of 1 µM suffix neutramer was added to the vector solution while 10 µL of 1 µM prefix neutramer and 10 µL of 1 µM PstI-SacI neutramer was added to the insert solution. Both mixtures were heated to 70°C on a heat block and cooled back down to room temperature.

GeneRead size selection purification was then conducted on each mixture to remove the neutramer-bound ends. Putting 2 μL of vector solution and 0.6 μL of insert solution into the corresponding 20 μL ligation mixtures, the five ligation tubes were left overnight. 1 μL of each ligation sample was then used for gel electrophoresis to observe for proper ligation. 3 μL of the vector and insert ligated solution was used for chemical transformation into Mach1 competent cells. Transformed cells had their plasmids extracted for gel electrophoresis and restriction mapping to confirm the plasmid's identity as the one predicted using Clone Manager.

Synthesis of RP4 oriT-T1-lacI-PT5-rpmH-sfBFP-gph-pUC57-mini-chlR:

The exact same procedure as the synthesis of T1-lacI-PT5-sfBFP-gph-IMBB2-pUC57-mini was used with only differences in solution volume in the digests and ligation. This time T1-lacI-PT5-sfBFP-gph-IMBB2-pUC57-mini served as the insert and RP4 oriT-pUC57-mini-chlR was used as the vector. The vector solution consisted of 50 μL of plasmid, 10 μL of 10x NEB CutSmart buffer, 2 μL EcoRI-HF, 2 μL XbaI-HF, and 36 μL deionized H_2O and insert solution consisted of 74 μL of plasmid, 10 μL of 10x NEB CutSmart buffer, 2 μL EcoRI, 2 μL SpeI-HF, 2 μL PstI-HF, and 2 μL AatII-HF. After overnight at 37°C, 10 μL 1 μM BioBrick prefix neutramer was added to the vector solution and 10 μL 1 μM BioBrick suffix neutramer along with 10 μL 1 μM AatII-EcoRI neutramer was added to the insert solution. GeneRead size purification was again performed on both vector and insert solutions to remove the neutramer bound ends. The ligation mixtures were made using 3.4 μL of vector solution and 0.3 μL of insert solution in each of the 5 tubes. After ligation, 1.12 μL of the vector-insert ligated sample was transformed into Mach1 competent cells. Once again, restriction mapping and gel electrophoresis was performed on the final plasmid to confirm its identity.

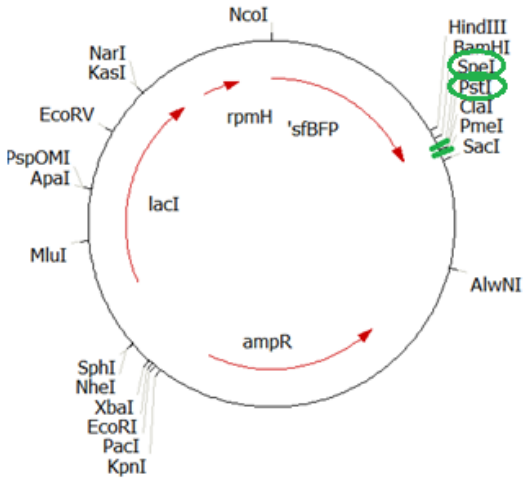


Figure 1- T1-lacI-PT5-rpmH-sfBFP-IMBB2-pUC57-mini (4517 bps) served as the vector and was cut at SpeI and PstI for gph to be inserted downstream there.

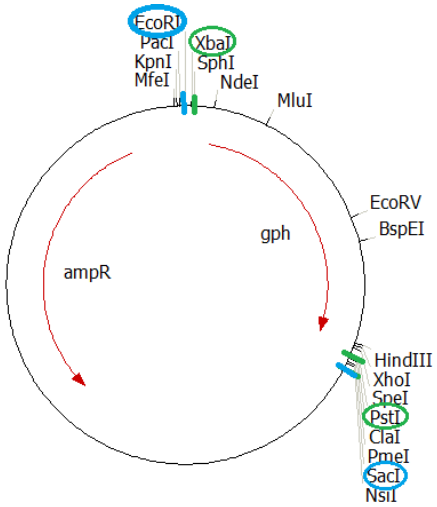


Figure 2- gph-IMBB2.4-pUC57-mini (2755 bps) served as the insert and was cut at XbaI and PstI for gph to be inserted downstream in T1-lacI-PT5-rpmH-sfBFP-IMBB2-pUC57-mini. Targeted restriction sites are shown in green while neutramer sites are shown in blue.

