Overexpression of a Leucine-Responsive Regulator Protein Gene of *Streptomyces nymphaeiformis* in *Streptomyces lividans* TK24

CALEB PALACIOS AND DR. STEPHEN BARON
Department of Biology, Bridgewater College, Bridgewater, VA 22812
2025

Polyhydroxyalkanoates (PHAs) are biodegradable polymers that can be substitutes for nonbiodegradable polymers, such as petroleum-based plastics. PHAs are degraded by PHA depolymerases, which are extracellular enzymes. *Streptomyces* sp. SFB5A produces a PHA depolymerase, and the enzyme is encoded by a *phaZ* gene. An *lrp* gene was found 2,700 bp upstream from *phaZ* and is suspected to be a transcriptional regulator, as well as a morphogenesis and secondary metabolism regulator. This *lrp* gene was isolated, amplified, and cloned into *E. coli* ET12567/pUZ8002 and extracted to make a recombinant plasmid, pIJ86-*lrp*. The plasmid was utilized in the intergeneric conjugation between *E. coli* ET12567/pUZ8002 + pIJ86 and *Streptomyces lividans TK24,* however, the trials were inconclusive and require further investigation.

Introduction

Polyhydroxyalkanoates (PHAs) are organic, water-insoluble polymers synthesized by many soil bacteria [1]. PHAs consist of long chains of 3-hydroxyalkanoate monomers. Polyhydroxybutyrate (PHB) is the most common PHA, composed of 3-hydroxybutyrate (3HB) monomers. PHA's have significant potential as fully biodegradable alternatives to petroleum-based plastics [1]. Many soil and water bacteria use secreted enzymes known as PHA depolymerases to degrade PHAs to their monomers, which are subsequently transported into the cell and metabolized for growth [2].

The genus *Streptomyces* is comprised of common, gram-positive soil bacteria that are well known for their ability to degrade biopolymers, including PHAs, and for their production of over 50% of all known antibiotics. This genus of bacteria is characterized by a high genomic %G+C base content (~70%). *Streptomyces nymphaeiformis*, the bacterium used in our lab [3], produces a PHB depolymerase when grown on PHB or 3HB, but not on glucose [4]. This observation suggests that transcription of its PHB depolymerase gene (*phaZ*) is regulated. However, the mechanisms for the transcriptional regulation of PHB depolymerase genes remain largely unknown. A better understanding of these regulatory mechanisms could facilitate strategies to either accelerate the decomposition of biodegradable plastic waste in landfills or inhibit decomposition, thereby enhancing the stability of biodegradable plastic products during storage.

The *phaZ* gene from *S. nymphaeiformis* has previously been cloned and sequenced [Fig. 1] [4]. The gene consists of a promoter (control) region, a signal sequence, and a coding sequence for the secreted enzyme. The signal sequence codes for a short signal peptide (~ 40 amino acids), which directs the enzyme to the cell membrane for secretion and is cleaved from the rest of the protein upon secretion. The secreted protein coding sequence of *phaZ* ends with a translation stop codon, followed by a suspected transcriptional terminator.

Figure 1. Structure of the PHB depolymerase gene (*phaZ*) of *S. nymphaeiformis* [4]. The regulatory sequences, promoter, and terminator are proposed based on sequence homologies.

A potential mechanism for regulation of the *phaZ* gene in *S. nymphaeiformis* is two-component signal transduction, a system that enables bacteria to alter gene expression in response to external stimuli [5]. In prokaryotes, these systems are commonly composed of two main proteins: a histidine kinase (HK) [5, 6] or serine/threonine protein kinase (STK) [7], and response regulator (RR), which function together in a phosphotransfer system. HKs and STKs, often transmembrane proteins, detect environmental signals, undergo autophosphorylation, and subsequently transfer a phosphate group to the RR, a cytoplasmic protein. The phosphorylated RR then triggers a cellular response, commonly through the altering of gene expression [Fig. 2] [5, 6, 7]. One potential stimulus for HK activation is nutrient availability, which may explain why the presence of PHB or more likely, 3HB could serve as a signal for the transcriptional activation of *phaZ* [6]. A known stimulus for STK activation in the Gram-positive bacterium, *Bacillus subtilis* is breakdown products of cell walls, such as muropeptides, which are generated during spore germination [7]. In fact, growth of *S. nymphaeiformis* on the cell wall sugar, N-acetylglucosamine in the absence of PHB induces a low but detectable level of PHB depolymerase activity (H. Blevins and S. Baron, unpublished data).

