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Extraction of Transcriptional Regulators for the Polyhydroxyalkanoate Depolymerase Gene from *Streptomyces nymphaeiformis*

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Abstract

Plastic waste has become an increasingly prevalent environmental pollutant. This problem is exacerbated by the inability of plastic to degrade under most natural conditions. In contrast, polyhydroxyalkanoates (PHAs) are biologically produced, plastic-like polymers that can be broken down and metabolized by bacteria. The bacterium *Streptomyces nymphaeiformis* can degrade the PHA, polyhydroxybutrate (PHB), using an extracellular PHB depolymerase, which is encoded by the *phaZ* gene. PHB depolymerase is synthesized only in the presence of PHB or its monomer, but not glucose, suggesting that transcription of *phaZ* is regulated, presumably by transcriptional regulatory proteins that bind to its promoter region. The DNA base sequence of *phaZ* has previously been determined. In the predicted promoter region, there are sequences with homology to binding sites for known transcriptional regulators from other bacteria. A modified pull-down assay using streptavidin magnetic beads was evaluated for success in elucidating potential regulatory DNA-binding proteins for the *phaZ* gene. However, the method was unsuccessful.

Introduction

Commonly used in a variety of settings from household purposes to industrial environments, synthetic polymers commonly known as petroleum-based plastics are used widely due to their resistance to degradation. However, this same resistance to degradation has led to adverse effects on the environment as plastics have begun to over accumulate; "typically, 40-80% of mega- and macro- marine debris items are plastics, much of it packaging, carrier bags, footwear, cigarette lighters and other domestic items"(1). Polyhydroxyalkanoates (PHAs), are biologically produced, plastic-like polymers. While PHAs are currently more expensive to produce than petroleum-based plastics, their use could slow down the accumulation of plastic waste. PHAs are polymers of "R(-)-3-hydroxyalkanoic acid (HA) monomers ranging from C3 to C14 carbon atoms" composed of different chains that can be saturated or unsaturated, straight or branched (2). PHAs, which accumulate as granules within the cytoplasm of cells, are produced to serve as "intracellular carbon and energy storage compounds" (3). There are known to be nearly 125 different monomer units (HAs) of PHAs, resulting in many polymers with various unique properties and uses (4). The PHA most studied has been polyhydroxybutrate (PHB), which is composed of 3-hydroxybutyrate (3HB) monomers. PHAs are produced intracellularly by bacteria when they are in an environment which does not provide stable growing conditions; poor environments would lack key nutrients such as nitrogen, phosphorus, or magnesium, but have excess carbon. PHAs are then utilized as a source of energy once growth conditions are stable. However, when PHA-producing bacteria die and lyse, the PHAs become available for use as an energy source by PHA-degrading bacteria (5).

The ability of microorganisms to degrade PHAs is dependent on the release of PHA depolymerases, of which there are two types, extracellular and intracellular. Intracellular depolymerases function to degrade PHAs stored within the cell (5). Extracellular PHA depolymerases (e-PHAD) are secreted into the surrounding medium by PHA-degrading bacteria to hydrolyze extracellular PHAs into water soluble monomers (5). Advancement in the understanding of the genetic mechanisms that result in the synthesis of PHA depolymerases could be useful in accelerating the breakdown of biodegradable plastics in environments such as landfills.

The genus *Streptomyces* is characterized as Gram positive, filamentous soil and water bacteria that produce a variety of antibiotics. *Streptomyces nymphaeiformis* degrades PHB and produces a PHB depolymerase when it is grown in the presence of PHB or the monomer 3HB, but not in the presence of glucose (6). This indicates that transcription of the gene which codes for PHB depolymerase (*phaZ)* in this organism is regulated. A long-term goal of the research in Dr. Baron's lab is to determine the molecular mechanism for the regulation of *phaZ* in *S. nymphaeiformis*.