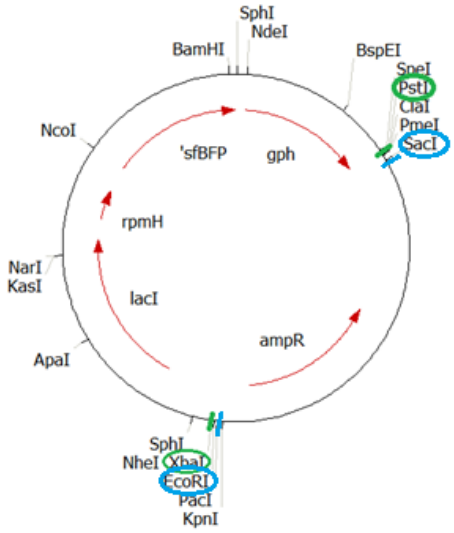


Figure 3 - T1-lacI-PT5-sfBFP-gph-IMBB2-pUC57-mini (5348 bps) served as the insert and was cut at XbaI and PstI for gph segment to be inserted downstream of RP4 oriT-pUC57-mini-chlR. Targeted restriction sites are shown in green while neutramer sites are shown in blue.

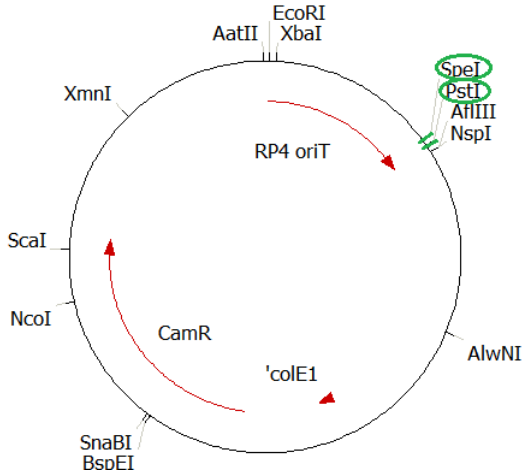


Figure 4- RP4 oriT-pUC57-mini-chlR (2684 bps) served as the vector and was cut at SpeI and PstI for gph to be inserted downstream after the RP4 oriT region.

RP4 oriT-T1-lacI-PT5-rpmH-sfBFP-gph-pUC57-mini-chlR (6066 bps):

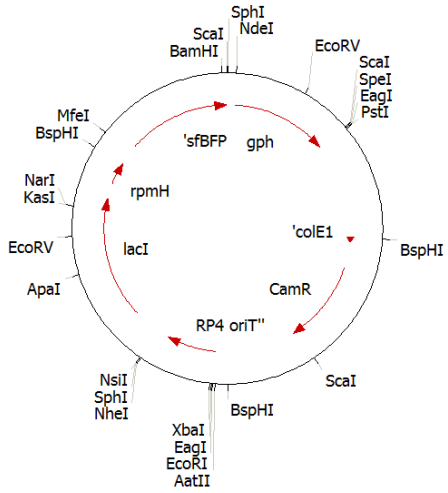


Figure 5- The final product RP4 oriT-T1-lacI-PT5-rpmH-sfBFP-gph-pUC57-mini-chlR (6066 bps).

P1 Transduction:

We used phage transduction as a means of extracting the Δ*serB* gene and inserting this gene into another strain with the Δ*mutS* gene to create our double mutant (Moore, 2011). The strain whose gene was extracted was called the donor and the strain to which the phages would to deliver through homologous recombination the gene was called the recipient (Liljeström et al, 1985). 2 mL of overnight culture of donor strain was grown and diluted 100-fold into LB with 10 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.2% glucose. Cells were grown at 37°C until early log phase (about 2 hours) and then 40 µL of P1 phage lysate was added to the culture. Culture was left to grow until lysis occurred, signified by a halting of growth. 100 µL of chloroform was added to the lysate and then the solution was centrifuged at 13,000 rpm for 2 minutes. The supernatant was transferred to a new tube and 4 drops of chloroform were added to store the tube at 4°C. 2 mL of recipient strain that was grown overnight was centrifuged at 6000 rpm for 2 minutes and resuspended in 500 µL of LB with 10 mM $MgSO_4$, and 5 mM $CaCl_2$. 100 µL of P1 lysate was then added to 100 µL of recipient cells and 100 µL of LB was added to another 100 µL of

recipient cells, as a negative control. Both mixtures were incubated at 37°C for 30 minutes and then plated onto selective media.

