A Study of Protein-Protein Interactions in *Salmonella* Typhimurium

by

Zenan Tao

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Graduate Supervisory Committee:

Yixin Shi, Chair
Heather Bean
Xuan Wang

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ABSTRACT

The Multiple Antibiotic Resistance Regulator Family (MarR) are transcriptional regulators, many of which forms a dimer. Transcriptional regulation provides bacteria a stabilized responding system to ensure the bacteria is able to efficiently adapt to different environmental conditions. The main function of the MarR family is to create multiple antibiotic resistance from a mutated protein; this process occurs when the MarR regulates an operon. We hypothesized that different transcriptional regulator genes have interactions with each other. It is known that *Salmonella pagC* transcription is activated by three regulators, *i.e*., SlyA, MprA, and PhoP. Bacterial Adenylate Cyclase-based Two-Hybrid (BACTH) system was used to research the protein-protein interactions in SlyA, MprA, and PhoP as heterodimers and homodimers in vivo. Two fragments, T25 and T18, that lack endogenous adenylate cyclase activity, were used for construction of chimeric proteins and reconstruction of adenylate cyclase activity was tested. The significant adenylate cyclase activities has proved that SlyA is able to form homodimers. However, weak adenylate cyclase activities in this study has proved that MprA and PhoP are not likely to form homodimers, and no protein-protein interactions were detected in between SlyA, MprA and PhoP, which no heterodimers have formed in between three transcriptional regulators.

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Zenan Tao

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CHAPTER 1

INTRODUCTION

Due to the wide range of environmental conditions bacteria have to be able to efficiently adapt to their changing environment. It is vital for the cell's survival to be able to monitor the changes that occur around it: such as, pH, temperature, concentrations of toxic substrates, and osmotic activity (1). This is commonly accomplished using the two-component system. The two-component signal transduction occurs when a stimulus is received and passed to a response regulator in order to adapt to the environmental changes (2). This two-component signal transduction often involves a histidine protein kinase, which is later transferred to an aspartate residue after the regulator protein has taken action (3). The transmembrane proteins channel takes the input from the stimulus to the intracellular responses that adapt to it (1).

Protein-protein interactions play an important role in the cells ability to adapt to its environment (4). Currently, research is being done in order to further understand the protein-protein interactions that occur in living organisms. These protein-protein interactions are being analyzed and recorded in order to create the ultimate interaction map known as the interactome map (4).

MarR Family

Multiple Antibiotic Resistance Regulator family (MarR) is known as a transcriptional regulator, which plays an important role in molecular biology. MarR family is found in Escherichia coli and many other species. The process of the transcriptional regulation is to transcript DNA to RNA in order to maintain the cell's

gene activities (5). When MarR family regulates an operon, a multiple antibiotic resistance is created from a mutated protein The MarR family is primarily in charge of controlling antibiotic efflux pumps as well as the expression of genes (7) and the regulation of metabolic enzymes (5). This specific class of transcriptional regulators function primarily by controlling an operon that affects the drug efflux pumps (8). The operon encodes the efflux pumps which are then responsible for transporting and eliminating toxic substances.

Efflux pumps are transport proteins, present in microorganisms (9), that are responsible for the elimination of toxic substances. Their job is so find toxic substrates within the cell and transport them into the external environment (8). These pumps were first observed in Escherichia coli (9). Efflux pumps are not only able to transport toxins outside of the cell, but they also have mechanisms that allow them to lower antibiotic concentration and also enhance mutation accumulation (10). Antibiotic resistance can be accomplished by either decreasing the targets affinity for binding to the antibiotic, or by decreasing the concentration of the antibiotic that is present within the cell (9).

There are five major efflux transporters: major facilitator (MF), multidrug and toxic efflux (MATE), resistance-nodulation-division (RND), small multi-drug resistance (SMR), and ATP binding cassette (ABC) (8). These transporters can be associated with antibiotic resistance when over-expression occurs. This over-expression can occur with the activation of a transcriptional regulator such as MarA (8). Over-expression of multidrug efflux pumps has been linked to drug resistance (10). This is due to the cells ability to withstand and survive antibiotic pressure (8). Along with this, the cells are able to develop more mutations in the target site related genes (8).

When MarR family regulates an operon, a multiple antibiotic resistance is created from a mutated protein (5). The MarR family also controls mutations in certain proteins that lead to multiple antibiotic resistance (5). Antibiotic resistance is described as an antibiotics inability to reach its microbial target (9). This class of transcriptional regulators are commonly found within organisms showcasing a larger genome size. The larger the genome size the more MarR homologs can be found within that system. MarR homologs are abundantly found in free-living, complex organisms rather than organisms containing a reduced genome size. They are not found in abundance within organisms found in restricted niches or those who have a small genome size (7). Their presence within the system has shown to play a key role in allowing an organism to adapt to its changing environment.

The ability to adapt to a changing environment comes generally from the MarR family's capability to regulate gene expression. These transcriptional regulators function by repressing gene activity. Uncommonly, some MarR homologs have shown a potential to activate transcription, although most do not. Gene expression is most commonly regulated through several ways. It can either be regulated physically through ligand binding or chemically through the oxidation of specific cysteines (5). A conformational change occurs when the ligand bind resulting the attenuation of DNA (7). Ligand binding competes with RNA polymerase resulting in an obstruction that does not allow for transcriptional elongation to occur (5).

Transcription will shut of as the concentration of the MarR homologs increases. As the concentration of MarR decreases then transcription will increase. The nature of this mechanism allows for a more sensitive response to the binding of the ligands (5).

The MarR family of transcriptional regulators demonstrate an interesting structure that facilitates their gene regulation. The MarR family of transcriptional regulators display a winged helix-turn-helix (wHTH) DNA-binding domain (7). As shown in figure 1. They exist as dimers that commonly bind to palindromic sequences (5), as shown in figure 1. These palindromic sequences reside in cognate promoters (7). DNA-binding affinity is controlled by the interlock of the amino- and carboxy-terminal helices that determines the distance between the DNA recognition helices (5).

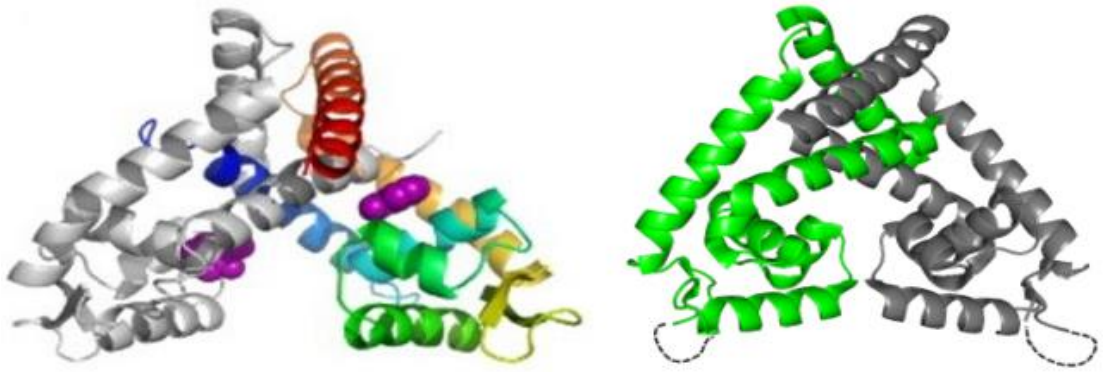

Figure 1: MarR family transcriptional regulator structure and SlyA dimer structure. Left illustrated above is the winged helix-turn-helix (wHTH) DNA- binding domain that is characteristic of the MarR family of transcriptional regulators (7). These transcriptional regulators exist as dimers that bind to palindromic sequences that reside in cognate promoters (5). The illustrated above in the right image is the SlyA dimer structure. The SlyA dimer binds to palindromic sequences as shown in the structure (27).

SlyA

SlyA is a MarR family transcriptional regulator belonging to the Enterobacteriaceae family (11). It was originally observed in Salmonella typhimurium where it was identified to be a key element for the survival of the bacteria in macrophages (12) as well as for the resistance to oxidative stress within bacteria (13). *slyA* has also been found to be a contributing factor in the regulation of certain PhoP-dependent genes (13).

In Escherichia coli *slyA* is used for the expression of a cryptic hemolysin (12) as well as cryptic cytolysin (6). More than 130 *slyA* homologs have been found in both bacteria and archaea (14). *slyA* has the same crystal structure and wHTH DNA-binding as the rest of the MarR family (15).

Proteins that derive from the MarR/SlyA family are responsible for expressing virulence genes (15). *slyA* also plays a key role in changing the cell surface in order to protect the cell from toxic compounds that the host produces (6). The regulation of *slyA* occurs through promoter occlusion (6). This allows *slyA* to be a gene that represses its own expression (6). As shown in figure 2.

