Heterologous complementation of isolated and environmental DNA into *Escherichia coli* using lambda phage

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Motivation and Introduction

Arsenic is a relatively uncommon element with an unfortunate and pernicious knack for existing in environments necessary to human life. In subsurface environments, arsenic is regularly transferred between solid and aqueous phases and can be removed or added to bioavailable reservoirs like drinking water or rice grains.[1,2] This cycling is mediated by both geochemistry and biology. Of particular concern is arsenic's trivalent form, arsenite, due to its significant toxicity.[3] However, more attention is being paid to the importance of organic arsenic in these systems; its occurrence in drinking water and accumulation in rice grains are a growing concern. Yet, a comprehensive understanding of how environmental arsenic is biologically methylated and demethylated does not exist, especially in anaerobic environments.

Alongside transforming arsenic species between their inorganic counterparts arsenate and arsenite, many microbes also possess the ability to methylate arsenic via the *arsM* gene.[4] This is generally considered a detoxifying mechanism and can account for a significant amount arsenic cycling in some groundwater environments.[5–7] Microorganisms can also demethylate arsenic species using the *arsI* gene and have been shown to do so in aerobic environments.[8–10] However, in aerobic environments, the demethylation of arsenic is not well understood.

This document serves as a completion report on a modest, multi-week project I have conducted here at the MBL Microbial Diversity Short Course during the summer of 2017. However, my motivation for pursing this project goes beyond this course; it is my aim to use the methods I have practiced, developed, optimized, and summarized hereto in order to pursue a greater understanding of the metabolic and genomic components of in situ, anaerobic microbial arsenic demethylation.

To this end, I have learned how to employ heterologous complementation techniques using lambda phage as a vector. Using DNA from both an isolated bacterium and an environmental sample, I have successfully transfected genes governing the production of an amino acid essential for cell wall growth (diaminopimelic acid) into an *E. coli* auxotroph that is deficient of such a gene. It is my hope that this method will be an analog for exploring other environmental DNA (Figure 1), specifically that of genomes responsible for the aforementioned demethylation process.

Methods

Methods for high molecular weight DNA extraction, plasmid extraction, and phage transfection were followed with high fidelity according to each kit's protocol. Procedures and deviations are summarized here but for comprehensive methods the protocols should be gathered from each company's website and/or individual kit.

Sample collection for insert DNA. Vibrio owensii was isolated from an intertidal stream (Trunk River estuary: 41°32'3.43"N, 70°38'29.73"W) and cultured in a sterile salt water media until enriched in LB for DNA extraction. Samples from Cedar Swamp (41°31'37.96"N, 70°39'15.16"W) were collected in July 2017 and stored in an aluminum foil-covered 1 L glass Ball and stored at room temperature.

Plates, media, and spotting. All agar plates and media were either filter or autoclave sterilized. Spotting and relocating of cultures was done using a butane flame-sterilized inoculation loop.

Extraction and digestion of pstb-LAFR5 plasmid. A Promega PureYield™ Plasmid Miniprep System was used to extract the pstb-LAFR5 cosmid (p-LAFR5) from *E. coli* strain JM109. Extracted plasmids were digested and linearized using ScaI-HF and BamHI-HF restrictive enzymes at 37^{o}C overnight.

Extraction and digestion of insert DNA. DNA was extracted from both the vibrio isolate and Cedar Swamp environmental sample using a Zymo Research Quick-DNA Fecal/Soil Microbe Midiprep Kit (catalog No. D6110). DNA was digested using the Sau3AI restriction enzyme for 30 min. This was half the recommended time to fully digest and donoe so to maximize both digestion and concatemerization. Extracted DNA base pair size was estimated through loading DNA into a 1.5% agarose gel and 1x TAE buffer with Sybr Safe DNA stain and running electrophoresis at 200 mA for 1 hour. Digested DNA sizes and efficacy of digestion enzymes were determined though a time series digestion where samples were collected and the enzymes inactivated (65^{o}C) at 1, 5, 10, 15, 30, 45 min, and 1 hour.