The regions of *phaZ* coding for PHB depolymerase, as well as 169 DNA base pairs upstream from the coding region have been cloned and sequenced (6) The upstream sequence contains likely -10 and -35 consensus *Streptomyces* promoter sequences to which RNA polymerase may bind to the DNA for initiation of transcription (Fig. 1) (7). In addition, this region contains several direct and inverted based repeats which are thought to be binding sites for activators and repressors which regulate the transcription of *phaZ* (Fig. 1). Similar repeats control transcription of the chitinase gene in *Streptomyces lividans* (8). The *phaZ* promoter region also contains sequences homologous to binding sites for known regulatory proteins from other bacteria, such as SpoIIID of *Bacillus subtilis* which activates genes in response to carbon starvation (5), and RhIR of *Pseudomonas aeruginosa,* which activates genes in response to high cell densities (7). Both sequences could provide active binding sites for transcriptional activators or repressors that regulate *phaZ.*

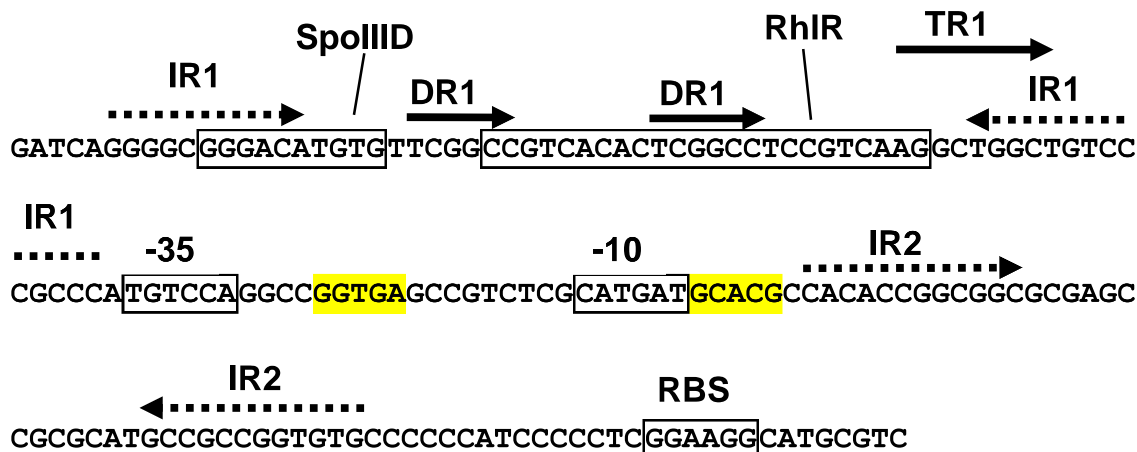


Figure 1. Diagram of the promoter region of *phaZ*. The -35 and -10 consensus sequences are boxed. The short sequences homologous to the sequences found in SpoIIID and RHIR are indicated. Inverted repeats (IR1 and IR2), a direct repeat (DR1), and a tandem repeat (TR1) are indicated by arrows.

The method of streptavidin pulldown assay can be utilized for the detection of potential DNA binding regulatory proteins (9). This method uses a biotin labeled DNA sequence suspected of binding regulatory proteins, which is then tightly bound to streptavidin, a biotin-binding molecule, which in turn is covalently bound to magnetic beads. The beads with the bound labeled DNA are then mixed with a cell extract and incubated. After incubation a magnetic field can be used to collect the beads, which are then washed with a buffer to remove unbound or non-specifically bound proteins. The DNA binding proteins are released by boiling and can be visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The goal of this research project was to extract transcriptional regulators of *phaZ* from cell extracts of *Streptomyces nymphaeiformis.* Specific objectives were: 1) to amplify the *phaZ* promoter region via PCR; 2) bind the labeled probe to streptavidin magnetic beads; 3) grow cells of *S. nymphaeiformis* and prepare cell extract; 4) perform streptavidin pulldown; and 5) evaluate binding of proteins to the beads using SDS-PAGE.