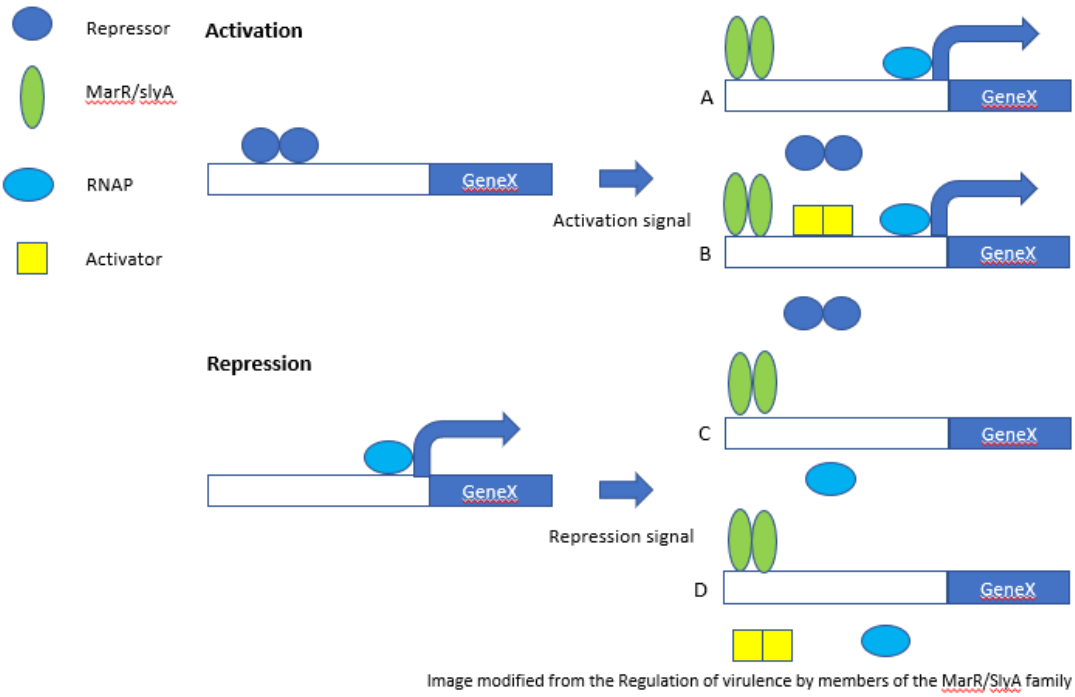

Figure 2: Activation and Repression of MarR/SlyA family transcriptional regulators. A. MarR/SlyA family competes with a repressor binding on the promoter to allow a transcription. B. MarR/SlyA family enabling an activator and competes with a repressor binding on the promoter to allow a transcription. C. MarR/SlyA family excludes RNAP and binds to the promoter. D.MarR/SlyA family competes with an activator protein on binding, excludes RNAP and prevents the transcription. Image is modified from the Regulation of virulence by members of the MarR/SlyA family.

MprA

MprA is a transcriptional regulator that shares a c-terminal wHTH DNA-binding structure with the rest of the MarR family (16). The *mprA* gene is responsible for controlling the expression of various Escherichia coli genes (17).

When transcribed the increase of the MprA serves as a regulatory mechanism for some genes. When increased it inhibits MccC7 production but blocks proU osmoinduction (17). The MprA is produced when during the stationary phase (18). *mprA* contains two recognition motifs that when bound illustrate important thymine residues (19). Further research needs to be conducted to determine the function of proteins encoded by *mprA*-dependent genes (19).

PhoP

PhoP is a transcriptional regulator found in Salmonella (20). This particular gene works commonly in a two-component system to provide resistance to different antimicrobial peptides (21). The two-component system that PhoP is a potent regulator that is able to perform diverse actions within the cell. It allows Salmonella to produce a lethal infection (tested in mice), adapt to conditions where Mg(2+) are provided, and create resistance to an array of antimicrobial peptides (22).

This transcription regulator works alongside SlyA to control gene expression. Specifically, in the expression of *slyA* itself; where SlyA is the repressor and PhoP is the activator of SlyA (6). The two-component system of the PhoP transcriptional regulator has shown to enhance resistance of both E. coli and Salmonella to toxic substances (23).

Protein-Protein Interaction

Proteins are important in living organism and cells because they control biological activity. Some proteins are independently, which include their only functions. However, most proteins control biological systems by interacting with other proteins. There are different type of protein interactions includes Protein-DNA Interaction, Protein-RNA Interaction, Protein-Cofactor Interaction, Protein-Ligand Interaction and Protein-Protein Interaction.

Protein-Protein Interaction is defined by physical contacts between two or more protein molecules that occur in vivo level, including living organism and cells (4). Protein-Protein interaction only occurs when two or more proteins interact together directly (24). However, functional interactions shouldn't be considered as Protein-Protein interaction because those functional interactions widely exist in living organisms, and includes interactions between genes, protein, and metabolites. Protein-Protein Interaction can be separated into two different types, stable interactions and transient interaction, both can be strong or weak. Common experimental methods to research Protein-Protein Interactions are co-immunoprecipitation, Pull-down assays, Far-western blot analysis, and Two-Hybrid Screening.

Co-Immunoprecipitation

Co-Immunoprecipitation is a straightforward and rapid method to detect protein-protein interactions. This method identifies protein-protein interactions when the interactions are stable and strong. As shown in figure 3. Co-Immunoprecipitation uses target protein-specific antibodies to locate and identify proteins that are bound with the target protein. This method located physiologically relevant interactions that are bound to the target protein. As shown in figure 3.

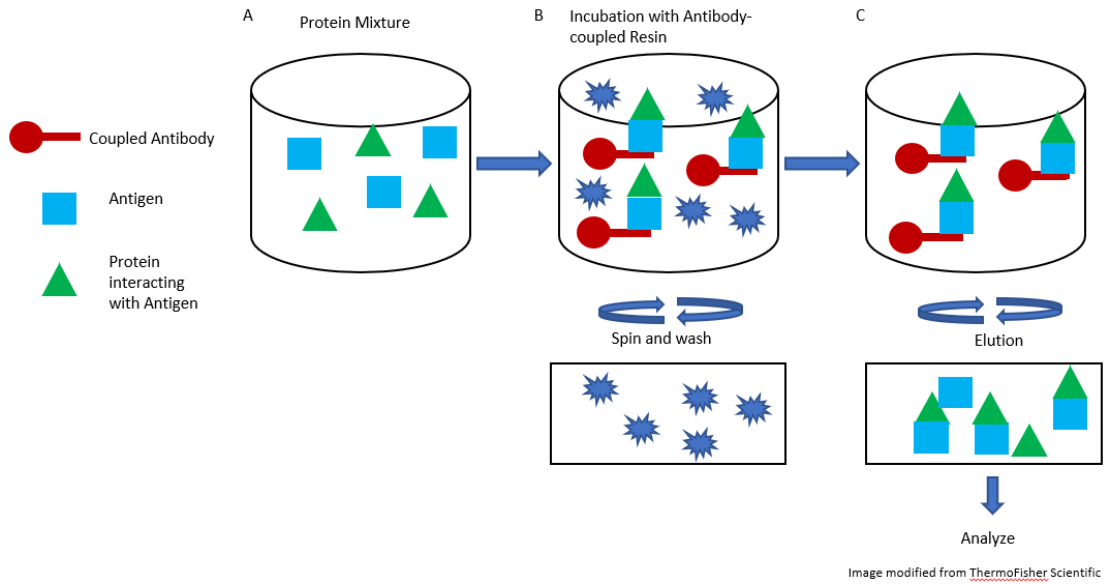

Figure 3: Principle of Co-immunoprecipitation (co-IP). A. A protein mixture was prepared. B. The protein mixture was incubated with Antibody-coupled Resin. The immune complex is then precipitated. An antibody-binding protein is immobilized. C. Purified Antibody-binding protein was eluted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Image was modified from Thermofisher Scientific.

Pull-down assays

Pull-down assay is a method commonly used to detect the physical interaction of two or more proteins in vitro. Pull-down assay is similar to Co-Immunoprecipitation. However, rather than using antibodies, the pull-down assay uses bait proteins to capture the proteins. This method is another form of affinity purification that involves affinity chromatography. Affinity chromatography is used in Pull-down assay to shorten the time for purifying proteins. As shown in figure 4.

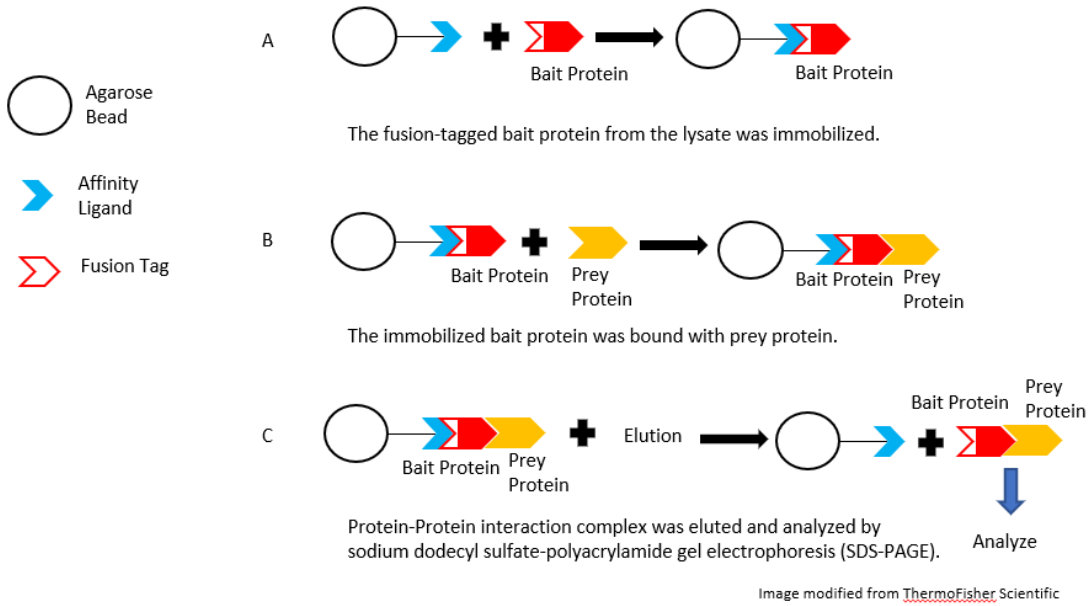

Figure 4: The principle of Pull-down assay. A. The fusion-tagged bait protein from the lysate was immobilized with an affinity ligand. B. The immobilized bait protein was bound with prey protein. C. Protein-Protein interaction complex was eluted and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Image was modified from Thermofisher Scientific.