Competent Cell Formation:

Prior to transforming our designed plasmid, the double mutant strain must be made competent to accept the foreign DNA (Inoue et al, 1990). Strains of our double mutant *E. coli*, with and without the RP4 plasmid, were grown overnight at 37°C in 2 mL liquid cultures with 2 μL of kanamycin, tetracycline, and/or ampicillin. Each culture was then diluted 100-fold, 0.5 mL into 50mL of fresh LB with the same antibiotics, into a flask and allowed to grow at 37°C until mid-log phase. The optical density of the samples were measured using a spectrophotometer and once the optical density reached 0.4-0.6 (took about three hours of growth), the cultures were transferred to 50 mL falcon tubes and centrifuged at 3000 rpm for 10 minutes at 4°C using the JA-10 rotor. The supernatant was then disposed of and the cells were gently re-suspended in 20 mL of ice-cold TB buffer. After resuspension, cultures were spun again at 3000 rpm for 10 minutes at 4°C using the JA-10 rotor. Supernatant was then dumped and cells were re-suspended in 4 mL of ice cold TB buffer along with 280 μL of dimethyl sulfoxide, or DMSO. 200 μL aliquots of cells were then placed into 600 μL tubes on dry ice and then frozen at -80°C.

Creation of Δ*serB* Δ*mutS* with T1-lacI-PT5-sfBFP-gph-IMBB2-pUC57-mini:

Δ*serB* keio strain was grown overnight to serve as the donor strain and Δ*mutS* BMH 71-18 served as the recipient strain for P1 transduction. After transduction, the new double mutant was made competent using the aforementioned protocol. The competent Δ*serB* Δ*mutS* strain was then transformed with the T1-lacI-PT5-sfBFP-gph-IMBB2-pUC57-mini plasmid.

Creation of Δ*serB* Δ*mutS* with RP4 plasmid and RP4 oriT-T1-lacI-PT5-rpmH-sfBFP-gph-pUC57-mini-chlR

We used the double mutant created by the P1 transduction earlier and conjugated the RP4 plasmid into it. The same competent cell procedure was performed to make the double mutant with RP4 competent. Finally, the RP4 oriT-T1-lacI-PT5-rpmH-sfBFP-gph-pUC57-mini-chlR plasmid was transformed into the Δ*serB* Δ*mutS* with RP4.

Aspartate Motility Media:

In order to select for mutations that rescue Δ*serB,* a minimal media plate was designed with aspartate acting as a chemoattractant to motivate the fittest cells to migrate outside the center of the plate (Mesibov and Adler, 1972). For 400 mL, 0.25% agar (1g) was added in 300 mL of deionized H_2O and autoclaved for 1 hour. A separate flask containing 4.51g of Difco M9 minimal media was added to 80 mL deionized H_2O and autoclaved separately for an hour. After autoclaving, both solutions were mixed together and allowed to cool to room temperature. 8ml of 20% glucose was filter pipetted into the mixture along with 800 μL of 1M $MgSO_4$, 40 μL of $CaCl_2$, and 200 μL of Isopropyl β-D-thiogalactopyranoside (IPTG). 400 μL of 1000x kanamycin, tetracycline, and ampicillin were also added to ensure selectivity and preservation of the plasmids in our strains. 0.016g of aspartate (3×10^{-4} M) was added last as the chemoattractant.

ΔserB Dropout Media:

For 1 L, 0.25% agar (2.5g) was added to 400 mL of deionized H_2O and autoclaved for 1 hour. Skipping serine, 0.1302g of leucine and then 0.0261g of the rest of the 18 amino acids were added to 600 mL of deionized H_2O. The amino acid solution was then filter sterilized and

inserted into the autoclaved agar solution. 1 mL of 1000x kanamycin, tetracycline, and ampicillin was then added last.

Motility Selection:

We used motility as a means of selecting for the fittest mutants as the fittest mutants would Using either the aspartate or dropout media, we pipetted 3 μL of our strain onto the center of the semisolid plate. Cells are allowed to grow until they reach the ends of the plate where the ones that reach the ends are extracted using a pipette and re-grown in liquid culture. Plasmids will then be extracted and transformed into a new batch of the same cell types for re-plating.

Semi-continuous evolution:

We sought to create 4 types of Δ*serB* Δ*mutS* with RP4 strains each with different plasmids: 1 type with RP4 oriT-T1-lacI-PT5-rpmH-sfBF-gph-pUC57-mini-chlR (as our experimental strain), 1 type with Plac-lacO-rfp-TT-IMBB2s-pUC57-chlR (as our negative control), 1 type with pCDF-Ptac-lacOc-ampC ADC-33 (provides ceftotaxime resistance) and the last type with pCDF-Ptac-lacOc-aph(3')-Via (provides amikacin resistance). Both the experimental and negative control strains were to be evolved under the same semisolid motility plates and the ceftotaxime and amikacin strains were to be used to select for conjugation recipients and against donors.

Results

Figure 6 – The semisolid M9 minimal media with aspartate plate with RP4 K-12 E.coli at day 5. Both the RP4 K-12 and ΔserB, ΔmutS with gph strains looked exactly the same from day 2 to 5. All the cells appeared to be clumped in the center, which is evidence of growth, but no migration occurred.