Materials and Methods

Objective 1. The full promoter region *phaZ* has been cloned into a plasmid, S82 (6). DNA probes were synthesized by polymerase chain reaction, using plasmid S82 as template and forward and reverse primers (EMSA1-F, EMSA1-R, Table 1) that flank 182 bp of the promoter region. The forward primer was labeled with biotin at its 5' end during synthesis (MWG Biotech, Louisville KY) to ensure the resulting DNA probe would be biotin-labeled. Identical unlabeled primers were used to produce unlabeled DNA probes. PCR reaction tubes contained 12.5 pmol each of EMSA1-F and EMSA1-R primers, 5% dimethyl sulfoxide, one Illustra PureReady-to-Go™ PCR bead (Cytiva), and 8.4 ng of S82 plasmid all in a final volume of 25 µL. The PCR schedule was as follows: initial denaturation at 94 °C for 3 min; 30 cycles of 94 °C for 1 min, 54 °C for 30 sec, 72 °C for 1 min; and final incubation at 72 °C for 5 min. PCR product was analyzed on a 2% agarose minigel in 1X tris-acetate-EDTA (TAE) buffer (10) at 80 V to ensure alignment with predicted length of 182 bp. DNA size was estimated using Step Ladder DNA size standards (Sigma-Aldrich, St. Louis MO). The concentration of extracted DNA was measured using a Multiskan SkyHigh™ microplate reader equipped with a µDrop™ plate.

Forward Primer	5'-ATCGGTCTACTCGATCAG-3'
Reverse Primer	5'-ATCACGCATGCCTTCCGA-3'

Table 1. ESMA Primer Sequences. Biotin labeled and unlabeled primers contained the same sequence, but the labeled forward primer has biotin attached to its 5' end.

Objective 2: Biotinylated *phaZ* promoter DNA (the bait) was mixed with streptavidin coated magnetic beads in 2X binding and washing buffer (2X B&W) (10 mM Tris-Cl, 1 mM EDTA, 2M NaCl, pH 7.5), then incubated at 30 °C with agitation. After 15 minutes a magnetic field was applied for 3 min, and the supernatant removed. A sample (2 µL) of the supernatant was used to quantify the DNA concentration, using a Multiskan SkyHigh Microplate Spectrophotometer equipped with a µDrop™ plate (ThermoFisher Scientific). Treatment with the streptavidin

magnetic beads was expected to remove all or most of the biotinylated DNA from solution. The bait-labeled magnetic beads were stored in 1X B&W buffer at 4°C. Binding of the bait to the streptavidin beads was evaluated using a Chemiluminescent Nucleic Acid Detection Module (#89880, Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, samples (0.5 µL) of untreated bait and supernatant from after mixing of bait with streptavidin beads were spotted onto a 1 x 5 cm strip of Immobilon-NY+ charged nylon (#INYC20200, Millipore, Bedford MA), followed by cross-linking with ultraviolet light, incubation with streptavidin-horseradish peroxidase conjugate, washing, and development with luminol/peroxide solution. Chemiluminescence was detected with an iBright Imager (ThermoFisher Scientific).