Far-Western Blot

Far-Western blot is a direct method to analyze protein-protein interaction by using tagged bait proteins and prey proteins. These proteins separated through gel electrophoresis, at which point a protein-protein interaction can be detected. Far-Western blot allows the study of protein-protein interactions processed without using antigen-specific antibodies. As shown in figure 5.

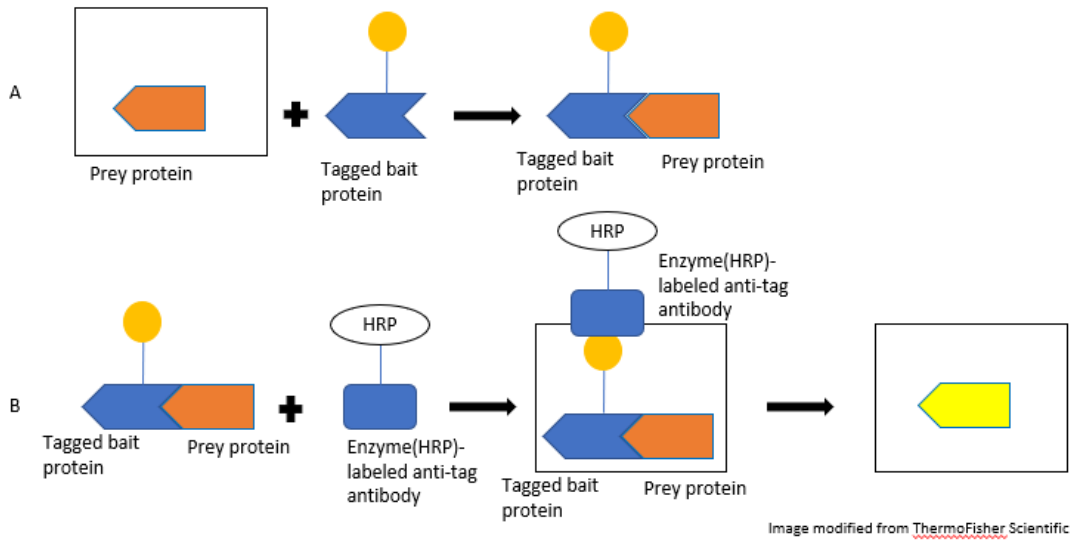

Figure 5: The principle of Far-western blot analysis. A. Prey proteins were separated by gel electrophoresis and interacts with tagged bait protein. B. Enzyme (HRP) with antibody was targeted on the bait tag of bait protein for labeling the interaction. radiolabeled bait protein that is detected by exposure to film. Image was modified from Thermofisher Scientific.

Two-Hybrid Screening

Two-Hybrid Screening is an accessible method to most labs without sophisticated equipment to provide a direct way to detect and research protein-protein interactions in vivo. The principle of Two-Hybrid Screening is to detect physical interactions between proteins, that protein-protein interactions can be discovered though this method. As shown in figure 6.

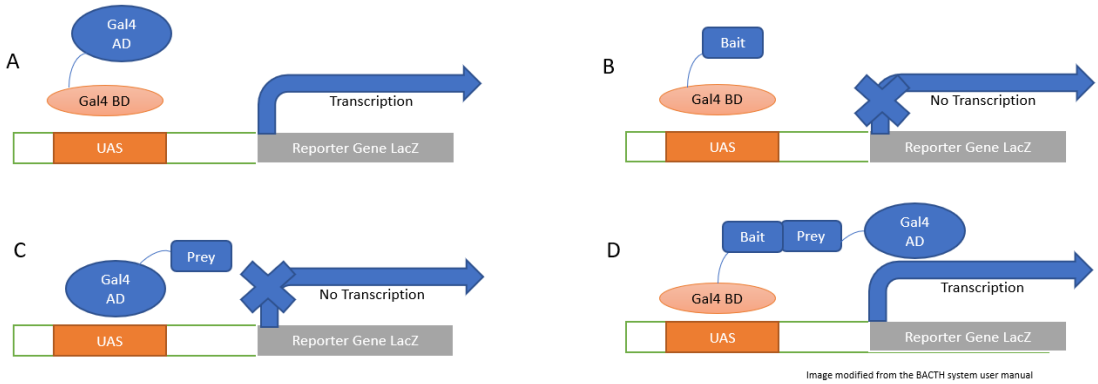

Figure 6: The principle of Two-Hybrid Screening. A. Gal4 transcriptional factor gene produces two domain proteins (BD and AD). Those proteins are essential for the transcription of the reporter gene (lacZ). B. A fusion protein Bait with Bal4BD were prepared and the transcription of reporter gene (lacZ) was not activated. C. A fusion protein Prey with Bal4AD were prepared and the transcription of reporter gene (lacZ) was not activated. D. Transcription of the reporter gene (lacZ) occurred when Gal4BD+Bait interacts with Gal4AD+Prey. Image was modified from the BACTH system user manual.

Bacterial Adenylate Cyclase Two-Hybrid (BACTH) System

The focus of this paper is to explore the protein-protein interactions of two hybrid proteins with the same recipient bacteria Escherichia coli DHM1 cells. This is done by using the BACTH system kit. The four fragments were pKT25, pKNT25 (both of which contained the kanamycin resistance gene), pUT18, and pUT18c (both of which contained the ampicillin resistance gene).

Bacterial Adenylate Cyclase Two-Hybrid (BACTH) System is a scientific system used to detect protein-protein interactions in Escherichia coli by using bacterial adenylate cyclase in vivo. Plasmids that carry T25 (pKT25 and pKNT25) or T18 (pUT18 and pUT18C) fragments were used to combine as fusions with three transcriptional regulator genes (*slyA*, *mprA* and *phoP*), due to different antibiotic resistances (Ampicillin resistance on T18 fragment and Kanamycin resistance on T25 fragment), to detect the co-expression of proteins in those transcriptional regulators. The reporter strain DHM1 that carries both T18 and T25 fragments can be grown on

selection plates with X-Gal. The X-Gal selection plates were used to detect the expression of lacZ if adenylate cyclase was activated. By running the β-galactosidase enzymatic activities assay, the interaction between those proteins can also be detected. As shown in figure 7.

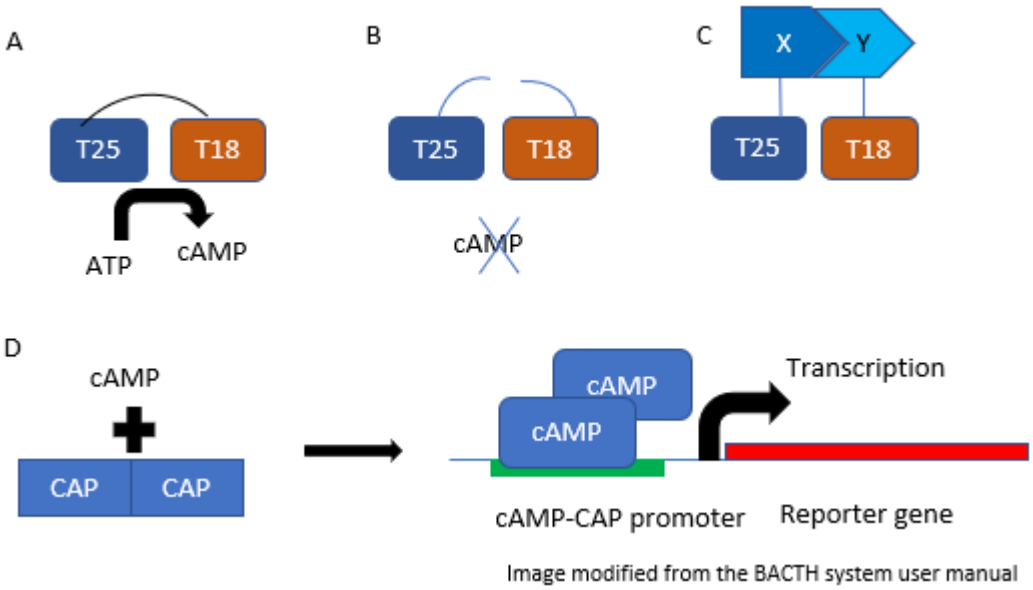

Figure 7: Shown above is the BACTH system that was used over the course of the experiment. A. Catalytic domain of adenylate cyclase (CyaA) from Bordetella pertussis combines two complementary fragments, T25 and T18. B. By separating two complementary fragments, T25 and T18 are not active. C. Two complementary fragments (T25 and T18) are used to interact with two polypeptides (X and Y). A functional complementation occurs due to the two protein interactions between two fragments. D. cAMP (Cyclic AMP) was produced and binds to the CAP (Catabolite Activator Protein). The cAMP/CAP complex regulator of gene transcription in Escherichia coli. Image was modified from the BACTH system user manual.

Plasmids

The catalytic domain of adenylate cyclase (CyaA) from Bordetella pertussis combines two complementary fragments (T25 and T18) that fused with two proteins that interacted with each other (Gouzei K, 1998). cAMP was produced when two-hybrid proteins interact with each other, which cause the functional complementation between two complementary fragments (T25 and T18). Transcription of catabolic operons was activated when cAMP binds with the CAP, which forms characteristic

phenotypes. Four plasmids that carry T25 fragment (pKT25 and pKNT25) or T18 fragment (pUT18 and pUT18C) were used and either N or C-termini on both fragments allow genetic fusions due to protein interactions.