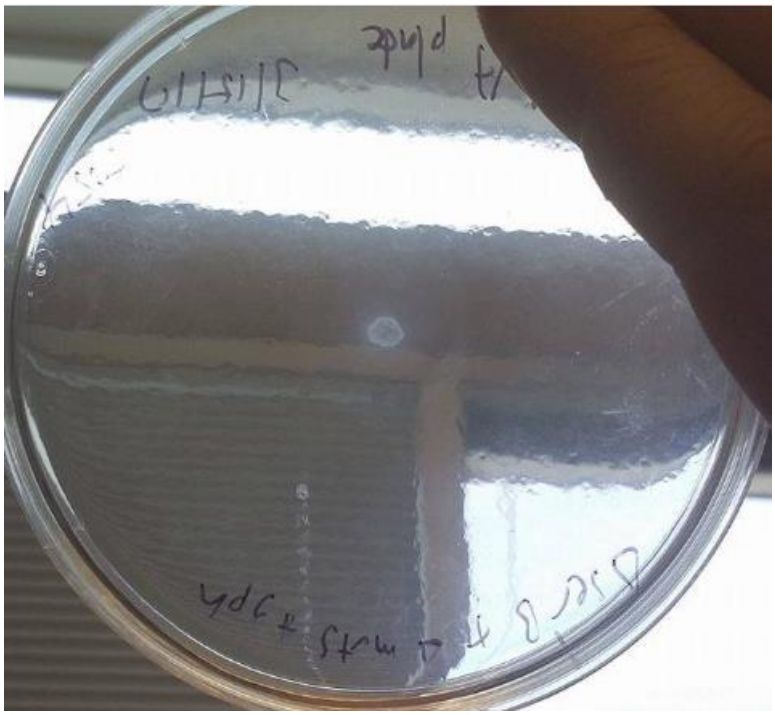


Figure 7- The semisolid ΔserB dropout media plated with ΔserB, ΔmutS with gph at day 7. Growth and migration were difficult to see during first few days but on the 7th day, a large cloudy ring formed around the center. This indicated that the cells are sparse and are in the process of migration.

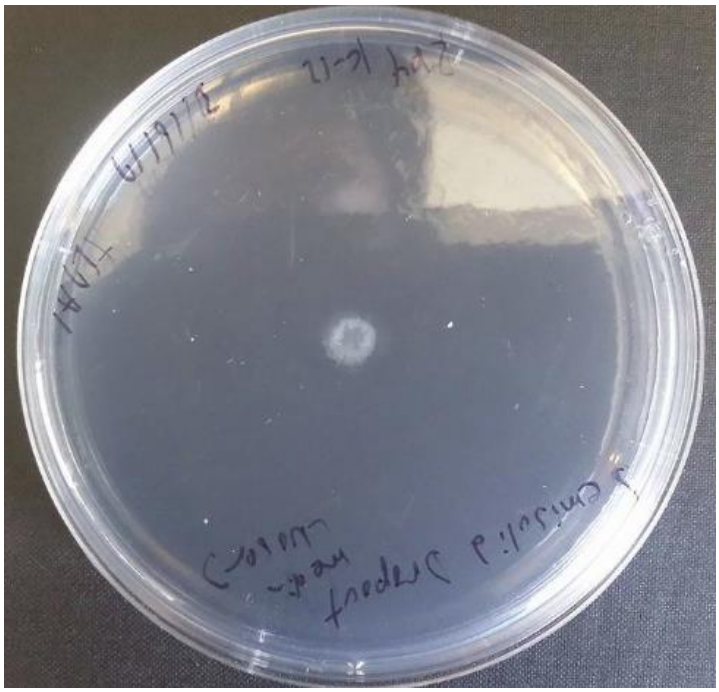


Figure 8- The semisolid ΔserB dropout media plated with RP4 K-12 E.coli at day 6 (positive control). The cells are very dense in the center and are slowly migrating away. We note that the cells are dense are wild-type yet they did not migrate as far as our experimental strain. This could potentially be attributed to the presence of the RP4 plasmid and its metabolic burden on the cells.