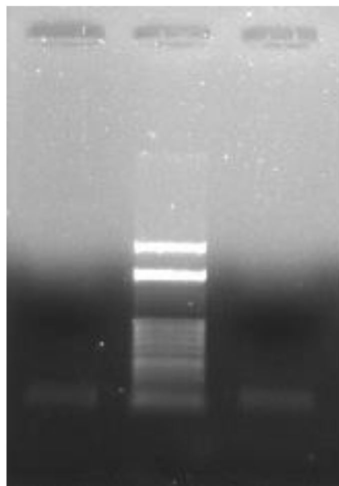
Objective 3: *S. nymphaeiformis* was grown in a defined medium containing 0.2% w/v PHB (SNC-PHB), as described (6). PHB depolymerase activity in culture supernatants was determined using a qualitative slide assay and a quantitative turbidimetric assay (6). Cells were harvested by centrifugation (5,000 x g, 10 min, 4 °C) and resuspended in an equal volume of Buffer A (20 mM Tris-Cl, 5 mM EDTA, 50 mM NaCl, 1 mM 2-mercaptoethanol, pH 8.0) In one method (Trial 1), cell homogenate was prepared by mixing cells with 0.1 mm glass beads, freezing the mixture at -80°C, grinding in a mortar and pestle, and sonication for 30 min (30 sec pulses, 30 sec pauses). In another method (Trial 2) homogenate was prepared by passing cell pellet resuspension through a French pressure cell at 15000 psi twice. Homogenates from Trials 1 and 2 were clarified by centrifugation, resulting in cell extracts. Proteins bound to nucleic acids were removed by precipitation with 1% w/v polyethyleneimine (PEI), followed by centrifugation (30,000 x g, 10 min, 4 °C); the pellet was then extracted twice with 200 mM NaCl in 20 mM Tris-Cl pH 7.5. The supernatant from the extractions were made to 80% saturation in ammonium sulfate. Precipitated protein was pelleted by centrifugation (30,000 x g, 10 min, 4 °C), and the pelleted proteins then redissolved in a minimal volume of Buffer A. Protein concentration of the redissolved protein (PEI extract) was then determined using the bicinchoninic acid (BCA) assay (Thermo Scientific™, catalog #23225).

Objective 4: Methods used for this objective were modified from Sui et al. (9). Pulldown reactions contained 12 µg of PEI extract and 10 µL of bait-labeled magnetic beads in a final volume of 20 µL and were incubated at 30 °C for 1 hour. Sheared salmon sperm DNA (competitor DNA) (4 µg) were added to a parallel sample as a specificity control (12). An additional control included cell extract incubated without competitor DNA and with magnetic beads not labeled with the bait. A magnetic field was applied to pellet the magnetic beads. The supernatant, which contained non-specific proteins was removed. The pellet was washed four times with protein binding buffer (20 mM Tris (pH 7.5), 20 mM KCl, 10% v/v glycerol, 5 mM $MgCl_2$, and 1 mM 2-mercaptoethanol) at 2.5 times the initial volume of reaction mixture. Pellets were resuspended in 10 µL of ultrapure water plus 10 µL of 2X Laemmli SDS-PAGE sample buffer (13). The mixture was boiled for 5 minutes and microfuged to pellet magnetic beads. The supernatant containing proteins released from the beads was subjected to SDS-PAGE.

Objective 5: Proteins released from the streptavidin magnetic beads were analyzed by SDS-PAGE on Mini-PROTEAN TGX Stain-Free Precast Gels (12%T) (Bio-Rad Laboratories, Hercules CA) using the system of Laemmli (13). Proteins were visualized using a Bio-Rad Gel-Doc™ Imaging

system and by silver staining (14). Migration of sample proteins was analyzed in comparison to molecular weight markers to estimate sizes.

Results and Discussion



The first objective was to amplify the *phaZ* promoter region of *S. nymphaeiformis.* The methods outlined were successful based on the results of agarose gel electrophoresis. The *phaZ* promoter region has a length of 182 bp, and the agarose gel exhibited faint bands at approximately 200 bp (Fig. 2). After purification of the PCR product high concentrations were regularly achieved, with the highest being 99.6 µg/mL.

Figure 2. Agarose gel electrophoresis of PCR product. Well contents left to right, unlabeled probe, step ladder, labeled probe.

The labeled *phaZ* probe successfully bound to streptavidin magnetic beads as shown by chemiluminescence imaging. Untreated bait DNA showed considerable chemiluminescent signal, while the supernatant after incubation of the bait with streptavidin magnetic beads completely lacked signal (Fig. 3).

Figure 3. Chemiluminescent trial results. L, untreated bait DNA; S, supernatant after incubation of bait with streptavidin magnetic beads. Darkness represents chemiluminescent signal.