As a derivative of plasmid pSU40, plasmid pKT25 produces a kanamycin resistance. It was also known that plasmid pKT25 carries a complementary fragment T25. When lac promoter controlled by transcription, T25 fragment was expressed. By inserting a multicloning site sequence (MCS) to the 3' end of T25, the in-frame genetic fusion can be constructed at the C-terminal end of the T25 polypeptide.

plasmid pKNT25 is also a derivative of plasmid pSU40, which express a kanamycin resistance. By inserting a multicloning site sequence (MCS) to the 5' end of the T25, the in-frame genetic fusion can be constructed at the N-terminal end of the T25 polypeptide.

plasmid pUT18 produces an ampicillin resistance because it is a derivative of plasmid pUT19. plasmid pUT18 carries a complementary fragment T18. When lac promoter controlled by transcription, T18 fragment was expressed. By inserting a multicloning site sequence (MCS) to the 5' end of T18, the in-frame genetic fusion can be constructed at the N-terminal end of the T18 polypeptide.

Similar to plasmid pUT18, the plasmid pUT18C is also a derivative of plasmid pUT19, which express the ampicillin resistance. plasmid pUT18C also carries the T18 complementary fragment. By inserting a multicloning site sequence (MCS) to the 3' end of the T18, the in-frame genetic fusion can be constructed at the N-terminal end of the T18 polypeptide. As shown in figure 8.

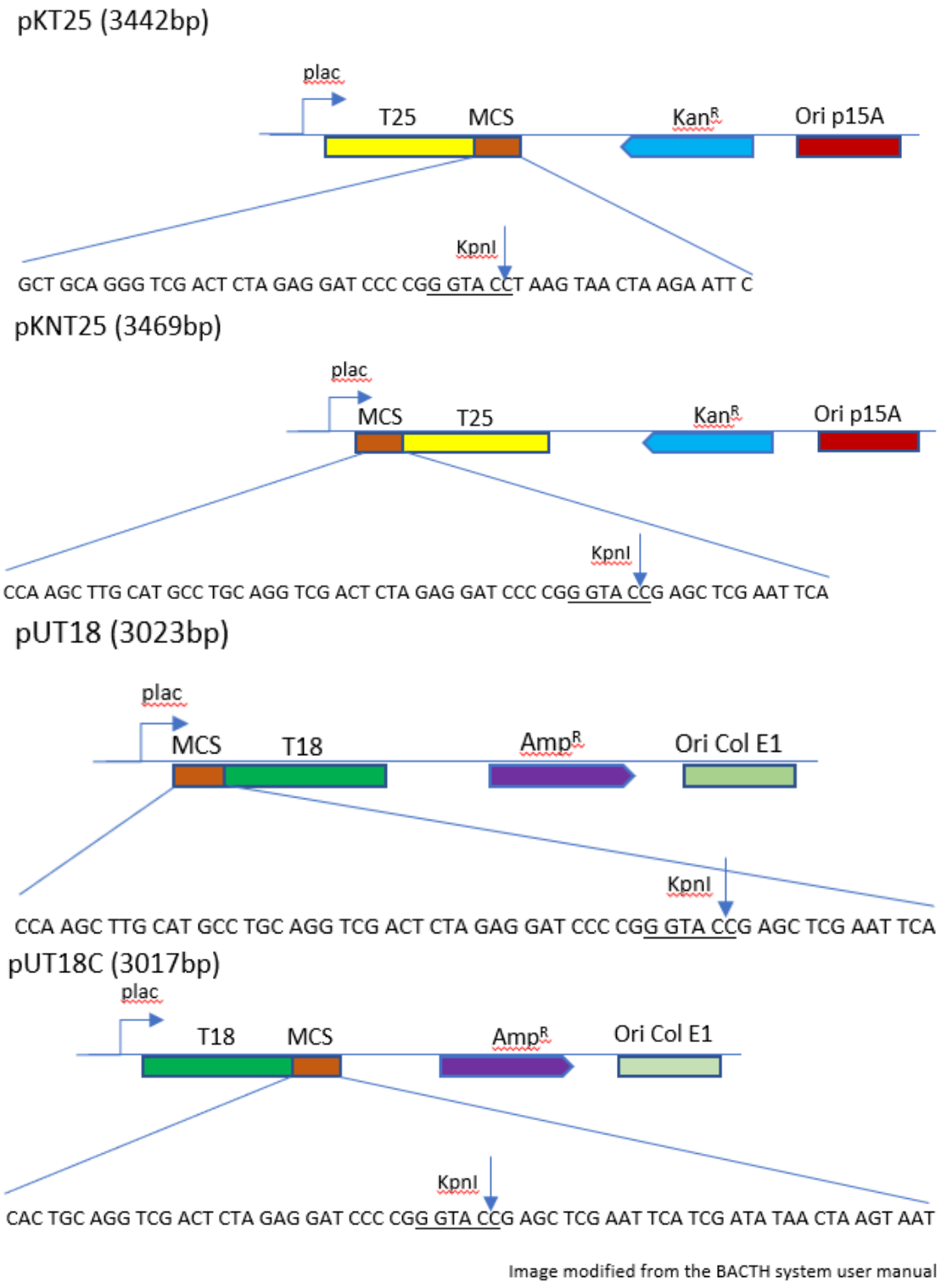

Figure 8: Plasmid maps of pKT25, pKNT25, pUT18, and pUT18C. Image was modified from the BACTH system user manual.

CHAPTER 2

MATERIALS AND METHODS

Cloning Vectors and Genes

Four distinct plasmids carrying T25 (pKT25 and pKNT25) and T18 (pUT18 and pUT18C) fragments were used in this experiment to ligase with three transcriptional regulator genes (*slyA*, *mprA*, *phoP*). These plasmids allow for protein-protein interaction in both N-terminal and C-terminal on both T25 and T18 fragments. The pKT-Zip and pUT-Zip plasmids were used as a positive control after combining them into the reporter strain DHM1. Negative controls for this experiment were dependent on different combinations, which used only one transcriptional regulator gene that had been ligated with one of the plasmid and combined with another plasmid carrying fragments without a gene combination.

SlyA, MprA and PhoP are three transcriptional regulators in MarR family. Their genes were used to ligate the four plasmids (pKT25, pKNT25, pUT18, and pUT18C) by using the technology of molecular cloning to research the protein-protein interactions between each other.

Polymerase Chain Reaction (PCR) and Gene Purification

Polymerase Chain Reaction (PCR) is a technology that widely used in molecular biology. The advantage of PCR is the ability to amplify and test genes in vivo rather than testing it in a live organism. Three transcriptional regulator genes (*slyA*, *mprA*, and *phoP*) were amplified by using PCR, as shown in Table 1. After the PCR, those three amplified genes were run using a 1% 0.5X TBE agarose gel with the

amount of 2uL. This was done to check for fragment size under UV light. Products with positive results were purified using 0.1X volume of 3M NaAC and 2X volume of 100% pure ethanol into -20ºC freezer for 15 minutes. After incubation they were each centrifuged, and the pellet was washed with 70% ethanol, and resuspended by TE.

Table 1: The PCR Reaction System and Program Settings.

PCR Reaction System		PCR Program		
Reagents	Amount	Name	Temperature	Time
5X PCR Buffer	20μL	Initialization	94°C	5mins
gDNA	2μL	Denaturation	94°C	30s
25mM dNTP (with MgCl2)	0.8μL	Annealing	X°C	30s
Forward Primer	0.2μL	Elongation	72°C	40s-1min (Back to Denaturation with 34 cycles total)
Reverse Primer	0.2μL	Final Elongation	72°C	7mins
DNA Taq Polymerase	3.33μL	Final Hold	4°C	Indefinite
Distilled Water	up to 100μL			

Phenol Alkaline Plasmid Isolation

Plasmids were isolated by using Phenol Alkaline. Pellets containing the plasmids were resuspended with GTE solution with lysozyme, reacted with NaOH/SDS solution, and they were incubated in an ice bath after the addition of KaC solution. Phenol was used to extract the proteins, and chloroform was added to remove the phenol from the solution. Later, products were purified using 2X volume

of 100% pure ethanol with 0.1X volume of 3M NaAC and placed into the -20ºC freezer for 15 minutes. After incubation the products were centrifuged, and the pellets were washed with 70% ethanol, and then resuspended by TE with RNASE.

DNA Isolation

In restriction digest, restriction enzyme work as molecular scissors that cut DNA on specific restriction sites. The restriction enzyme KpnI (BioLabs), was used to digest four DNA vectors (pKT25, pKNT25, pUT18, and pUT18C). Three target PCR products (*slyA*, *mprA*, and *phoP*) were incubated in 37ºC for 3 hours to produce cohesive ends, as shown in Table 2. All digestion products were run through an agarose gel (concentration of 1% 0.5X TBE) in order to check the fragment size under UV light. Positive results were extracted from the gel and purified using OMEGA E.Z.N.A Gel Extraction Kit. After which, 1µg of vector DNAs and 3µg of insert DNAs were ligated together. Creating a solution with a total volume of 10µL includes 2µL 5X ligation Buffer and 0. 5µL T4 Ligase (5 Weiss U/µL). Nuclease-free water was used to bring the total volume up to 10µL if necessary. As shown in Table 3. The reaction was kept at room temperature overnight.

Competent cells (Top10) were washed using autoclaved water and 15% glycerol and stocked with 15% glycerol. Then, 10µL of ligation products were mixed with 200µL, and incubated on the ice bath for 30 minutes. After which, a 90s heat shock was conducted in 42ºC. After a 3-minute incubation period on the ice bath, a 500µL LB broth was added into the mixture and incubated for 1 hour in 37ºC shaker. When the incubation concluded, a 150µL culture was streaked on LB plates with either Kanamycin or Ampicillin antibiotics (50 µg/mL Kanamycin or 50 µg/mL Ampicillin). These plates were incubated at 37ºC for 12-15 hours.