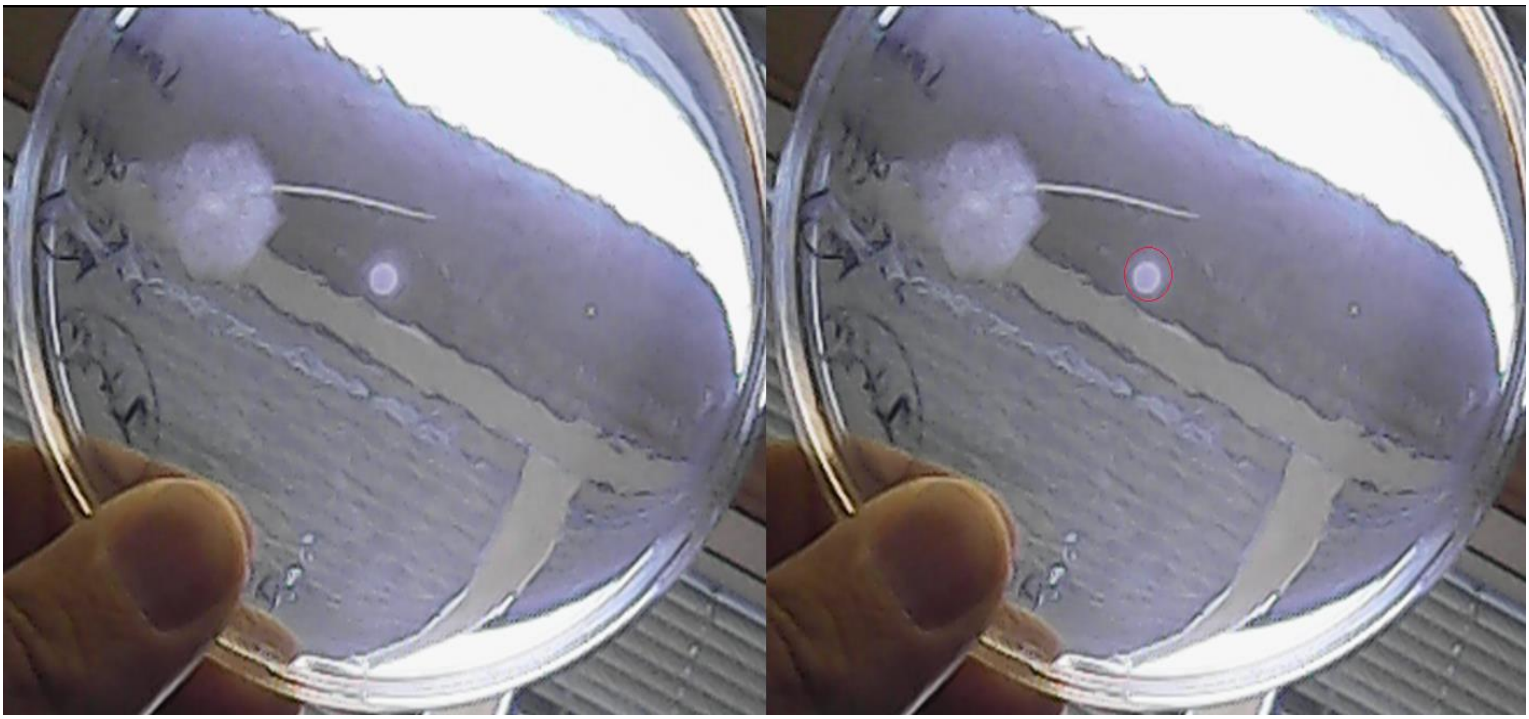


Figure 9- After growing the double mutant on serine dropout media until a white halo appeared, the outermost strains were isolated and regrown onto a new serine dropout media plate. This first generation double mutant was grown at the same time at 30°C as the original double mutant and within three days, the first generation double mutant grew denser and migrated outwards quicker. Both left and right pictures are of the first generation double mutant but the right photo indicates the circumference of the strain's migration with the red circle. Contamination was present on the left side of the plate but did not affect the evolution of the strain.

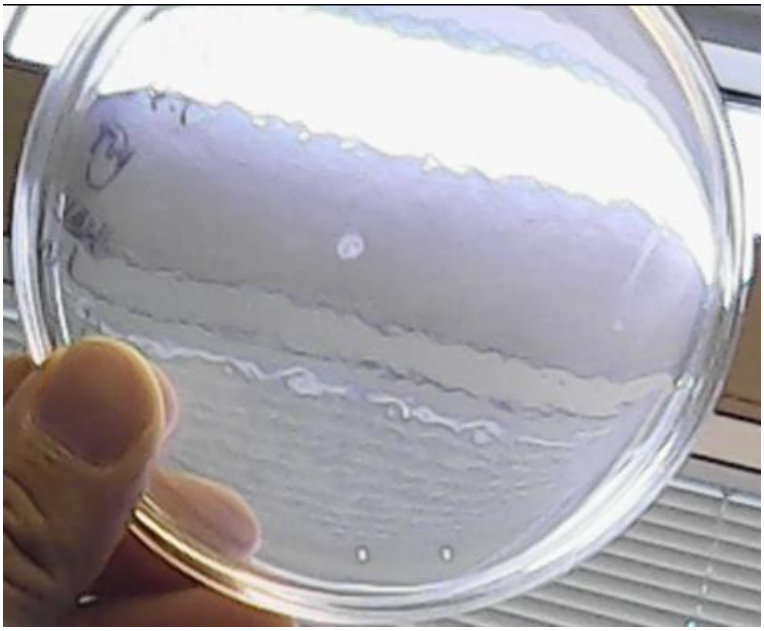


Figure 10- Growth of the original double mutants (ΔserB, ΔmutS with gph) on serine dropout media at the same time as the first generation double mutants. The white circle indicates the point where the cells were spotted but no white halo was present around it signifying no migration yet.