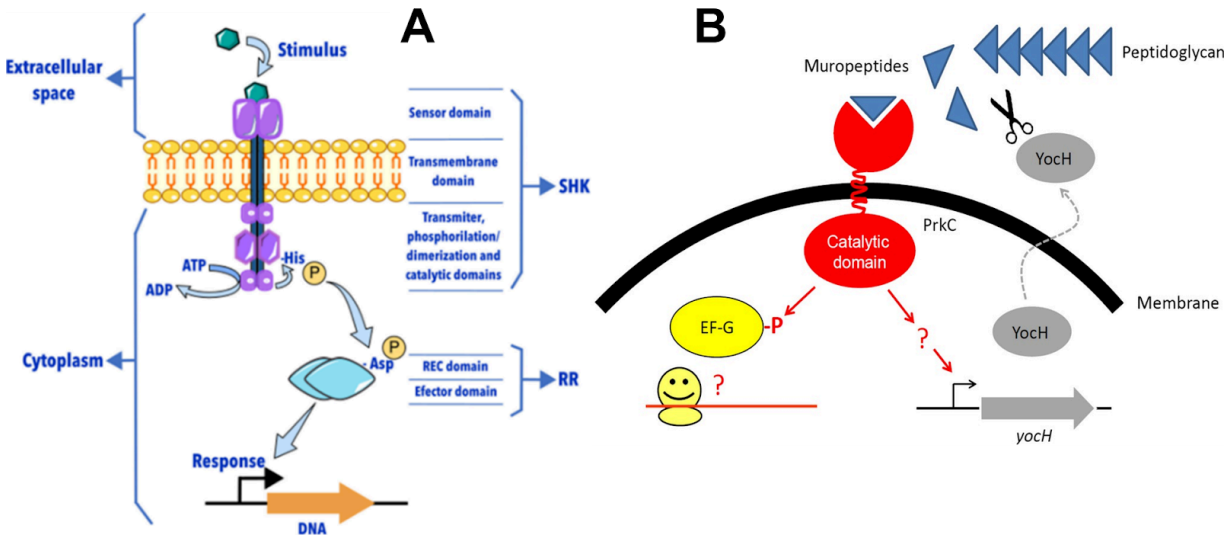


Figure 2. Schematic representation of two types of bacterial two-component signal transduction mechanisms. **A,** System with a sensory histidine kinase [6]. The HK detects an external stimulus and undergoes autophosphorylation. A phosphate group, derived from ATP, is then transferred to the RR, which subsequently activates gene expression as part of the regulatory response. **B,** System from *Bacillus subtilis* with a sensory serine/threonine kinase (PrkC) [7]. PrkC senses muropeptides from cell wall breakdown, autophosphorylates, and presumably transfers a phosphate group to a response regulator of the *yocH* gene, which encodes a muropeptidase.

The genome of *S. nymphaeiformis* has been sequenced [4], allowing for the precise localization of the *phaZ* gene. Approximately 2,700 base pairs (bp) upstream of *phaZ*, is a putative *lrp* gene encoding a leucine-responsive regulator protein, which we suspect to encode a transcriptional regulator for *phaZ*, perhaps a response regulator [Fig. 3]. Additionally, downstream of *lrp* and 250 bp upstream of *phaZ,* there is a potential gene encoding a serine/threonine protein kinase *(4abt)*. We suspect that the *4abt and lrp* may

encode a sensory kinase and response regulator respectively, potentially participating in two-component signal transduction regulation of the *phaZ* [Fig. 3].

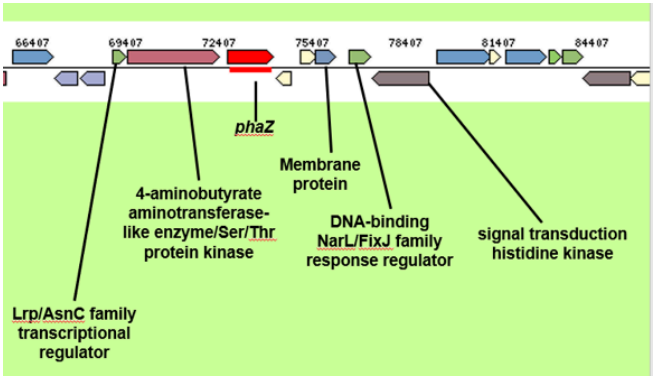


Figure 3. Genomic context of *phaZ*. The coding sequence for the secreted PHB depolymerase protein is highlighted in red. Directly upstream of *phaZ* is the gene potentially encoding a serine/threonine protein kinase (*4abt*), shown in purple. Further upstream, the putative *lrp* gene, which encodes a leucine-responsive regulatory protein, is depicted in green.