Table 2: Restriction Digest Reaction

Restriction Digest Reaction System

Reagents	Amount
10X Digest Buffer	5µL
Vector DNAs or PCR produts	1µg
KpnI	0.5µL
Distilled Water	up to 50µL

Table 3: Ligation Reaction

Ligation Reaction System

Reagents	Amount
5X Ligation Buffer	2µL
Insert DNAs	3µg
Vector DNAs	1µg
T4 Ligase	0.5µL
Distilled Water	up to 10µL

Confirmations

Colonies were selected and streaked on LB plates with either Kanamycin or Ampicillin antibiotics. Isolated single colonies were used to run the ID-PCR as a confirmation for identification of the correct fragment insert. Single colonies (8 isolated colonies/DNA) were added into a 10µL (total volume) mixture combined with 5X PCR Buffer, 25mM dNTP with MgCl2, forward primer, reverse primer,

DNA Taq Polymerase and distilled water, as shown in Table 4. After the PCR, all samples were pipetted into the 1% 0.5X TBE agarose gel for 30 mins. By using the UV light, clear binds showed up as positive results, which confirmed that the insert DNAs (*slyA*, *mprA*, *phoP*) were successfully inserted into the vector DNAs (pKT25, pKNT25, pUT18, pUT18C).

Table 4: ID-PCR Reaction System and Program Settings

PCR Reaction System (8 isolated colonies/DNA)		PCR Program		
Reagents	Amount	Name	Temperature	Time
5X PCR Buffer	16μL	Initialization	94°C	5mins
25mM dNTP (with MgCl2)	0.64μL	Denaturation	94°C	30s
Forward Primer	0.16μL	Annealing	X°C	30s
		Elongation	72°C	40s-1min
Reverse Primer	0.16μL			(Back to Denaturation with 34 cycles total)
DNA Taq Polymerse	2.67μL	Final Elongation	72°C	7mins
Distilled Water	up to 80μL	Final Hold	4°C	Indefinite

Colonies that were used as templates in the ID-PCR reaction were re-inoculated in 5 mL LB broth with either Kanamycin or Ampicillin antibiotics (50 µg/mL Kanamycin or 50 µg/mL Ampicillin) overnight in a 37°C shaker. Overnight cultures were used to isolate plasmids by using the phenol alkaline plasmid isolation method. After that, isolated plasmid DNAs were used in ID-Digestion with KpnI (BioLabs) as a restriction enzyme, as shown in Table 5. A 20µL mixture containing 10X Digest Buffer, plasmid DNAs, KpnI and distilled water were placed on a 37°C water bath for 2 hours. Then, the digestion products were run onto a 1% 0.5X TBE agarose gel to check positive results. Plasmids that showed positive results were sent to a sequencing lab for a professional and thorough sequencing analysis.

Table 5: Restriction Digest Reaction

restriction digest reaction system

Reagents	Amount
10X Digest Buffer	2µL
Plasmids	10µL
KpnI	0.2µL
Distilled Water	up to 20µL

Two-hybrid Screening and β-galactosidase Enzymatic Activities Assay

lacZ is a marker gene that is commonly used in gene expression and regulation. When two transcriptional regulators work together to activate lacZ gene, then β-galactosidase is encoded. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is a substrate of β-galactosidase. When β-galactosidase is encoded, X-Gal yields a blue color in affected colonies. IPTG acts as an inducer, it

induces the β-galactosidase to function more efficiently. After the plasmid DNAs, carrying the T25 and T18 fragments, had settled as a combination, and had been inserted into the reporter strain DHM1, by using electric shock, transformation products were streaked on LB plates with both Kanamycin and Ampicillin antibiotics (50 µg/mL Kanamycin,50 µg/mL Ampicillin) at 37ºC. After the overnight inoculation, colonies were re-streaked on LB plates containing both Kanamycin and Ampicillin antibiotics, IPTG, and X-Gal (50 µg/mL Kanamycin,50 µg/mL Ampicillin) as a screening test. Positive results yielded the blue color on colonies.

Colonies were inoculated into 1 mL LB broth contained both Kanamycin and Ampicillin antibiotics (50 µg/mL Kanamycin,50 µg/mL Ampicillin) on a 37ºC shaker overnight. From the overnight cultures, 12 µL were re-inoculated into 600 µL LB broth contained IPTG, Kanamycin and Ampicillin antibiotics (0.5 M IPTG, 50 µg/mL Kanamycin,50 µg/mL Ampicillin). β-galactosidase enzymatic activities assay was run at different times during the incubation (4 hours, 12 hours, 24 hours, and 48 hours) to test the protein-protein interaction.

CHAPTER 3

RESULTS

PCR Reactions and Plasmid Isolation

PCR reactions were used to amplified three transcriptional regulator genes (*slyA*, *mprA*, and *phoP*). A total of 35 cycles of denaturation, annealing, and elongation were repeated in the PCR reaction. After the PCR reaction concluded, amplified genes were running on agarose gel. *slyA*, *mprA*, and *phoP* showed positive results due to the comparable sizes of the experimental bands and the theoretical.

Four plasmids (pKT25, pKNT25, pUT18, pUT18C) were isolated by using the method of phenol alkaline plasmid isolation. The products were run on agarose gel and all plasmids showed positive results that matched their expected sizes. As shown in figure 9.

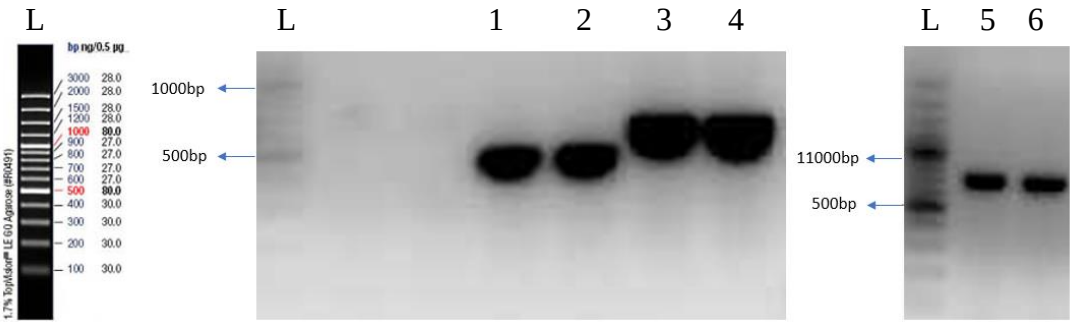

Figure 9: PCR results of *slyA*, *mprA*, and *phoP*. Three DNAs were amplified by PCR reaction. PCR product were run onto the 1% 0.5X agarose gel. L=100bp DNA Ladder, 1,2=*slyA* (441bp), 3,4=*mprA* (531bp), and 5,6=*phoP* (675bp). All DNAs showed strong bands on the gel and were matched with published gene sizes.

Restriction Digest

PCR products were purified and resuspend in TE solution. All plasmids and PCR products were digested by using KpnI. It was confirmed that all amplified DNAs and plasmid DNAs have only one restriction site. For this reason, the result showed

only one clear bind that matched the size of each DNAs that acted as the true positive result. After the reaction, all digestion products were run on the 1% 0.5X agarose gel for 30mins. The result has showed that all digestion products were positive with their expected size. As shown in figure 10 and figure 11.

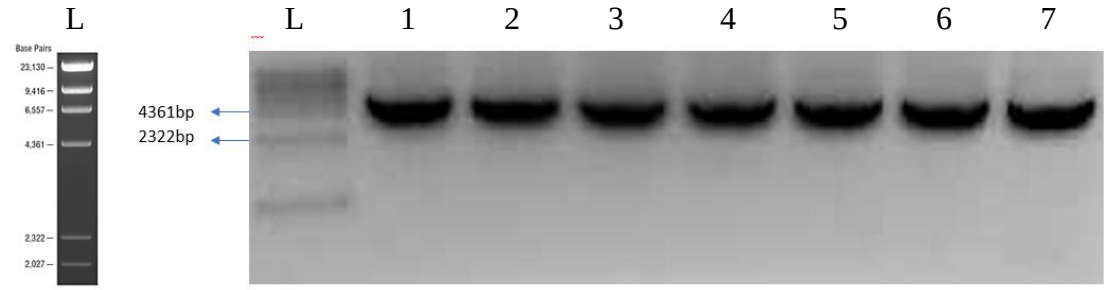

Figure 10: Restriction digest result of pKT25, pKNT25, pUT18, and pUT18C. Four plasmid DNAs were well digested by using KpnI. Digestion products were run on the 1% 0.5X agarose gel. L=Lambda DNA/Hind III Marker ladder, 1,2= pKNT25 (3469bp), 3,4,5= pKT25 (3442bp), 6=pUT18 (3023bp), 7= pUT18C (3017bp). All plasmid DNAs showed strong bands on the gel and were matched with published plasmid DNA sizes

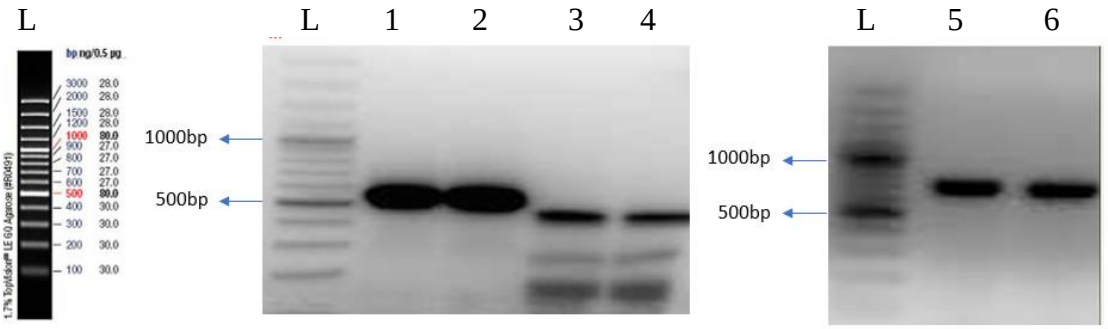

Figure 11: Restriction digest result of *slyA*, *mprA*, and *phoP*. Three DNAs were well digested by KpnI. Digestion products were run on the 1% 0.5X agarose gel. L= 100bp DNA Ladder, 1,2=*mprA* (531bp), 3,4=*slyA* (441bp), and 5,6=*phoP* (675bp). All DNAs showed strong bands on the gel and were matched with published gene sizes.