Packaging lambda phage for DNA insertion. Using an Agilent Gigapack® III Gold Packaging Extract that included 4 modules designed to package recombinant lambda phage, I inserted ligated and Wild-Type (WT) DNA into lambda phage. To package, a lambda phage package was thawed and 0.05 - 0.2 μg of WT DNA was added to the packaging extract and incubated at room temperature (RT) for 2 hours. After 2 hours, 500 uL of SM buffer (Appendix A) and 20 uL of chloroform were added to the packaged extract solution. After gently mixing, the solution was spun to separate the chloroform; the supernatant containing the packaged phage was removed and stored at 4C.

Preparation of host bacteria. Two auxotrophs were prepared in liquid cultures, a diaminopimelic acid (DAP)-deficient *Escherichia coli* (*E. coli*) auxotroph and a tryptophan (Tryp) *E. coli* auxotroph. The DAP-negative mutant was cultured in LB broth (Appendix A) and amended with DAP to a 300 μM concentration. The Tryp-negative mutant was cultured in M9 media (Appendix A) alongside an amendment of tryptophan (300 μM). These bacteria were incubated in the dark at 37^{o}C and

shaken at ≈200 rotation per min. Cultures were prepared for lambda phage infection by an additional incubation in LB broth amended with 10 mM MgSO4 and 0.2% maltose. Once that enrichment reached an optical density at 600 nm (OD_{600}) between 0.5 and 1, cells were concentrated cells via microcentrifuge and resuspended in 10 mM MgSO4*7H2O to a OD_{600} of 0.5 and used immediately for phage transfection. In preparation for the WT phage control experiment, a non-auxotrophic *E. coli* strain, VCS257, was also cultured using this method but without an additional amendment of DAP or Tryp.

Transfection of *E. coli* strains by lambda phage. Packaged lambda phage containing either WT or inserted DNA were aliquoted into an Eppendorf tube (25 uL) alongside 25 uL of the host bacteria strains at OD_{600} of 0.5 and allowed to incubate at RT for 30 min. Then 200 uL of LB broth was added to the reaction and allowed to sit at 37^{o}C and gently shaken every 15 min. Infected cells were microcentrifuged and resuspended in LB broth before plating.

Plating of lambda phage and transfected *E. coli*. Except for the Tryp *E. coli* transfection experiment which was plated on M9 agar (Appendix A), experiments were plated on LB agar. Before the plating of the transfected *E. coli*, some plates were amended with either DAP, Tryp, tetracycline, or a combination of tetracycline and either DAP or Tryp. Most plates were just LB-olny agar. Transfected tryp-auxotrophs were plated on M9 with controls plated on M9 amended with tetracycline and tryptophan. Transfected DAP-auxotrophs were plated on LB with experimental control groups plated on tetracycline and DAP. WT phage and VCS257 *E. coli* were plated on LB agar.

Results and Discussion

Phage packaging. The success of the phage package was tested using a simple positive control; WT lambda phage DNA was given to a recombinant lambda phage package and mixed with a non-auxotrophic, robust, *E. coli* strain (VCS257). A properly reassembled WT lambda phage would begin infecting host bacteria and enter either its lytic or lysogenic cycle. I tested the success of the phage's assimilation of the WT DNA by plating the lambda phage/*E. coli* mixture in 10^{0}, 10^{-2}, and 10^{-4} dilutions on LB agar plates.

The development of plaques on the plate treated with the 10^{-4} dilution mixture showed significant plaque development (Figure 2). A robust lawn of *E. coli* VCS257 developed on most of the plate, however, after ~40 hours many of the colonies were lysed. This is evidence of a successful packaging of the recombinant lambda phage with WT lambda phage DNA.

DNA Extraction. DNA was extracted with the intention of achieving the highest molecular weight DNA possible. It was my goal to begin working with the largest base pair DNA with which I could digest using the Sau3AI enzyme in a calibrated way. To calibrate, I conducted a timed digestion series where I removed and inactivated the digesting enzyme at specific times. By loading the larger base-pair DNA alongside the digested DNA into an agarose gel for electrophoresis, I could estimate how long I should digest my extracted DNA to maximize the size of the insert into the lambda phage (Figure 3).