Streptomyces lividans TK24 is often used as a host for overexpression of genes from other *Streptomyces* species [Kieser]. Foreign genes can be efficiently introduced into *S. lividans* TK24 by interspecific conjugation with *Escherichia coli.* The "shuttle" plasmid pIJ86 was designed to be replicated in both *E. coli* and *Streptomyces* [Fig. 3] [9]. It also has genes for proteins involved in bacterial conjugation, enabling transfer of the plasmid from *E. coli* to *Streptomyces* through a tube called the sex pilus. However, DNA replicated in *E. coli* is usually methylated at certain bases and unstable when introduced into *Streptomyces.* To avoid this problem, a non-methylating strain of *E. coli*, 12567/pUZ8002 [Kieser] is used as a host for pIJ86.

Materials and Methods

The workflow for this experiment was designed to overcome and manipulate the cloning of high GC content DNA and *Streptomyces* species. This process involved PCR amplification, intermediate cloning in *E.coli*, shuttle vector construction, transformation through a non-methylating *E.coli* host, and a final conjugation into *S. lividans.*

Primer Design and Sequence Analysis

Prior to in-lab experimentation, a lengthy bioinformatic analysis of the *lrp* gene sequence was conducted to ensure and facilitate cloning into the pIJ86 vector. The main challenge identified was the presence of the internal *BamHI* restriction site within the *lrp* genetic sequence. The multiple cloning sites of pIJ86 normally utilize *BamHI* and *HindIII* for cloning. Using a standard *BamHI* site in the forward primer would lead to an internal cleavage of the gene during the digestion step, rendering it non-functional. To circumvent this issue, the forward primer was designed to contain a *BglII* restriction site. The overhangs produced by *BglII* are compatible with *BamHI,* creating a hybrid sequence not detected by either enzyme. This strategy allows for the clone to remain intact and leads to efficient enzyme binding.

Genomic DNA Extraction and PCR Workup

Amplification was performed using Illustra™ PuReTaq™ Ready-To-Go™ PCR Beads, which provide a standardized mix of Taq DNA polymerase, dNTPs, and buffer components (10 mM Tris-Cl pH 9.0, 50 mM KCl, 1.5 mM MgCl2). To address the high GC content (approx. 70%), Dimethyl Sulfoxide (DMSO) was added to a final concentration of 5% (v/v). DMSO acts as a denaturant, lowering the melting temperature of the DNA and preventing the formation of stable secondary structures that inhibit DNA.

Specific Thermal cycling conditions were employed to ensure specific yield and product. Initial Denaturation proceeded at 94C for 5 minutes to ensure complete strand separation. Then, 10 cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min. The high annealing temperature in the initial cycle was selected to maximize primer binding to the GC-rich target. Finally, the PCR treatment followed 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min, with a final extension of 72C for 5 minutes. Following PCR amplification, the products were analyzed via electrophoresis on a 1.0% agarose gel in 1X TAE buffer containing 0.5 µg/mL ethidium bromide. Visualization was performed using a BioRad Gel Doc™ EZ Imager to confirm the presence of a single band of the expected size (~494 bp).

Cloning into pCR™4-TOPO™

To enhance cloning efficiency, an intermediate step using TOPO-TA was performed instead of a typical restriction digest. *Taq* Polymerase possesses a specific activity that adds a single adenosine (A) to the 3’ ends of PCR products. The vector is supplied with a single 3’-thymidine(T) overhangwhich is bound to topoisomerase I. This entire process allows for the rapid 5-minute ligation of the PCR product in the vector without the need for any ligase.

Transformation of *E.coli* DH5α

The TOPO-ligation mixture was then transformed into chemically competent E. coli DH5α cells using a standard heat-shock protocol, disrupting the membrane. Cells were incubated on ice for 45 minutes, heat shocked at 42 °C for 45 seconds to form pores, and recovered on ice for 10 minutes. SOC medium was added to stabilize cells and support plasmid uptake. Transformation cells were then incubated at 37 °C in a 200rpm Shaker for 90 minutes to allow for kanamycin

resistance gene expression, which was carried by the TOPO vector. Transformants were then plated on Tryptic Soy Agar (TSA) plates supplemented with 50 µg/mL Kanamycin.

Plasmid Isolation and Verification

Plasmid DNA was isolated from transformant cells using a Sigma GenElute Plasmid Miniprep Kit. To confirm the presence of the intended insert a diagnostic restriction digest was performed using *EcoRI* **.** Digests were then analyzed on a 1% agarose gel to verify the release of the *lrp* insert (-494bp) from the vector backbone (-3956 bp).

Construction of *lrp-pIJ86* Expression Vector

The construction of the final vector involved subcloning the *lrp* gene from the TOPO vector into the pIJ86 shuttle vector. Beginning with a restriction digest at 37 °C for 3 hours. The insert was subject to a double digest with *BglII* and *HindIII*. This released the *lrp* gene with a 5' *BglII* overhang and a 3' *HindIII* overhang. The shuttle vector was digested with the same enzymes, generating a vector with a 5' *BamHI* overhang and 3' *HindIII* overhang. Both the Insert and Vector were prepared and visualized before ligation.