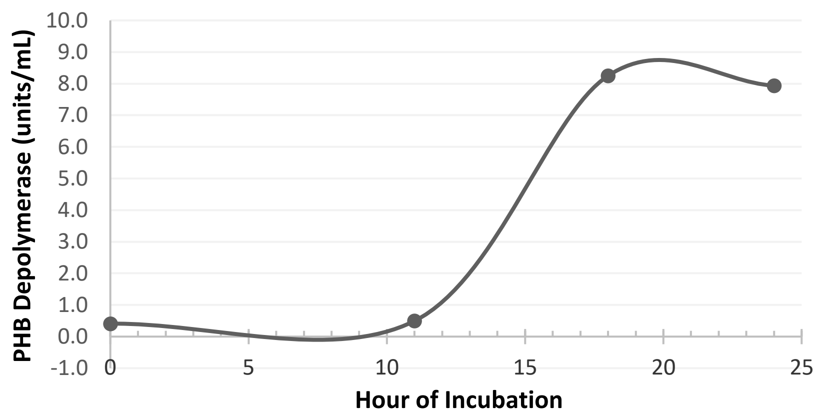
Cells of *S. nymphaeiformis* were grown for use in preparing cell extracts for analysis. However, during an initial attempt (Trial 1) to grow cells, growth and PHB degradation were unusually slow in the defined medium. A Gram stain conducted during the cell-growth indicated that the culture was pure, and the microscopy indicated the expected morphology of *S. nymphaeiformis.* Furthermore, a qualitative slide assay for PHB depolymerase in a 24 h culture supernatant did not show PHB depolymerase activity. The lack of enzyme activity could have resulted from delayed analysis, potentially allowing proteases to degrade any enzyme that would have been present. Nevertheless, these cells were utilized for cell extract preparation. Initially, a method requiring the grinding of the cells in a chilled mortar and pestle, followed by sonication to produce a cell lysate was utilized. A workable protein concentration of 25.9 mg/mL was indicated by a BCA assay.

For the second cell growth attempt (Trial 2), new cultures were prepared using a pure glycerol stock from 2018 which had been stored at -80 °C. When this culture was streaked onto defined agar medium containing PHB, good cell growth and PHB degradation were visible (Fig. 4), indicating good production of PHB depolymerase. Cultures prepared with the 2018 cells followed a growth curve more consistent with previously observed trends (6).

Figure 4. *S. nymphaeiformis* on defined PHB agar plate, showing visible PHB clearing.

Additionally, quantitative turbidimetric assays of PHB depolymerase assay were using culture supernatants obtained at time intervals 0, 11, 18, and 24 h during growth in defined PHB medium (Fig. 5A). Activity increased over time, with a maximum of 8 units/mL at 18 h, consistent with previous results (6). In addition to good PHB activity, at the conclusion of cell growth a gram stain (Fig. 5B) was performed which indicated the expected morphologies. Based on these observations it was expected that cell lysate from this cell growth would be more characteristic of *S. nymphaeiformis*, and would be more likely to contain *phaZ* DNA-binding proteins.

A



B

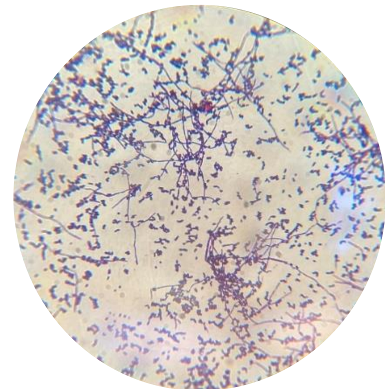


Figure 5. Growth and PHB depolymerase production by *S. nymphaeiformis* in defined medium containing 0.2% w/v PHB. **A,** PHB depolymerase activities; **B,** Gram stain of cells from 24 h (1,000X magnification, bright-field, oil immersion)

In preparation of extract from Trial 2 cells, a French pressure cell was used instead of the grinding and sonication used for Trial 1 cells. This greatly improved the observed protein concentration. Grinding and sonication resulted in a protein concentration in the cell extract of 1.87 mg/mL versus 7.27 mg/mL with French pressure cell lysis.