Confirmation

Plasmids were used as vector DNAs and were ligated with purified PCR products. After the transformation, vector DNAs were successfully ligated with insert DNAs and inserted in to the Top10 recipient cells to form colonies. Colonies were picked and streak on plates to form single isolated colonies. Four single colonies were used as a template in ID-PCR reaction to test the target DNAs. A strong positive

result confirmed that the target DNAs (*slyA*, *mprA*, *phoP*) were ligated with four plasmid DNAs (pKT25, pKNT25, pUT18, pUT18C). As shown in figure 12-fugure 15.

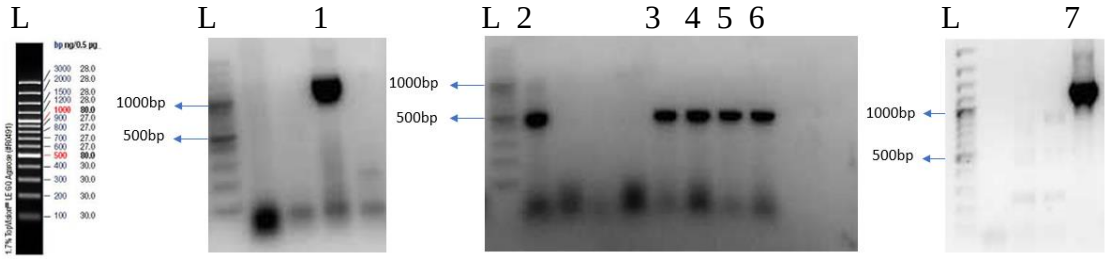

Figure 12: ID-PCR results of pKT25-*mprA*, pKNT25-*mprA*, pUT18-*mprA*, and pUT18C-*mprA*. The ID-PCR of *mprA* gene was ligated on four plasmid DNAs (pKT25, pKNT25, pUT18, pUT18C). After the PCR reaction, ID-PCR products were run on the 1% 0.5X agarose gel. L= 100bp DNA Ladder, 1=pKT25-*mprA* (1299bp), 2=pKNT25-*mprA* (648bp), 3,4,5,6=pUT18-*mprA* (648bp), and 7=pUT18C-*mprA* (1170bp). All DNAs matched the theoretical sizes with strong bands.

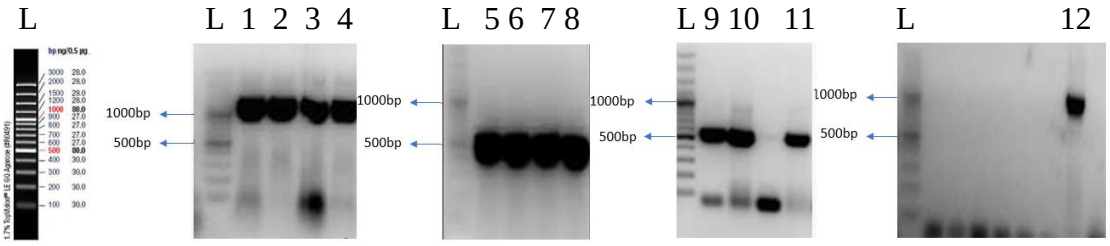

Figure 13: ID-PCR results of pKT25-*slyA*, pKNT25-*slyA*, pUT18-*slyA*, and pUT18C-*slyA*. The ID-PCR of *slyA* gene was ligated on four plasmid DNAs (pKT25, pKNT25, pUT18, pUT18C). After the PCR reaction, ID-PCR products were run on the 1% 0.5X agarose gel. L= 100bp DNA Ladder, 1,2,3,4=pKT25-*slyA* (1209bp), 5,6,7,8=pKNT25-*slyA* (558bp), 9,10,11=pUT18-*slyA* (558bp), and 12=pUT18C-*slyA* (1080bp). All DNAs matched the theoretical sizes with strong bands.

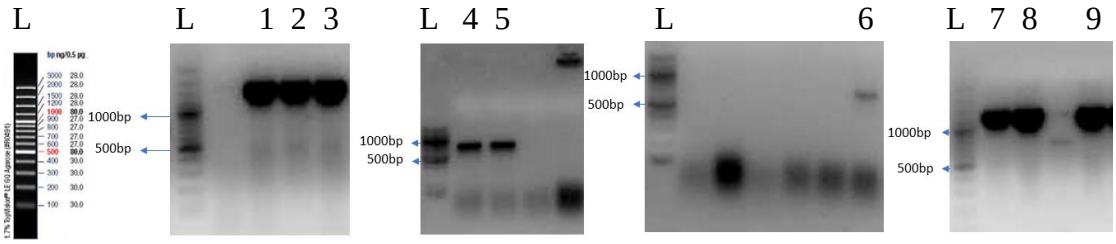

Figure 14: ID-PCR results of pKT25-*phoP*, pKNT25-*phoP*, pUT18-*phoP*, and pUT18C-*phoP*. The ID-PCR of *phoP* gene was ligated on four plasmid DNAs (pKT25, pKNT25, pUT18, pUT18C). After the PCR reaction, ID-PCR products were run on the 1% 0.5X agarose gel. L= 100bp DNA Ladder, 1,2,3=pKT25- *phoP* (1443bp), 4,5=pKNT25- *phoP* (792bp), 6=pUT18- *phoP* (792bp), and

7,8,9=pUT18C- *phoP* (1314bp). All DNAs matched the theoretical sizes with strong bands.