Gel Purification and Ligation

To prevent any unintended TOPO backbone product and undigested vector, the digestion products were separated on a 0.8% low-melting-point agarose gel containing SeeGreen™ dye. The bands corresponding to the *lrp* insert and pIJ86 vector were excised under blue light to minimize damage and retention of product. The gel slices were melted at 65 °C to prepare for ligation, which was performed using T4 DNA ligase. The reaction was incubated overnight in a Thermal Cycler and programmed to alternate between 10°C (30 seconds) and 30°C (30 seconds). This cycling of the ligation ensures efficiency, where the lower temperature promotes annealing of sticky ends, while the higher temperature optimizes the ligase activity.

Transformation into *E.coli* DH5α

The shuttle vector must be maintained in *E.coli* for verification and amplification; however, some future issues arose. *S. lividans* possesses a system that degrades DNA containing methyl groups at certain sequences. To overcome this barrier, the plasmid must pass through a methylation-deficient deficient *E.coli* strain before final transfer into *Streptomyces*. To start, the *lrp-pIJ86* ligation mixture was transformed into *E.coli* DH5. After transformation, the cells were selected onto TSA containing 50 µg/mL Apramycin. Colony PCR was performed to verify the presence of the insert. Positive clones were collected and cultured, with the plasmid DNA being extracted. A further digest was performed using *HindIII* and *KpnI*. To cut within the pIJ86 vector, *KpnI* was used, while *HindIII* cuts at the 3' end of the insert, which releases a 740 bp fragment when the gene is present and in correct orientation.

***E.coli ET12567/pUZ8002* Electroporation**

The validated lrp-pIJ86 plasmid was transferred into a non-methylating electrocompetent *E.coli* strain. This strain is dam-13::Tn9 dcm-6 hsdM, meaning it lacks Dam and Dcm methylases, producing unmethylated DNA suitable for Streptomyces. It also carries the pUZ8002 plasmid,

which is Kanamycin resistant. Electrocompetent cells were prepared by thorough washing in ice-cold 10% glycerol to remove any salt. To perform the electroporation, a BTX ECM 399 apparatus was set to 1800V in a 1mm gap cuvette. Immediately following the shock, cells were recovered in SOC medium and cultured onto LB agar containing Apramycin (50 µg/mL), Chloramphenicol (12.5 µg/mL, selects for ET12567), and Kanamycin (25 µg/mL, selects for pUZ8002). After growth, the product was upscaled with Colony PCR and visualized to confirm that *lrp* is present at 494 bp.

Interspecific Conjugation

The transfer of the lrp-pIJ86 plasmid from E. coli to S. lividans was achieved via interspecific conjugation, a method that bypasses the low efficiency of direct protoplast transformation in Streptomyces. The donor, E. coli ET12567/pUZ8002 containing the plasmid, was grown in an LB broth containing triple antibiotic selection and monitored for A_{600} absorbance on a spectrophotometer. When in log phase, cells were washed twice with LB and DI water to remove any antibiotics that would inhibit *S.lividans.* To prepare the destination for conjugation, spores of S. lividans TK24 (stock conc. 2.0 x 10^10 cfu/mL) were heat-shocked at 50°C for 10 minutes to induce germination. Approximately 10^8 donor cells were mixed with 10^8 recipient spores. The mixture was centrifuged, resuspended in a minimal volume, and plated on Mannitol Soya Flour (MSF) agar supplemented with 10 mM $MgCl_2$. The cation Mg2+ is critical for stabilizing the integrity of the mating bridges formed between the Gram-negative and Gram-positive bacteria. Plates were incubated at 30°C for 18 hours to allow for plasmid transfer and growth. After the mating period, the MSF plates were overlaid with 1mL of sterile water containing Nalidixic Acid (500 µg) and Apramycin (1 mg). This results in final plate concentrations of ~25 µg/mL Nalidixic Acid and ~50 µg/mL Apramycin. Nalidixic acid kills the donor *E.coli* strain by inhibiting DNA gyrase, while Apramycin selects for *S. lividans* exconjugants that have received the pIJ86 plasmid. Plates were then incubated for 7 days at 30 °C until colonies began to form and sporulation occurred.

***S.lividans* Characterization and Analysis**

To confirm the genotype of the *S.lividans* exconjugants, Colony PCR was performed. Due to the thick and complex cell wall of *Streptomyces*, a standard lysis is not sufficient enough. The exconjugants underwent a modified protocol which involved the resuspension of Mycelia in 50% DMSO, and an extended boiling step was employed to release the genomic DNA. PCR was performed using *lrp-specific* primers to verify the presence of a -494 bp band.