A larger DNA insertion into the DAP-auxotroph by the lambda phage is preferable over a smaller one for several reasons. Having a larger piece of DNA inserted into the cosmid and eventually into the *E. coli* increases the number of genes being inserted and therefore the likelihood of successfully transfecting the gene of interest. The counterpoint to this is approach is that too many genes may be inserted and if a successful mutant is identified, finding that gene may be difficult given the number of genes inserted into that cosmid. However, because this method accommodates gene complementation through the transfection of a cosmid, the cosmid has the advantageous characteristic of persisting through *E. coli* growth and replication allowing for extraction generations of *E. coli* later with the same collection of originally inserted genes. This is a crucial point as this persistent cosmid could, at some later time from some later *E. coli* generation, be further digested and transfected through the same method. In other words, beginning with high molecular weight allows the method to more quickly identify the broad region where a gene of interests exists; with that information and isolated region of the genome, the DNA can be further digested to narrow the gene of interest's location.

Vibrio Transfection. Growth was observed on all vibrio DAP-transfection control plates, all of which were treated with both DAP and tetracycline (Figure 4). These colonies confirm the successful infection of the tetracycline-resistant cosmid. The DAP was added to the control plates as a necessary source for most colonies that were likely not infected with the proper gene to produce DAP.

None of the remaining LB agar plates were amended with tetracycline or DAP. Forty hours after the transfected solution was plated, colonies began to develop (Figure 4). After 60 hours, ~22% of the plates exhibited colony growth. Each of these colonies represent a strain of *E. coli* that contains a tetracycline-resistant cosmid and a DAP gene which was been effectively expressed.

Two curious results of the successful DAP transfection in the ΔDAP-mutants were that 1) colonies appear to be grouped together on individual plates and 2) that the distribution of ΔDAP-mutants were not consistent with the dilutions utilized when plating. I hypothesize that this was because of an incomplete resuspension of the pelleted transfected *E. coli* in 10 mM MgSO4. To confirm that the ΔDAP-mutants were infected with the DAP gene and not plate contamination, I conducted several follow-up experiments.

Vibrio transfection confirmation. To confirm the successful transfection of the DAP gene I inoculated two balch tubes containing LB broth with colonies from the LB-only agar plates; in one balch tube I included an additional tetracycline amendment (Figure 5). The balch tubes were incubated at 37^{o}C and shaken for 24 hours. Both liquid media solutions showed growth, however, the LB/tetracycline solution exhibited more cell debris at the bottom of the tube than the LB-only solution. I hypothesize that this is because the tetracycline amendment stressed the *E. coli* and although it had a tetracycline resistance via the inserted cosmid, perhaps its insertion was not as seamless and therefore not as effective. The LB-only balch tube showed significant growth in 24 hours which little cell debris. These results further supprt that LB agar plate

colonies were indeed transfected with the cosmid and part of the vibrio genome containing the DAP gene.

For additional confirmation, I prepared an LB-agar plate with 200 uL of tetracycline and streaked out a ΔDAP-mutant on one side and the original DAP-auxotroph on the other – this agar plate was free of DAP (Figure 5); The plate was incubated for 24 hours at 37^{o}C. After 1 day the plate showed significant growth where the ΔDAP-mutant was streaked and no growth on the other side of the plate. This is consistent and offers more evidence that the E. coli was successfully infected with both the cosmid (tetracycline resistance) and vibrio's DAP gene.

Tryptophan Transfection. This part of the experiment could fairly be classified as a complete failure. Neither the control plates (M9+tryptophan+tetracycline) nor the M9-only plates exhibited growth. This could be because M9 is a difficult media for *E. coli* to grow on, especially in the presence of tetracycline (control plates). Another possibility is that I did not carry out this transfection properly.