Ajith et al. reported successfully using PEI to remove DNA-protein complexes from cell extracts of *Streptomyces peucetius* (11). Therefore, we used PEI to try to enrich for DNA-binding proteins in cell extracts of *S. nymphaeiformis*, followed by extraction of the PEI pellet and ammonium sulfate precipitation of the extracted proteins. During the process the PEI pellet extract

obtained with Trial 2 cell extract did not have the same color and odor as that obtained with Trial 1 cell extract. This may have been related to the differences observed during cell growth. At the conclusion of PEI purification with Trial 2 cell extract, the redissolved ammonium sulfate pellet had a relatively low concentration of 0.725 mg/mL, compared to 25.9 mg/mL with Trial 1 cell extract.

We were concerned that DNA binding proteins may not have bound to the PEI and instead remained in the supernatant after PEI treatment. Therefore, the PEI supernatant from Trial 2 was precipitated with ammonium sulfate and redissolved in a minimal volume of buffer. This solution had a protein concentration of 4.32 mg/mL as compared to the 25.9 mg/mL from the Trial 1 purification. As both Trial 2 concentrations were substantially lower than the Trial 1 concentration, cell lysate, purified PEI extract, and purified PEI product supernatant from Trial 2 were all utilized for the streptavidin pulldown assay. The streptavidin pulldown assays proceeded in an expected manner, as the pellets of streptavidin magnetic beads appeared to form properly after magnetization, and washes proceeded without issue.

Proteins bound to the bait-labeled streptavidin magnetics beads were analyzed by SDS-PAGE. Fluorescence imaging of the gel loaded with Trial 1 samples (Fig. 6A) exhibited a strong band at 14 kDa for all samples. This probably represented streptavidin monomers, since the mass of the streptavidin tetramer is 52 kDa. Diffuse protein bands were observed in all lanes. However, an expected *phaZ* DNA-binding protein would be expected to appear in lane 4 (“bait, no competition”) and possibly lane 5 (“bait, with competition”), but not in lanes 1 or 2 (“no bait, no competition”). In comparison to Fig. 6A, Fig. 6B (silver-stained gel) shows no bands, not even the standards step ladder. This suggested that during silver staining all proteins were washed from the gel. Therefore, samples from Trial 1 did not provide evidence of *phaZ* DNA-binding proteins.