Protein Extraction and SDS-PAGE

Following PCR confirmation, exconjugants were cultured in CRM broth containing Nalidixic Acid and Apramycin, at 200rpm, until a “cloudy snowball” growth was observed in morphology. Cells were then harvested by centrifuge, washed, and lysed by Sonication, using alternating 10-second pulses to prevent overheating. To acquire the total protein concentration in the lysate, a BCA(Bicinchoninic Acid) Assay was performed. After calculating standard curves and ideal concentrations at A_{562} plasmid prep was prepared for SDS-PAGE. Calculated protein loads were separated on a 12% SDS-polyacrylamide gel to categorize proteins in the 10-200 kDa range. The

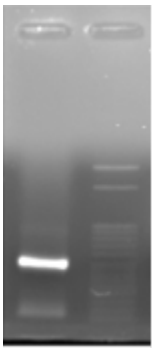
gel was stained with Coomassie Brilliant Blue to visualize the proteome and inspect for the overexpression of the ~16 kDa *lrp* protein.

RESULTS AND DISCUSSION

PCR Amplification and TOPO-TA Ligation

This research project began with the amplification of the *lrp* gene from *S.nymphaeiformis* genomic DNA. To ensure the accuracy and yield of the PCR reaction, the mix included 50% v/v Dimethyl Sulfoxide DMSO. This addition is due to the high-GC content of *Streptomyces* DNA. To facilitate direct cloning, the forward primer (lrpRC-F) incorporated a *BglII* site, and the reverse primer (lrpRC-R) used a *HindIII* site. The gel electrophoresis revealed a single prominent band at 480 bp, consistent with the expected size of *lrp* (Figure 1).

Figure 1. 1% Agarose gel of PCR product. Left lane, PCR product, 10 µL; right lane, Sigma StepLadder, 5.0 µL. A prominent band of the expected size, 480 bp, was observed (ladder bands were faint, but original viewing clearly showed the correct-sized piece).



Following amplification, the product was ligated into the pCR™4-TOPO™ vector. The reaction was then incubated and transformed into competent *E.coli DH5a.* On October 4, 2025, the transformation with TSA and Kanamycin yielded robust growth, with over 30 colonies on 50 µL plates. Ten Transformants were selected for overnight culture, with a plasmid extraction prepared the next day. On October 7th, an *EcoRI* restriction digest was performed to release insertion sites in the TOPO vector, and subsequent electrophoresis screening was evaluated.

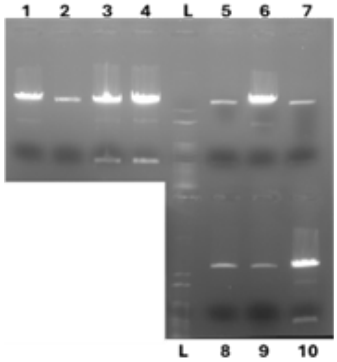


Figure 2. 1% agarose gel electrophoresis of restriction digests on plasmid extracts from TOPO-TA transformants. Numbers refer to transformant numbers; L = Wide range DNA markers.

Transformants 3, 4, 5, 7, and 10 showed the expected vector band of 2800 bp and insert of (480 + 17 = 497) bp. The corresponding

cultures were pooled into 1 tube, spun down, resuspended in 1 ml of spent culture broth, added 1 mL 50% v/v glycerol, froze for storage at -80°C. Plasmid DNA from transformants was pooled and quantified using µDrop plate and MultiSkan Sky plate reader. Concentration = 130 µg/mL; A260/280 = 1.93; A260/280 = 2.35.

Restriction Digest and Gel Purification

To prepare both the insert and vector for ligation, the pooled TOPO-*lrp* plasmid and the plJ86 vector were digested with BglII/HindIII and BamHI/HindIII, respectively. Visualization was performed and revealed expected sizes of 482 bp for the lrp insert and 5756 bp for the pIJ86 vector (Figure 3). The gel excision isolated both sets of DNA fragments to prepare for overnight ligation. Utilizing alternating temperatures, the ligation was designed to maximize sticky-end annealing (at 10 °C) and ligase activity (at 30 °C). This method increases the probability of generating a recombinant plasmid.

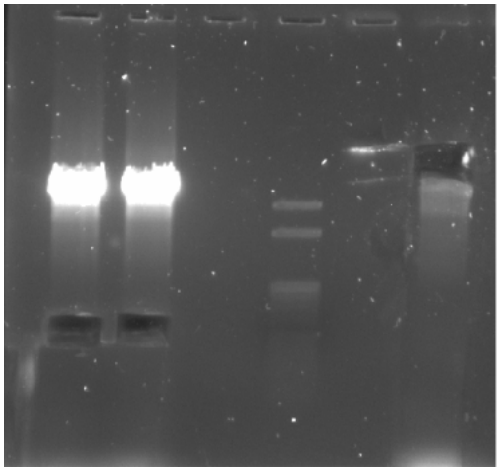


Figure 4. Cut SeeGreen Gel Electrophoresis. The ladder is in well 4. The lrp insert was cut from wells 1 and 2 around the 482 bp range. The pIJ86 vector was cut from well 6 at the 5756 bp range.