Environmental DNA Transfection. The environmental DNA transfection experiment's control LB agar plates amended with both DAP and tetracycline (in triplicate) all exhibited growth within 48 hours (Figure 6). This confirms that *E. coli* was successfully infected with the tetracycline-resistant p-LAFR5 cosmid. After 2 days, of all 16 LB-only plates that the Cedar Swamp transfections were plated, only 2 plates showed growth – each with a single colony (Figure 7). This indicates that at least one DAP gene, from the likely hundreds of organisms within the Cedar Swamp sample, was successfully inserted and expressed two E. coli ΔDAP-mutants. I would have liked to have confirmed the transfection via methods described earlier, but the course ended, the lab closed, and all plates were autoclaved.

Conclusions and a Look Ahead

The immediate objective of my mini-project for this course was to learn, develop, and optimize methods of heterologous complementation using lambda phage transfection. However, this project is part of a larger idea. This mini-project was conceived after discussing my ideas with Dianne Newmann and Kyle Costa regarding the knowledge gaps in anaerobic arsenic demethylating metabolisms. I view this project as a springboard to investigate an anaerobic arsenic-demethylating enrichment culture and explore anaerobic arsenic-demethylating genes. This project is only the beginning of a broader mission that I plan to work on over the coming years. Assuming I can enrich an arsenic-demethylating culture in my home laboratory, I will I apply this approach with an aim to further optimize and develop methods with specificity towards my gene of interest.

After a demethylating culture is developed, the next step will be to choose an appropriate host bacterium. An *arsC*-auxotroph *E. coli* will be a reasonable starting point to see if any arsenic-related genes can be transfected from either an arsenic contaminated soil or from an arsenic-demethylating enrichment culture. There will be a necessary addition to this method in order to investigate arsenic demethylation; any colonies shown to be transfected (i.e. grows in

tetracycline assuming the p-LAFR5 cosmid is used), will need to be transferred to a master well dish. From there, I will be able to culture colonies from the master well plate in other well plates and liquid media where I can employ a previously used end-point assays to quickly determine specific arsenic speciation changes.[11]

Figures

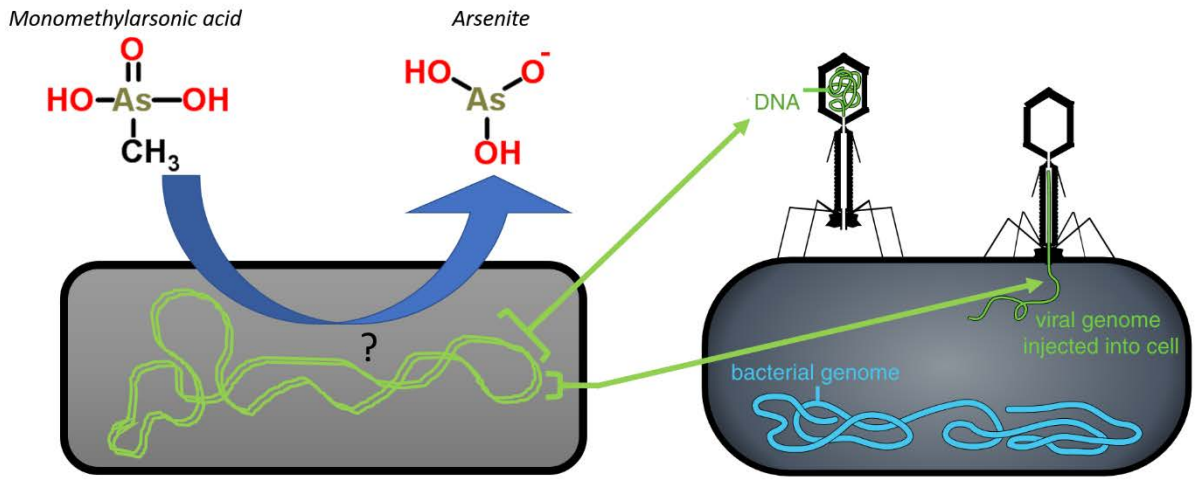

Figure 1. Conceptual schematic of motivating question and proposed future approach. Figure modified from Thomas Splettstoesser permission.