A week after plating, the aspartate motility media plates showed no signs of motility. Both the positive control, RP4 K-12, and Δ*serB*, Δ*mutS* with *gph* did not migrate out of the center of the plate where they were originally pipetted. The alternative Δ*serB* dropout media was used instead and after five days of growth, a circular ring formed around the center in the Δ*serB*, Δ*mutS* with *gph* plate, suggesting motility in progression. While the RP4 K-12 grew densely and migrated slightly more than the aspartate plates, they did not migrate as far as the Δ*serB*, Δ*mutS* with *gph* strains. Another trial was conducted putting the double mutant onto serine dropout media but after three days when signs of migration were immediately present—a white halo around the spotted area formed—the outermost cells were isolated and re-plated onto fresh serine dropout plates. When these first generation double mutants were grown alongside the original double mutants, the first generation double mutant had greater migration than the original ones by the third day.

For the semi-continuous evolution, all but the last plasmid—pCDF-Ptac-lacOc-aph(3')-Via—were successively transformed into Δ*serB* Δ*mutS* with RP4. Since the last strain was crucial for conjugation selection, no progress could be made until the plasmid is successively transformed.

Discussion:

Previous attempts of evolution via motility selection were used to select for antibiotic resistance and so our goal was to observe if the same procedure could be used to evolve mutation-suppressing genes in auxotrophic bacteria. The aspartate semisolid media design was derived from M9-minimal media recipe. However, we used 0.25% agar instead of 1.25% as recent experiments have shown this agar concentration to be optimal for facilitating motility and included aspartate to act as a chemoattractant for the cells to migrate outwards (Mesibov and Adler, 1972). Our first attempt of motility selection using the aspartate media showed evidence of cellular growth but no migration. We suspected that the root cause was due to the overabundance of nutrients in the plate, namely glucose, because an excess of chemoattractants decreases the cells' motivation to migrate and scavenge for resources (Topp and Gallivan, 2008). Other possible factors including agar viscosity were unlikely major causes of the immotility as the same agar concentration of 0.25% had been used with success in other unrelated motility experiments in our lab. This was confirmed when the ΔserB dropout media was designed with the same agar concentration yet signs of motility were evident after 3 days. The ΔserB dropout media recipe was inspired by the previous success of others using semisolid tryptone media for motility selections of antibiotic resistance (Wolfe and Berg, 1989). Since all amino acids but serine were included and 9 of these are well known to be chemoattractants, we excluded glucose to test if its excess was a primary cause of the immotility in the previous aspartate plate (Mesibov and Adler, 1972). Indeed the replacement of glucose with sparse amino acids increased motility, however we note that our motility rates were significantly slower than that of analogous motility assays. Topp and Gallivan's work in motility selection for β-galactosidase activity observed migration in 14 hours while our motility selection did not show signs of migration until

three days in. Even our K-12 Keio strain with RP4 serving as the positive control, did not migrate much faster, as migration was observed after two days, than our experimental strain which suggested that our media recipe could be further revised to promote motility. A natural idea would be to consider if our amino acid concentrations could be lowered as a means to motivate the cells to migrate outwards. Since this was likely the source of immotility in our aspartate plates and the threshold values for amino acid chemotaxis are well documented, a possible revision would be to optimize the concentrations of the 19 amino acids to near their threshold values (Mesibov and Adler, 1972). Our Δ*serB* dropout plate recipe was originally designed to observe whether motility selection could occur at all through this design and so we did not emphasize the optimality of the amino acid concentrations. On average our amino acid concentrations were about $4x10^{-4}$ M but chemoattractive amino acids are known to have chemotaxis thresholds at around 10^{-7} to 10^{-5} M, which suggested that we have room to lower our amino acid concentrations and optimize motility (Mesibov and Adler, 1972). Another possible factor for the slow speed at which our ΔserB ΔmutS strain migrated could be due to its metabolic burden in maintaining its 3 antibiotic resistance genes and plasmid. The relatively slow speed of our positive control, wild type K-12 with RP4, supports this observation because it suggested that being auxotrophic for serine metabolism didn't significantly delay motility. In fact, the wild type strain contained the RP4 plasmid in order to possess the necessary antibiotic resistances to grow in the dropout media but introducing the RP4 plasmid may have also introduced metabolic burdens that impaired its motility (Melynk et al, 2015). To further test this hypothesis in the future, a wild type K-12 strain without RP4 should be grown on a Δ*serB* dropout plate made without antibiotics and its speed of migration should be compared to that of our K-12 with RP4. If the K-12 without RP4 strain is found to migrate outwards in fewer than 2 days, then we can