Transformation into DH5a

On Oct 10, the ligation mix was transformed into E. coli DH5a. This intermediate host is methylation-competent (dam+/dcm+), which yields high transformation efficiency and allows for rapid screening and propagation of the plasmid. Transformation plates yielded high efficiency of colony formation; 10 different transformant colonies were selected for confirmation of pIJ86-*lrp* Clones through Colony PCR. Bold bands are indicative of a successful transformation and amplification of the lrp gene. The bands are around the 565 bp region, which aligns with the size of the lrp gene. Samples 4, 5, 8, and 9 amplified the most product, but amplified product is also seen in samples 1, 2, 3, and 10.

Figure 5: Gel Electrophoresis revealed the results of amplified lrp gene -565 bp.

The verified transformation *E. coli* cells were grown in LB broth with visible turbidity present, and cultures were then prepared for purification of the host bacterium. On October 20, 2025, a Purity concentration Analysis was performed on Plasmid Extracts, yielding a Concentration of 74.2 µg/mL with a 260/230 Purity of 1.82. With a subsequent Restriction Digest and Gel electrophoresis to all but prove the presence of the correct plasmid at 5496 bp.

Transfer to Non-Methylating *E. coli* ET12567/pUZ8002 Host

The validated plasmid was transferred into an electrocompetent *E. coli* strain that prevents the methylation of DNA. Electroporation was then performed between DH5a and ET12567/pUZ8002. The first Trial was declared a failure as the Negative Selectivity Control (Water + Cells plated on LB + Apr/Cm/Kan) showed confluent growth. The negative control should show zero growth. The cells were either already resistant to the antibiotics, contaminated, or the plates were degraded/not overlaid properly. For the following trial, New stock solutions were prepared, and fresh plates were poured to rule out intrinsic contamination of the stock. On October 23, 2025, successful electroporation was confirmed as all controls showed expected growth, validating the selective pressure. Experimental plates showed confluent growth, indicating successful transformation with isolated colonies. To determine if the electroporation

yielded our insert, 10 colonies were picked for Colony PCR. Throughout all samples, the gel analysis displayed incorrect bands throughout all samples and smearing across the gel.

Figure 6: Gel Electrophoresis of Colony PCR product, incorrect band placement indicates unsuccessful PCR reaction for the intended product of lrp.

When addressing the cause of these results, it may have been possible that the primers used during the reaction were causing an incorrect cut in the product. Further research points to Colony PCR being notoriously unreliable in ET12567/pUZ8002. The helper plasmid pUZ8002 is large (~55-60 kb) and low copy, but creates a complex DNA background that can cause smearing. The Primary Investigator pivoted from Colony PCR to plasmid extraction, where 10 transformants were pooled. The restriction digest of the extracts showed a prominent smear, which is typical when isolating plasmid DNA from ET12567, but displayed the 5496 bp plasmid (Figure 7). To bypass the visualization problem, a PCR was performed on the extracted plasmid pool, which yielded definite bands at -494 bp. This confirmed that the *lrp* gene was present in the library, even if the digest was messy.

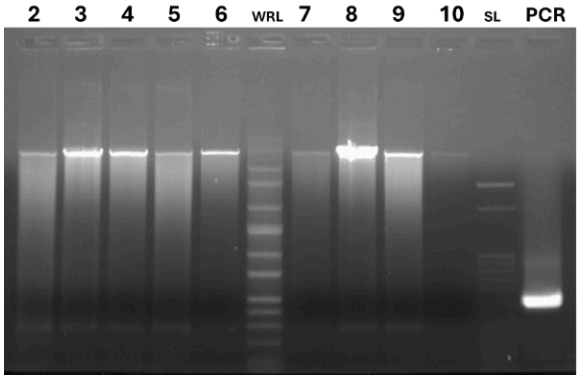


Figure 7: 1% Agarose gel electrophoresis of restriction digests and PCR product. Numbers indicate the transformant number (restriction digests). WRL, wide range DNA marker; SL, DNA StepLadder; PCR, PCR product from 101725 extracted plasmid #5.

To further confirm, a subsequent Colony PCR was performed on 10/27 using a modified procedure to successfully identify positive clones in lanes 3-10 (Figure 9).