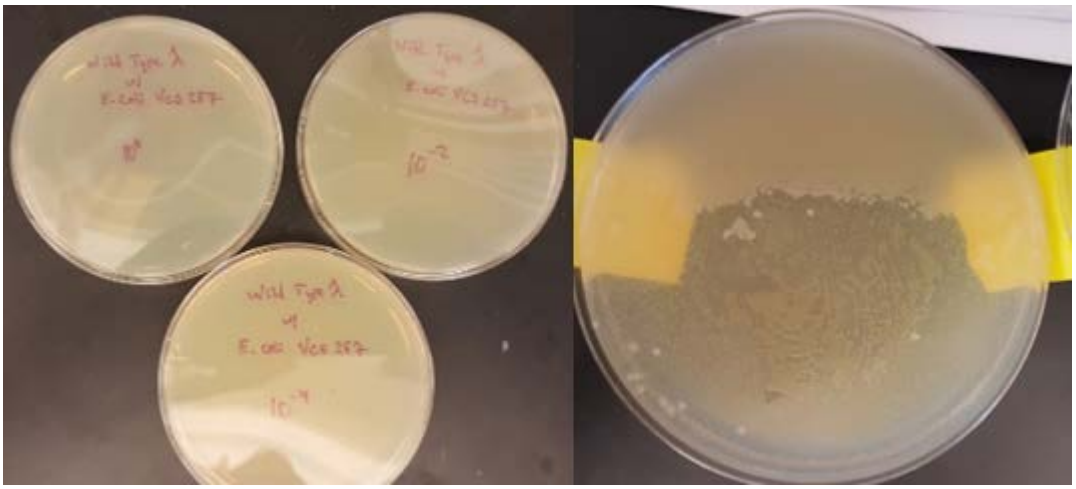

Figure 2. Phage control plates (left and right) after 36 hours of incubation.

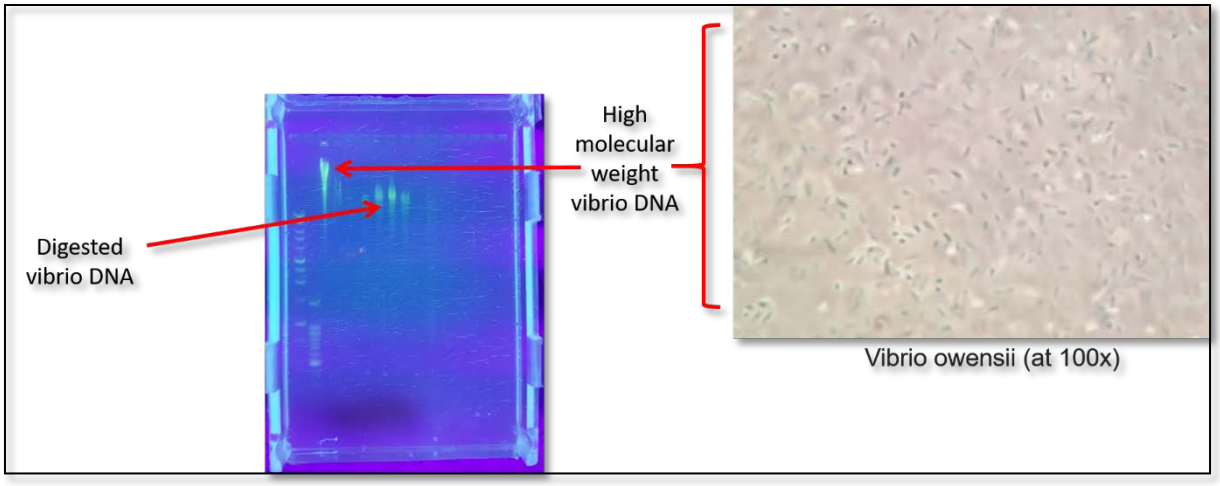

Figure 3. Left: Gel of 1.5% agarose and 1x TAE of both the large base pair extracted DNA in lane 3 and the digested DNA in lanes 5 through 9. Right: Vibrio owensii strain isolated from Trunk River in July 2017 and used for DNA extraction digestion calibration.