reasonably conclude that the RP4 plasmid and its antibiotic resistance genes impose a burden that delays motility in our strains. Removal of the chromosomal antibiotic resistances genes in our double mutant could then be performed using gene segments flanked by sequences homologous to the antibiotic resistance gene and its neighbors (Jasin and Schimmel, 1984). Despite the slow rate, the presence of migration and the asymmetry from which the cells expanded outward suggested that the population of cells were undergoing evolution. In particular, the regions where the cells migrated the farthest signified the accumulation of one or more beneficial mutations that allowed those cells to survive and move out to utilize more resources. While no cells have reached the ends of the plate yet, those that eventually do will be extracted and regrown on another fresh dropout plate. Ideally, this process will be repeated until migration rates become significantly quicker—when migration occurs days earlier than before. At this point, the *gph* plasmid from these cells must be isolated and retransformed into a fresh batch of Δ*serB* Δ*mutS* cells to eliminate any possible fitness advantages gained from chromosomal mutations. However with antibiotics that lose their efficacy in a few days—such as ampicillin whose activity decreases by 10% every hour at room temperature—leaving the cells to migrate until they reach the boundaries at the current rate would result in loss of the Gph plasmid since the fitness cost of maintaining the plasmid would outweigh the benefits in the absence of the antibiotics (Melynk et al, 2015). Thus in our following trial, we extracted the furthermost cells as soon as any visible sign of migration could be observed. This first evolved generation was then regrown at the same time as the original double mutant strain. Presence of denser colonies and greater migration in the first generation double mutants indicated that the motility selection succeeded in evolving the double mutants. Thus while we have not finished evolving our strain, we are confident that repeated application of our selection procedure will result in the strain

becoming fast enough to reach the ends of the selection plates; at this point, transformation of the plasmid must be conducted to conserve mutations in the *gph* gene while eliminating any chromosomal mutations. We expect the transformed strains to be less fit than the strains prior to transformation because the majority of beneficial mutations will occur in the chromosome. Nonetheless the minority of mutations that occur in the *gph* gene will be conserved and when the motility selection is repeated enough times, these mutations will significantly increase the Gph protein's ability to rescue Δ*serB* function. Although we are certain that our strategy would select for the evolution of more serine-catalyzing Gph proteins, future structural and kinetic assays must also be conducted to ascertain and elucidate the catalytic capabilities of the evolved Gph.

As for our attempt at semi-continuous evolution, our goal was to evolve the *gph* plasmid in Δ*serB* Δ*mutS* with RP4 and conjugate the plasmid into the same type of double mutant strain but with ceftotaxime resistance—pCDF-Ptac-lacOc-ampC ADC-33. This way by putting both cells under ceftotaxime and chloramphenicol (since the *gph* plasmid provides chloramphenicol resistance) selecting media after conjugation, we select for the delivery of *gph* from one Δ*serB* Δ*mutS* with RP4 strain to a new one without it. The idea behind creating the ceftotaxime and amikacin resistant strains of Δ*serB* Δ*mutS* with RP4 is so that the selective conditions can be changed to favor one strain or the other. In theory, this would allow us to evolve the *gph* plasmid by bouncing the plasmid to and fro the ceftotaxime and amikacin resistant strains, thereby eliminating chromosomal mutation accumulation without the need for transformation. However since we were unable to transform the amikacin resistant plasmid into Δ*serB* Δ*mutS* with RP4, we could not proceed to evolve the cells through conjugation. Our inability to attain transformants was not too surprising given that the cells in our semi-continuous scheme were to possess 3 plasmids and a total of 4 antibiotic resistance genes which would impose a heavy

metabolic burden on the cells (Melynk et al, 2015). While this fact may be discouraging, we are still optimistic that the final transformation can be achieved because the first three Δ*serB* Δ*mutS* with RP4 strains held a similar number of plasmids and antibiotic resistance genes, yet the *gph* plasmid was successfully transformed into them. Transforming the amikacin plasmid was deemed a failure because both the transformants and negative controls failed to grow on the amikacin plates. However the absence of growth could indicate that the amikacin concentration in the plates were too high and so a natural test could be to recreate plates with lower amikacin concentrations and attempt to grow the amikacin resistant strains on these plates. Overall, our semisolid dropout media appeared to be viable for using motility selection to evolve Gph. While not perfect, we anticipate that our motility selection with transformation method can be used for restricted evolution of other proteins. On the other hand, our semi-continuous model is not as promising but we have reason to believe that modifications to our current plate recipes and design will yield a successive approach.