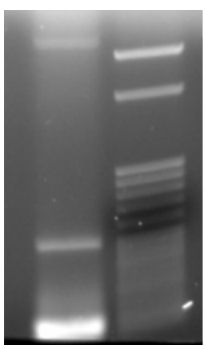


Figure 8: 1% Agarose gel electrophoresis of PCR product from combined plasmid extracts from transformants 2-10 (left lane) vs. 10 µL of DNA StepLadder (right lane). , Lanes 1-3 show expected bands at 740bp and 5496bp. Lanes 4-5 display incorrect base pair counts for the intended sample.

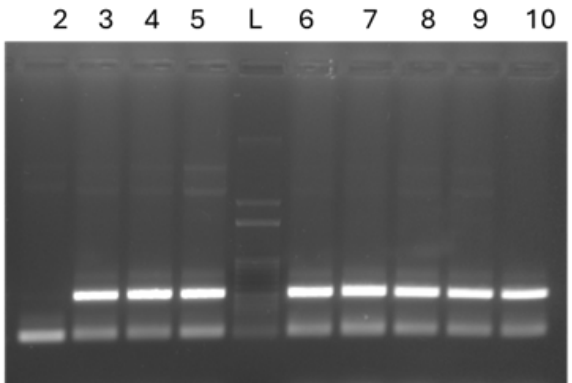


Figure 9: Gel Electrophoresis of Colony PCR product. Lanes 3-10 displayed correct band placement of 494bp.

Interspecific Conjugation

The donor *E. coli* [*lrp*+piJ86] cells (ET12567/pUZ8002) were mixed with heat-shocked *S. lividans* TK24 spores. Heat shock (50°C for 10 min) is critical to induce germination of the Streptomyces spores, making them receptive to plasmid transfer. Following the 18-hour incubation and antibiotic overlay (Nalidixic acid + Apramycin), the 10^-1 dilution plate and the (-) *E. coli* control plates showed a "film of bacteria," not concurrent with expected results. This indicates a failure of the counter-selection. Nalidixic acid targets DNA gyrase and is effective against Gram-negative bacteria like *E. coli*, while *Streptomyces* are naturally resistant. The overgrowth suggests that the ratio of *E. coli* to *Streptomyces* was too high in the low dilutions. The Nalidixic acid concentration (25 µg/mL) was insufficient for the density of the bacterial lawn, or the overlay was applied unevenly. The experiment proceeded with the higher dilution plates (10^-2, 10^-3). On these plates, no contamination was visible, and Streptomyces colonies were confirmed, appearing as white and red colonies (Figure 10).

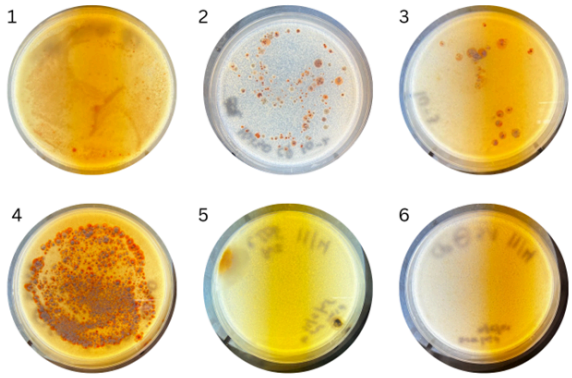


Figure 10: Dilution and Control Plates after 1 week. (Plate 1: 10-1) (Plate 2: 10-2) (Plate 3: 10-3) (Plate 4: (+) Control) (Plate 5: (-) Control E.coli) (Plate 6: (-) Control S. lividans)

S. lividans colonies are dry, leathery, embedded into the agar, and often produce a soil-smelling geosmin. These distinct “snowball” like colonies were plucked and streaked onto fresh (Apr + Nal) plates to establish pure cultures.

S. lividans Colony PCR

Initial attempts to screen the *S. lividans* exconjugants via Colony PCR failed, yielding no bands (Figure 11).

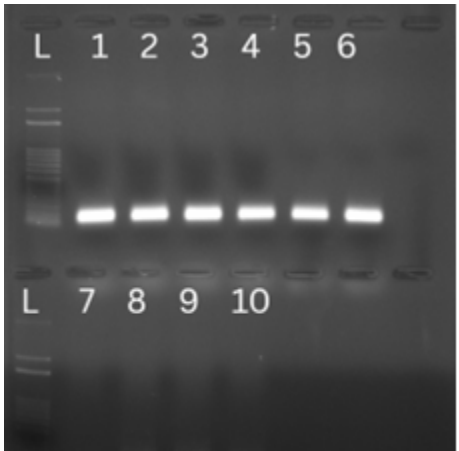


Figure 11: Colony PCR results for Conjugation, all 10 lanes displayed incorrect product placement.