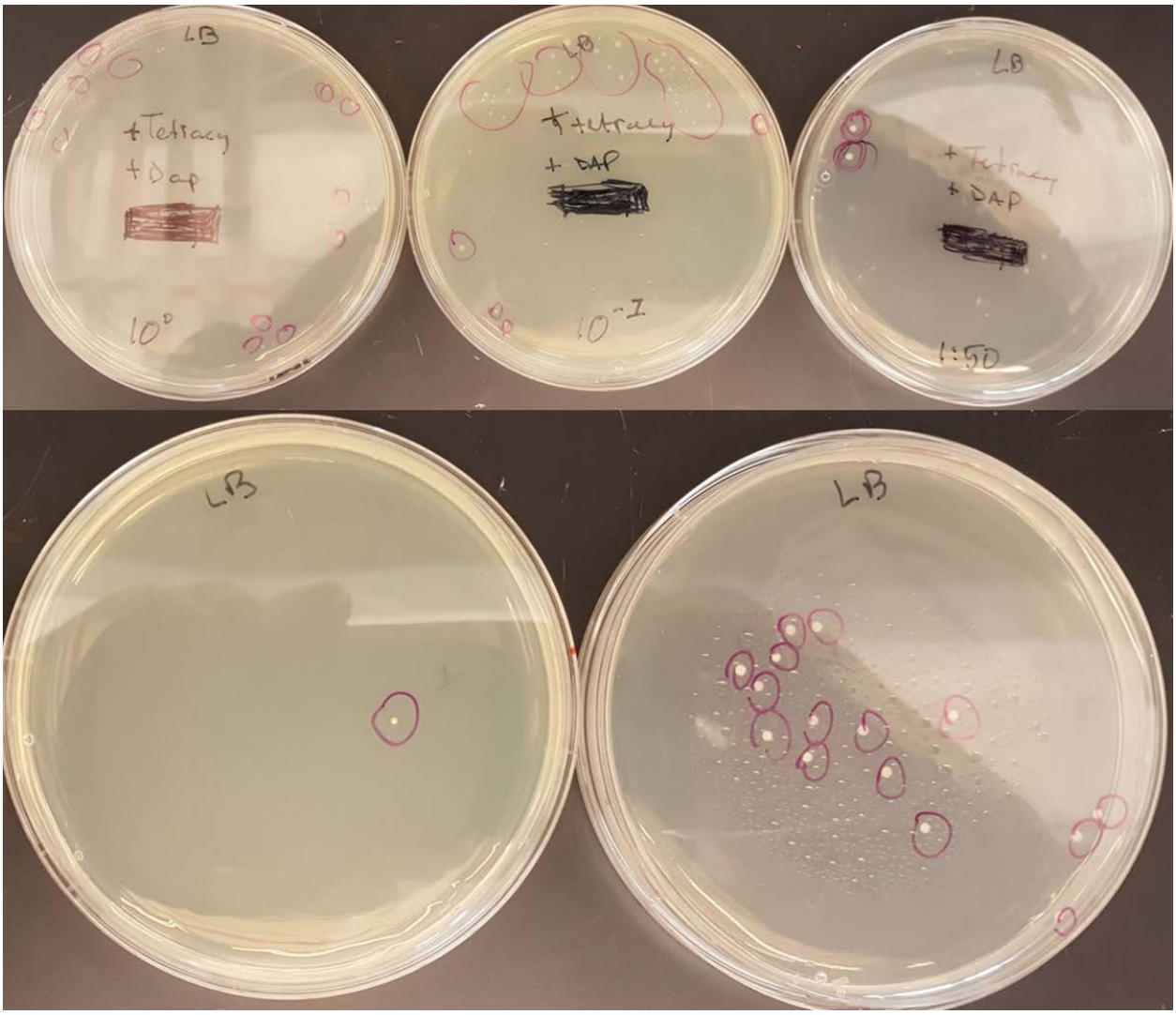

Figure 4. Vibrio complementation experiment: Top: control plates. Bottom: plates with no DAP amendment and colony growth indicating a successful transfection of the DAP gene into the E. coli auxotroph.