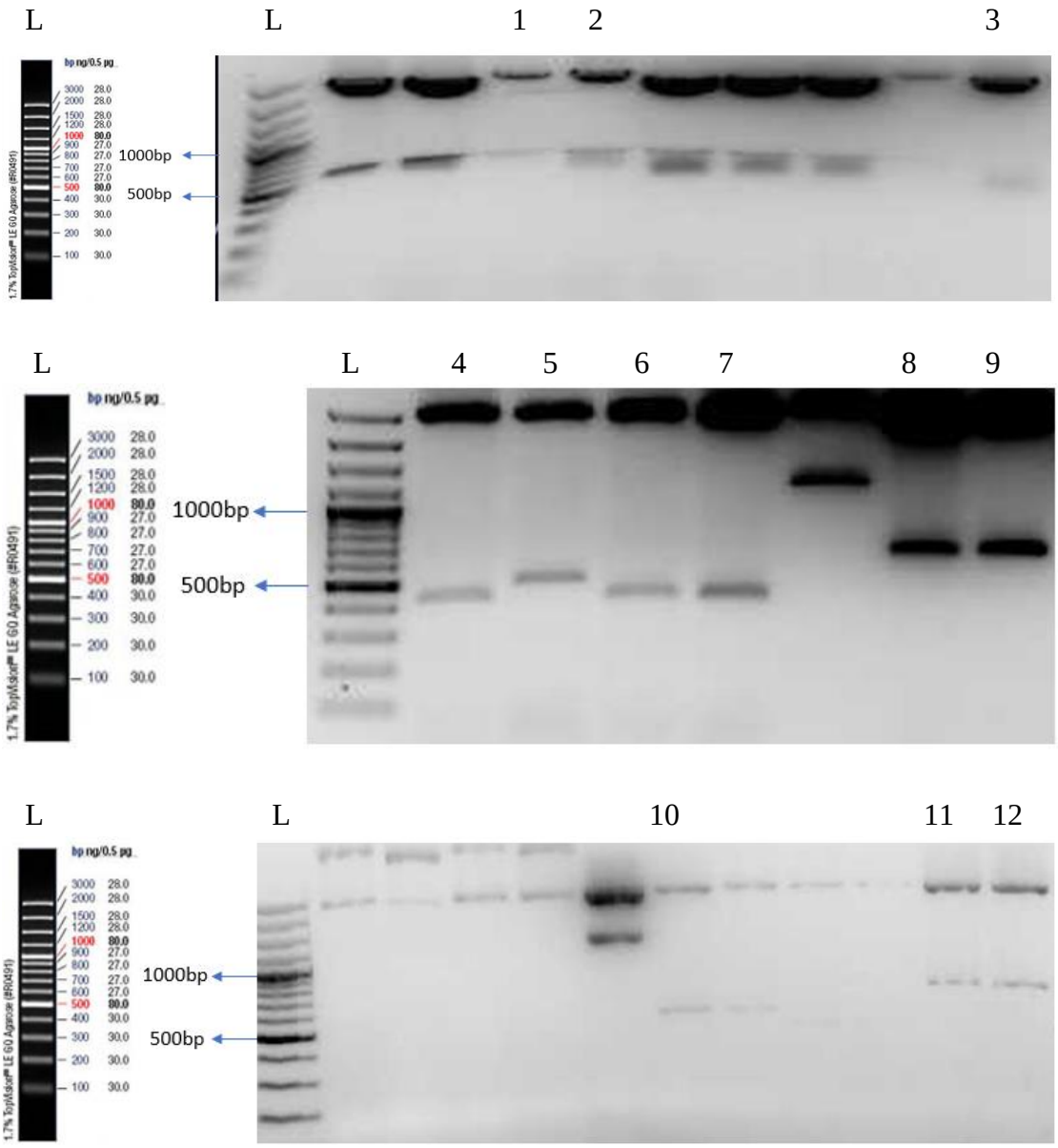

Figure 15: ID-Digestion results. Three DNAs that ligated on four plasmid DNAs (pKT25, pKNT25, pUT18, pUT18C) were well digested by KpnI. Digestion products were run on the 1% 0.5X agarose gel. L= 100bp DNA Ladder, 1= pUT18 (3023bp) and *mprA* (531bp), 2= pUT18C (3017bp) and *mprA* (531bp), 3= pUT18C (3017bp) and *slyA* (441bp), 4= pKT25 (3442bp) and *slyA* (441bp), 5= pKT25 (3442bp) and *mprA* (531bp), 6= pKNT25 (3469bp) and *slyA* (441bp), 7= pUT18 (3023bp) and *slyA* (441bp), 8= pKT25 (3442bp) and *phoP* (675bp), 9= pKNT25 (3469bp) and *phoP* (675bp), 10= pKNT25 (3469bp) and *mprA* (531bp), 11= pUT18 (3023bp) and *phoP* (675bp), 12= pUT18C (3017bp) and *phoP* (675bp). All DNAs matched the published sizes with strong bands.

Gene Sequencing

Plasmids that demonstrated positive results on both ID-PCR and ID-Digestion were sent to the sequence lab for a detailed comparison with sequences existing within the database. Results showed no differences between all nucleotide sequences and published sequences. Therefore, all insert DNAs were successfully ligated on target plasmid DNAs and were used on the two-hybrid screening. As shown in figure 16-figure 27. The β-galactosidase enzymatic activities assay was conducted by getting two-hybrid combinations from the transformation.

```
pKT25-slyA ACCGGAATCG CCACTAGGTT CTGATCTGGC ACGGTTGGTG CGCATTTGGC GTGCTCTGAT 60
      slyA attggaatcg ccactaggtt ctgatctggc acggttggtg cgcatttggc gtgctctgat 60

pKT25-slyA TGACCATCGC CTCAAGCCTC TGGAATTGAC GCAGACACAT TGGGTCACGT TGCACAATAT 120
      slyA tgaccatcgc ctcaagcctc tggaattgac gcagacacat tgggtcacgt tgcacaatat 120

pKT25-slyA TCATCAATTG CCGCCTGACC AGTCGCAGAT TCAATTGGCT AAGGCGATAG GCATTGAGCA 180
      slyA tcatcaattg ccgcctgacc agtcgcagat tcaattggct aaagcgatag gcattgagca 180

pKT25-slyA GCCATCGCTG GTACGCACGT TGGATCAACT TGAAGATAAG GGGCTAATTT CGCGGCAAAC 240
      slyA gccatcgctg gtacgcacgt tggatcaact tgaagataag gggctaattt cgcggcaaac 240

pKT25-slyA CTGCGCCAGC GATCGTCGCG CTAAGCGGAT TAAACTGACC GAAAAAGCGG AGCCGCTGAT 300
      slyA ctgcgccagc gatcgtcgcg ctaagcggat taaactgacc gaaaaagcgg agccgctgat 300

pKT25-slyA CGCTGAGATG GAAGAGGTCA TTCATAAAAC GCGCGGTGAA ATTTTGGCTG GGATTTCTTC 360
      slyA cgctgagatg gaagaggtca ttcataaaac gcgcggtgaa attttggctg ggatttcttc 360

pKT25-slyA AGAGGAGATT GAGCTTCTGA TTAAACNN-T CGCCAANCNN GAACACAATA TTATGGAATT 419
      slyA agaggagatt gagcttctga ttaaacttat cgccaaactt gaacacaata ttatggaatt 420

pKT25-slyA GCACTCTCAC GATTGA 435
      slyA gcactctcac gattga 436
```

Figure 16: pKT25-*slyA* sequencing result. The alignment between pKT25-*slyA* gene sequencing result and published *slyA* gene.

```
pKNT25-slyA GAATCGCCAC TAGGTTCTGA TCTGGCACGG TTGGTGCGCA TTTGGCGTGC TCTGATTGAC 60
      slyA gaatcgccac taggttctga tctggcacgg ttggtgcgca tttggcgtgc tctgattgac 60

pKNT25-slyA CATCGCCTCA AGCCTCTGGA ATTGACGCAG ACACATTGGG TCACGTTGCA CAATATTCAT 120
      slyA catcgcctca agcctctgga attgacgcag acacattggg tcacgttgca caatattcat 120

pKNT25-slyA CAATTGCCGC CTGACCAGTC GCAGATTCAA TTGGCTAAAG CGATAGGCAT TGAGCAGCCA 180
      slyA caattgccgc ctgaccagtc gcagattcaa ttggctaaag cgataggcat tgagcagcca 180

pKNT25-slyA TCGCTGGTAC GCACGTTGGA TCAACTTGAA GATAAGGGGC TAATTTCGCG GCAAACCTGC 240
      slyA tcgctggtac gcacgttgga tcaacttgaa gataaggggc taatttcgcg gcaaacctgc 240

pKNT25-slyA GCCAGCGATC GTCGCGCTAA GCGGATTAAA CTGACCGAAA AAGCGGAGCC GCTGATCGCT 300
      slyA gccagcgatc gtcgcgctaa gcggattaaa ctgaccgaaa aagcggagcc gctgatcgct 300

pKNT25-slyA GAGATGGAAG AGGTCATTCA TAAAACGCGC GGTGAAATTT TGGCTGGGAN TTCTTCAGAG 360
      slyA gagatggaag aggtcattca taaaacgcgc ggtgaaattt tggctgggat ttcttcagag 360

pKNT25-slyA GAGATTGAGC TTCTGANTAA ACTTATCGCC AAACTTGAAC ACAATATTAT GGAATTGCAC 420
      slyA gagattgagc ttctgattaa acttatcgcc aaacttgaac acaatattat ggaattgcac 420

pKNT25-slyA TCTCACGATT GA 432
      slyA tctcacgatt ga 432
```

Figure 17: pKNT25-*slyA* sequencing result. The alignment between pKNT25-*slyA* gene sequencing result and published *slyA* gene.

```
pUT18-slyA GAATCGCCAC TAGGTTCTGA TCTGGCACGG TTGGTGCGCA TTTGGCGTGC TCTGATTGAC 60
      slyA gaatcgccac taggttctga tctggcacgg ttggtgcgca tttggcgtgc tctgattgac 60
pUT18-slyA CATCGCCTCA AGCCTCTGGA ATTGACGCAG ACACATTGGG TCACGTTGCA CAATATTCAT 120
      slyA catcgcctca agcctctgga attgacgcag acacattggg tcacgttgca caatattcat 120
pUT18-slyA CAATTGCCGC CTGACCAGTC GCAGATTCAA TTGGCTAAAG CGATAGGCAT TGAGCAGCCA 180
      slyA caattgccgc ctgaccagtc gcagattcaa ttggctaaag cgataggcat tgagcagcca 180
pUT18-slyA TCGCTGGTAC GCACGTTGGA TCAACTTGAA GATAAGGGGC TAATTTCGCG GCAAACCTGC 240
      slyA tcgctggtac gcacgttgga tcaacttgaa gataaggggc taatttcgcg gcaaacctgc 240
pUT18-slyA GCCAGCGATC GTCGCGCTAA GCGGANTAAA NTGACCGAAN NNGCGGAGCC GCTGATCGCT 300
      slyA gccagcgatc gtcgcgctaa gcggattaaa ctgaccgaaa aagcggagcc gctgatcgct 300
pUT18-slyA GAGATNGNNG AGNTCANNTC ATAAAACGCG CGGTGNNAAT TTTGGCTGGG ATTTCTTCAG 360
      slyA gagatggaag aggtcat-tc ataaaacgcg cggtga-aat tttggctggg atttcttcag 358
pUT18-slyA AGGAGATTGA GCTTCTGATT AAACTTATCG CCAAACTTGA ACACAATATT ATGGAATTGC 420
      slyA aggagattga gcttctgatt aaacttatcg ccaaacttga acacaatatt atggaattgc 418
pUT18-slyA ACTCTCACGA TTGA 434
      slyA actctcacga ttga 432
```