Streptomyces are notorious for having a thick, multilayered peptidoglycan cell wall that is resistant to lysis through boiling.

Simply adding mycelia to a PCR mix rarely releases enough DNA. Furthermore, *Streptomyces* grow into the agar; picking colonies inevitably transfers agar, which contains polysaccharides that are potent inhibitors of *Taq* polymerase. The protocol was corrected to use the Plasmid extract from the Liquid Culture. On November 17, cells were grown in CRM broth and treated with Lysozyme (10 mg/mL) before lysis. PCR performed on this clarified lysate successfully amplified the 494 bp band but displayed a primer/dimer (Figure 12).

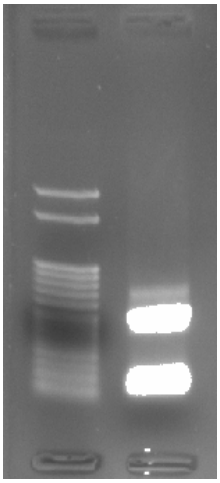


Figure 12: Plasmid Extract DNA visualized under Gel Electrophoresis, displaying lrp PCR product expected at 494bp.

Protein Expression and SDS-PAGE

Verified clones were cultured in CRM broth, harvested, lysed via sonication, and quantified for protein concentration via BCA Assay. The SDS-PAGE gel on 11/24 showed no unique protein bands in experimental lanes compared to control; the expected (~16 kDa) protein was absent (Figure 13).

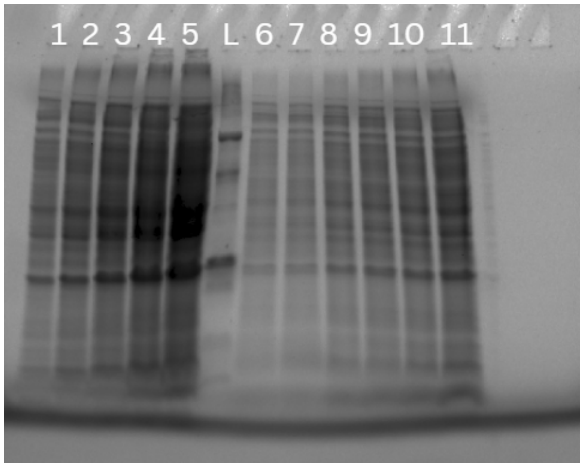


Figure 13: SDS-PAGE of exconjugant S.lividans TK24 [lrp + piJ86] in lanes 6-11, Control was present in lanes 1-5.

To investigate this lack of protein, plasmid DNA extracted for SDS-PAGE was subjected to a restriction digest using *Kpn1* and *HindIII* and subsequent visualization of the product yielded no presence of the insert.(Figure 14).

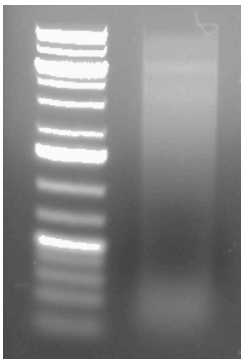


Figure 14: Restriction digest of S.lividans plasmid extract. No bands visualized.

The plasmid isolated from the protein expression culture contained the pIJ86 backbone (Apramycin resistance is confirmed) but lacked the *lrp* gene. The results of this experiment yield a clone that is genotype positive but phenotype negative, requiring further research and testing to evaluate the issues at hand.

Mechanisms of Instability

The discrepancy between the positive genotype confirmed on Nov 17 and the negative phenotype/missing gene on Nov 24 is the defining question of this project. This is not a failure of technique, but a biological response of the host organism that must be evaluated further. If the *S. nymphaeiformis* Lrp protein is toxic to *S. lividans* TK24, or if it acts as a "molecular monkey wrench," the host cell is placed under extreme selective pressure. Since the plasmid carries the Apramycin resistance gene, the cell cannot simply discard the plasmid to survive in the antibiotic-containing media, meaning some form of the insert is present and salvageable.

Alternatively, the population may have undergone a micro-evolution in the culture flask. Rare variants that undergo homologous recombination or deletion events—excising the toxic *lrp* insert while retaining the antibiotic resistance marker—gain a massive growth advantage. These "cheater" cells, no longer worried by the production of the toxic protein, replicate faster than the intact clones. Over the course of the multi-day incubation required for Streptomyces growth (often 5-7 days), these deletion mutants overtake the culture, and struggling positive exconjugants are outcompeted.

By the time the culture was harvested on Nov 24, the population was dominated by cells carrying the empty or mutated vector. This explains why the plasmid prep showed the vector backbone but no insert, and why the SDS-PAGE showed no protein for Lrp. 16sDNA sequencing is recommended to ensure the insert was not mutated and to pinpoint any errors that may have occurred.

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