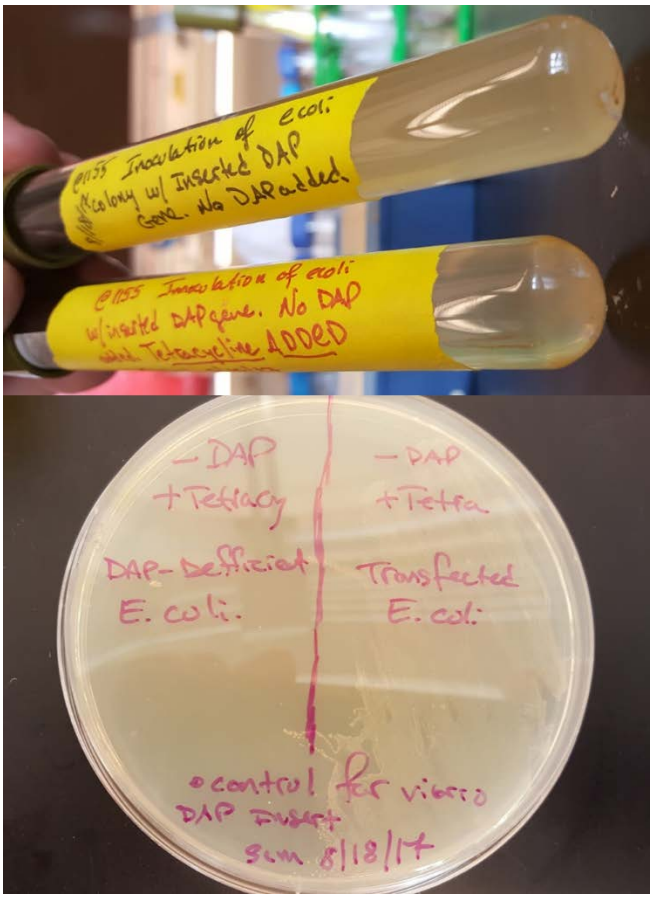

Figure 5. Two follow-up experiments to confirm that the cultures growing on the LB/no-DAP plates were transfected with the DAP gene. Top: Liquid enrichments of isolates from LB plates. The top balch tube is LB-only and the bottom was LB + tetracycline - the antibiotic which the cosmid has an inherent resistance. Bottom: LB agar plate (no DAP). The right side of the plate was streaked with transfected E.coli whereas the left was streaked with the DAP-auxotroph. Growth on the right is further confirmation of a successful DAP-gene transfection.

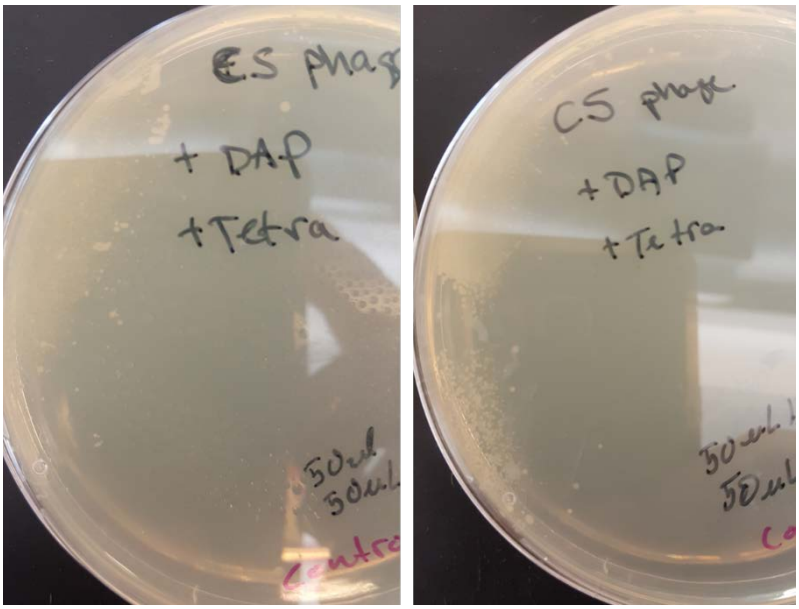

Figure 6. Control agar plates for the attempted transfection of environmental data. Both plates have been amended with tetracycline and DAP to allow for selective growth of only E. coli with the tetracycline resistant cosmid transfected by the lambda phage. Growth can be seen on both plates indicating a relatively successful 1) ligation of Cedar Swamp DNA with the p-LAFR5 cosmid and 2) tranfection of the cosmid by the lambda phage.