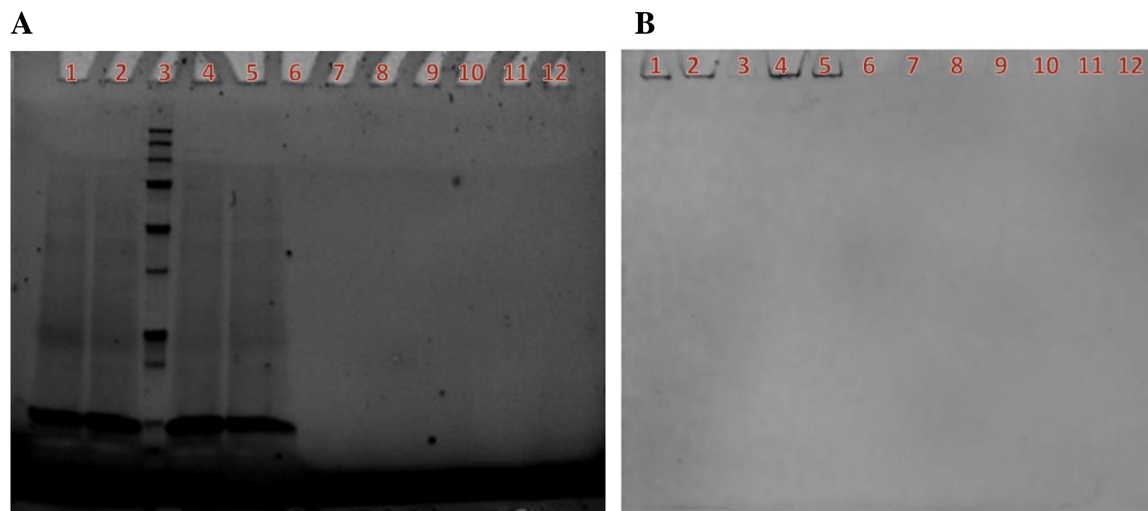


Figure 6. Protein SDS-PAGE Gel Imaging of Affinity Purification Products. Well contents from left to right (both A and B): “no bait, no competition”, “no bait no competition” replicate, BIO RAD Precision Plus Protein Unstained Standard, “bait, no competition”, “bait, with competition”. Panel A shows the unstained SDS-Page gel. Panel B shows the SDS-Page gel after being exposed to silver stain procedures.

Samples from Trial 2 also did not provide additional insights. None of the “bait” lanes exhibited visible bands that were not also visible in the “no bait” control lanes (Fig. 7C). As with the case seen in the process of silver staining, Coomassie Blue staining was unable to increase resolution of potential protein bands, and in fact resulted in decreased band resolution. Of note, once again the strong bands were observed at 14 kDa expected to be streptavidin monomer. With this continued observation, a potential concern with the modified pull-down methodology is the possibility that the streptavidin monomer bands could be masking potential promoter protein bands.

A

	A w/o “bait” w/o comp	B w/ “bait” w/o comp	C w/ “bait” w/ comp
1 Purified Extract	1A	1B	1C
2 Purified PEI Supernatant	2A	2B	2C
3 Cell lysate	3A	3B	3C

B

Well 1	1A
Well 2	1B
Well 3	1C
Well 4	Standard
Well 5	2A
Well 6	2B
Well 7	2C
Well 8	Standard
Well 9	3A
Well 10	3B
Well 11	3C
Well 12	No sample

C

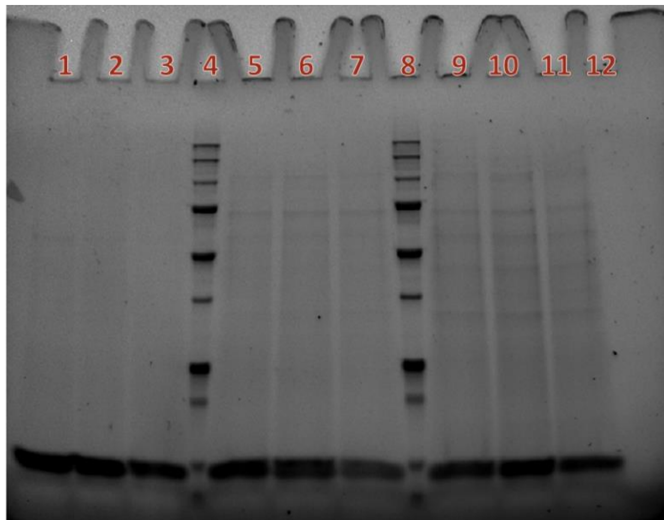


Figure 7. Protein SDS Page Imaging of Affinity Purification Products (Attempt 2). Panel A shows the labeling utilized for the samples. Panel B shows the loading pattern utilized. Panel C shows the unstained SDS-PAGE Gel.

Conclusion and Future Work

The use of a modified-pull down assay using magnetic streptavidin beads was unsuccessful in elucidating the potential *phaZ* DNA-binding proteins. A potential issue in the outlined method is the possibility that in the SDS-PAGE imaging *phaZ* DNA-binding proteins could be disguised by the bands at 14 kDa, which are expected to result from streptavidin monomer. In future work an electrophoretic mobility shift assay could be utilized to eliminate the inclusion of streptavidin, and potentially reveal any protein bands that could be disguised.

Acknowledgements

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