References

1) Soto, Sara M. “Role of Efflux Pumps in the Antibiotic Resistance of Bacteria Embedded in a Biofilm.” *Virulence* 4.3 (2013): 223–229. *PMC*. Web. 10 Apr. 2017.

2) Miller, Brian G., and Ronald T. Raines. "Identifying Latent Enzyme Activities:Â Substrate Ambiguity within Modern Bacterial Sugar Kinases ." *Biochemistry* 43.21 (2004): 6387-392. Web.

3) Bornscheuer, Uwe T., and Romas J. Kazlauskas. "Catalytic Promiscuity in Biocatalysis: Using Old Enzymes to Form New Bonds and Follow New Pathways." *ChemInform* 36.5 (2005): n. pag. Web.

4) Patrick, Wayne M. et al. “Multicopy Suppression Underpins Metabolic Evolvability.” *Molecular biology and evolution* 24.12 (2007): 2716–2722. *PMC*. Web. 10 Apr. 2017.

5) Yip, S. H.-C., and I. Matsumura. "Substrate Ambiguous Enzymes within the Escherichia Coli Proteome Offer Different Evolutionary Solutions to the Same Problem." *Molecular Biology and Evolution* 30.9 (2013): 2001-012. Web.

6) Lezin, George et al. “A One-Step Miniprep for the Isolation of Plasmid DNA and Lambda Phage Particles.” Ed. Martin G. Marinus. *PLoS ONE* 6.8 (2011): e23457. *PMC*. Web. 10 Apr. 2017.

7) Sleight, Sean C., and Herbert M. Sauro. "BioBrick Assembly Using the In-Fusion PCR Cloning Kit." *Synthetic Biology Methods in Molecular Biology* (2013): 19-30. Web.

8) Shetty, Reshma P., Drew Endy, and Thomas F. Knight. "Engineering BioBrick Vectors from BioBrick Parts." *Journal of Biological Engineering* 2.1 (2008): 5. Web.

9) Schuler, Mary A., and Raymond E. Zielinski. "Restriction Mapping of Plasmid DNA." *Methods in Plant Molecular Biology* (1989): 1-17. Web.

10) Matsumura, Ichiro. "Semi - Automated Cloning of Restriction Fragments into and out of Plasmid Polylinkers." *Supplementary Material for a Biotechniques Report* (2016): 1-16. Web.

11) Froger, Alexandrine, and James E. Hall. "Transformation of Plasmid DNA into E. Coli Using the Heat Shock Method." *Journal of Visualized Experiments* 6 (2007): n. pag. Web.

12) Ito, Hisao, Kousaku Murata, and Akira Kimura. "Transformation of Yeast Cells Treated with 2-mercaptoethanol." *Agricultural and Biological Chemistry* 47.7 (1983): 1691-692. Web.

13) Moore, Sean D. "Assembling New Escherichia Coli Strains by Transduction Using Phage P1." *Methods in Molecular Biology Strain Engineering* (2011): 155-69. Web.

14) Liljeström, Peter, Minna Pirhonen, and E.tapio Palva. "In Vivo Transfer of Chromosomal Mutations onto Multicopy Plasmids by Transduction with Bacteriophage P1." *Gene* 40.2-3 (1985): 241-46. Web.

15) Inoue, Hiroaki, Hiroshi Nojima, and Hiroto Okayama. "High Efficiency Transformation of Escherichia Coli with Plasmids." *Gene* 96.1 (1990): 23-28. Web.

16) Mesibov, Robert, and Julius Adler. "Chemotaxis Toward Amino Acids in Escherichia Coli." *Journal of Bacteriology* 112.1 (1972): 315-26. Web.

17) Wolfe, A. J., and H. C. Berg. "Migration of Bacteria in Semisolid Agar." *Proceedings of the National Academy of Sciences* 86.18 (1989): 6973-977. Web.

18) Topp, Shana, and Justin P. Gallivan. "Random Walks to Synthetic Riboswitchesâ€ ” A High-Throughput Selection Based on Cell Motility." *ChemBioChem* 9.2 (2008): 210-13. Web.

19) Jasin, M, and P Schimmel. “Deletion of an Essential Gene in Escherichia Coli by Site-Specific Recombination with Linear DNA Fragments.” *Journal of Bacteriology* 159.2 (1984): 783–786. Print.

20) Melnyk, Anita H, Alex Wong, and Rees Kassen. “The Fitness Costs of Antibiotic Resistance Mutations.” *Evolutionary Applications* 8.3 (2015): 273–283. *PMC*. Web.