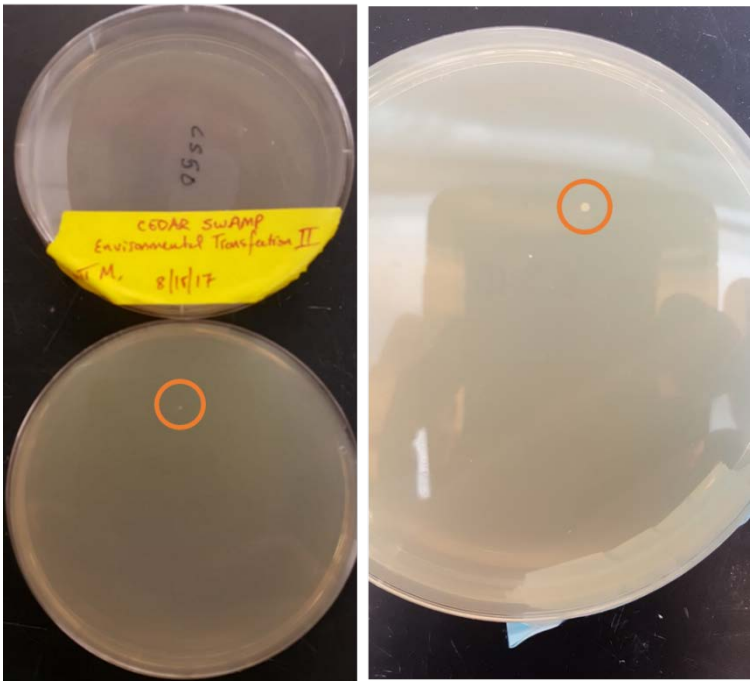

Figure 7. Results from the environmental transfection. Cedar Swamp DNA on LB/no-DAP plates grew successful colonies.

Appendix A

Media preparation and reagents

(from Gigapack III Gold Packaging Extract, Gigapack III Plus Packaging Extract, and Gigapack III XL Packaging Extract Protocol)

LB Agar (per Liter) 10 g of NaCl; 10 g of tryptone; 5 g of yeast extract; 20 g of agar. Adjust pH to 7.0 with 5 N NaOH Autoclave Pour into petri dishes (~25 ml/100-mm plate)

LB Broth (per Liter) 10 g of NaCl 10 g of tryptone 5 g of yeast extract Add deionized H_2O to a final volume of 1 liter Adjust to pH 7.0 with 5 N NaOH Autoclave

M9 Medium (per Liter) 6.0 g of dibasic sodium phosphate (Na_2HPO_4); 3.0 g of monobasic potassium phosphate (KH_2PO_4); 1.0 g of ammonium chloride (NH_4Cl); 0.5 g of NaCl; 1.0 ml of 1 M $MgSO_4$; 2.0 g of glucose; 0.1 ml of 1 M $CaCl_2$; 1.0 ml of 1 M thiamine-HCl.

M9 agar (per Liter) 6.0 g of dibasic sodium phosphate (Na_2HPO_4); 3.0 g of monobasic potassium phosphate (KH_2PO_4); 1.0 g of ammonium chloride (NH_4Cl); 0.5 g of NaCl; 1.0 ml of 1 M $MgSO_4$; 2.0 g of glucose; 0.1 ml of 1 M $CaCl_2$; 1.0 ml of 1 M thiamine-HCl; 20 g of agar.

SM Buffer (per Liter) 5.8 g of NaCl 2.0 g of $MgSO_4 \cdot 7H_2O$ 50.0 ml of 1 M Tris-HCl (pH 7.5) 5.0 ml of 2% (w/v) gelatin Add deionized H_2O to a final volume of 1 liter Autoclave

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