Figure 18: pUT18-*slyA* sequencing result. The alignment between pUT18-*slyA* gene sequencing result and published *slyA* gene.

```
pUT18C-slyA ACCGGAATCG CCACTAGGTT CTGATCTGGC ACGGTTGGTG CGCATTTGGC GTGCTCTGAT 60
      slyA attggaatcg ccactaggtt ctgatctggc acggttggtg cgcatttggc gtgctctgat 60
pUT18C-slyA TGACCATCGC CTCAAGCCTC TGGAATTGAC GCAGACACAT TGGGTCACGT TGCACAATAT 120
      slyA tgaccatcgc ctcaagcctc tggaattgac gcagacacat tgggtcacgt tgcacaatat 120
pUT18C-slyA TCATCAATTG CCGCCTGACC AGTCGCAGAT TCAATTGGCT AAAGCGATAG GCATTGAGCA 180
      slyA tcatcaattg ccgcctgacc agtcgcagat tcaattggct aaagcgatag gcattgagca 180
pUT18C-slyA GCCATCGCTG GTACGCACGT TGGATCAACT TGAAGATAAG GGGCTAATTT CGCGGCAAAC 240
      slyA gccatcgctg gtacgcacgt tggatcaact tgaagataag gggctaattt cgcggcaaac 240
pUT18C-slyA CTGCGCCAGC GATCGTCGCG CTAAGCGGAT TAAACTGACC GAAAAAGCGG AGCCGCTGAT 300
      slyA ctgcgccagc gatcgtcgcg ctaagcggat taaactgacc gaaaaagcgg agccgctgat 300
pUT18C-slyA CGCTGAGATG GAAGAGGTCA TTCATAAAAC GCGCGGTGAA ATTTTGGCTG GGANTTCTTC 360
      slyA cgctgagatg gaagaggtca ttcataaaac gcgcggtgaa attttggctg ggatttcttc 360
pUT18C-slyA AGAGGAGATT GAGCTTCTGA TTAAACNN-T CGCCAAACTT GAACACAATA TTATGGAATT 419
      slyA agaggagatt gagcttctga ttaaacttat cgccaaactt gaacacaata ttatggaatt 420
pUT18C-slyA GCACTCTCAC GATTGA 435
      slyA gcactctcac gattga 436
```

Figure 19: pUT18C-*slyA* sequencing result. The alignment between pUT18C-*slyA* gene sequencing result and published *slyA* gene.

```
pKT25-mprA GGATAGTTCG TTTACGCCCA TTGAACAAAT GCTAAAATTT CGCGCCAGCC GTCACGAAGA 60
      mprA ggatagttcg tttacgccca ttgaacaaat gctaaaattt cgcgccagcc gtcacgaaga 60
pKT25-mprA CTTTCCTTAT CAGGAAACTC TTTTGACTCG TCTTTGTATG CACATGCAAG GCAAGCTCCT 120
      mprA ctttccttat caggaaattc ttttgactcg tctttgtatg cacatgcaag gcaagctcct 120
pKT25-mprA GGAAAACCGC AATAAGATGC TGAAGGCTCA AGGGATTAAC GAGACGTTGT TTATGGCGTT 180
      mprA ggaaaaccgc aataagatgc tgaaggctca agggattaac gagacgttgt ttatggcgtt 180
pKT25-mprA GATTACGCTG GAGTCTCAGG AAAACCACAG TATTCAGCCG TCTGAATTAA GCTGCGCGCT 240
      mprA gattacgctg gagtctcagg aaaaccacag tattcagccg tctgaattaa gctgcgcgct 240
pKT25-mprA GGGTTCATCT CGCACCAATG CGACACGCAT TGCAGATGAG CTGGAAAAAC GCGGCTGAAT 300
      mprA gggttcatct cgcaccaatg cgacacgcat tgcagatgag ctggaaaaac gcggctggat 300
pKT25-mprA TGAGCGTCGT GAGAGCGATA ACGACCGCCG TTGCCTGCAT CTGCAATTAA CGGAGAAAGG 360
      mprA tgagcgtcgt gagagcgata acgaccgccg ttgcctgcat ctgcaattaa cggagaaagg 360
pKT25-mprA CCAGGCATTT TTGCAAGAAG TGCTTCCGCC TCAGCATCAT TGTCTGCATC AACTCTGGTC 420
      mprA ccaggcattt ttgcaagaag tgcttccgcc tcagcatcat tgtctgcatc aactctggtc 420
pKT25-mprA ATCACTTAGC ACAGCCGAAA AAGATCAACT TGAGCACATC ACTCGTAAAC TCCTGACGCG 480
      mprA atcacttagc acagccgaaa aagatcaact tgagcacatc actcgtaaac tcctgacgcg 480
pKT25-mprA TCTTGATCAA ATGGAGCAGG AAGGCACTGT TCTTGAGGCG CTGCGCCGN 529
      mprA tcttgatcaa atggagcagg aaggcactgt tcttgaggcg ctgcgctaa 529
```

Figure 20: pKT25-*mprA* sequencing result. The alignment between pKT25-*mprA* gene sequencing result and published *mprA* gene.

```
pKNT25-mprA GGATAGTTCG TTTACGCCCA TTGAACAAAT GCTAAAATTT CGCGCCAGCC GTCACGAAGA 60
      mprA ggatagttcg tttacgccca ttgaacaaat gctaaaattt cgcgccagcc gtcacgaaga 60

pKNT25-mprA CTTTCCTTAT CAGGAAACTC TTTTGACTCG TCTTTGTATG CACATGCAAG GCAAGCTCCT 120
      mprA ctttccttat caggaaattc ttttgactcg tctttgtatg cacatgcaag gcaagctcct 120

pKNT25-mprA GGAAAACCGC AATAAGATGC TGAAGGCTCA AGGGATTAAC GAGACGTTGT TTATGGCGTT 180
      mprA ggaaaaccgc aataagatgc tgaaggctca agggattaac gagacgttgt ttatggcgtt 180

pKNT25-mprA GATTACGCTG GAGTCTCAGG AAAACCACAG TATTCAGCCG TCTGAATTAA GCTGCGCGCT 240
      mprA gattacgctg gagtctcagg aaaaccacag tattcagccg tctgaattaa gctgcgcgct 240

pKNT25-mprA GGGTTCATCT CGCACCAATG CGACACGCAT TGCAGATGAG CTGGAAAAAC GCGGCTGAAT 300
      mprA gggttcatct cgcaccaatg cgacacgcat tgcagatgag ctggaaaaac gcggctggat 300

pKNT25-mprA TGAGCGTCGT GAGAGCGATA ACGACCGCCG TTGCCTGCAT CTGCAATTAA CGGAGAAAGG 360
      mprA tgagcgtcgt gagagcgata acgaccgccg ttgcctgcat ctgcaattaa cggagaaagg 360

pKNT25-mprA CCAGGCATTT TTGCAAGAAG TGCTTCCGCC TCAGCATCAT TGTCTGCATC AACTCTGGTC 420
      mprA ccaggcattt ttgcaagaag tgcttccgcc tcagcatcat tgtctgcatc aactctggtc 420

pKNT25-mprA ATCACTTAGC ACAGCCGAAA AAGATCAACT TGAGCACATC ACTCGTAAAC TCCTGACGCG 480
      mprA atcacttagc acagccgaaa aagatcaact tgagcacatc actcgtaaac tcctgacgcg 480

pKNT25-mprA TCTTGATCAA ATGGAGCAGG AAGGCACTGT TCTTGAGGCG CTGCGCCGG 529
      mprA tcttgatcaa atggagcagg aaggcactgt tcttgaggcg ctgcgctaa 529
```

Figure 21: pKNT25-*mprA* sequencing result. The alignment between pKNT25-*mprA* gene sequencing result and published *mprA* gene.

```
pUT18-mprA GGATAGTTCG TTTACGCCCA TTGAACAAAT GCTAAAATTT CGCGCCAGCC GTCACGAAGA 60
      mprA ggatagttcg tttacgccca ttgaacaaat gctaaaattt cgcgccagcc gtcacgaaga 60

pUT18-mprA CTTTCCTTAT CNNGNAACTC TTTTGACTCG TCTTTGTATG CACATGCAAG GCAAGCTCCT 120
      mprA ctttccttat caggaaattc ttttgactcg tctttgtatg cacatgcaag gcaagctcct 120

pUT18-mprA GGAAAACCGC AATAAGATGC TGAAGGCTCA AGGGATTAAC GAGACGTTGT TTATGGCGTT 180
      mprA ggaaaaccgc aataagatgc tgaaggctca agggattaac gagacgttgt ttatggcgtt 180

pUT18-mprA GATTACGCTG GAGTCTCAGG AAAACCACAG TATTCAGCCG TCTGAATTAA GCTGCGCGCT 240
      mprA gattacgctg gagtctcagg aaaaccacag tattcagccg tctgaattaa gctgcgcgct 240

pUT18-mprA GGGTTCATCT CGCACCAATG CGACACGCAT TGCAGATGAG CTGGAAAAAC GCGGCTGAAT 300
      mprA gggttcatct cgcaccaatg cgacacgcat tgcagatgag ctggaaaaac gcggctggat 300

pUT18-mprA TGAGCGTCGT GAGAGCGATA ACGACCGCCG TTGCCTGCAT CTGCAATTAA CGGAGAAAGG 360
      mprA tgagcgtcgt gagagcgata acgaccgccg ttgcctgcat ctgcaattaa cggagaaagg 360

pUT18-mprA CCAGGCATTT TTGCAAGAAG TGCTTCCGCC TCAGCATCAT TGTCTGCATC AACTCTGGTC 420
      mprA ccaggcattt ttgcaagaag tgcttccgcc tcagcatcat tgtctgcatc aactctggtc 420

pUT18-mprA ATCACTTAGC ACAGCCGAAA AAGATCAACT TGAGCACATC ACTCGTAAAC TCCTGACGCG 480
      mprA atcacttagc acagccgaaa aagatcaact tgagcacatc actcgtaaac tcctgacgcg 480

pUT18-mprA TCTTGATCAA ATGGAGCAGN AAGGCACTGT TCTTGAGGCG CTGCGCCGG 529
      mprA tcttgatcaa atggagcagg aaggcactgt tcttgaggcg ctgcgctaa 529
```

Figure 22: pUT18-*mprA* sequencing result. The alignment between pUT18-*mprA* gene sequencing result and published *mprA* gene.

```
pUT18C-mprA GGATAGTTCG TTTACGCCCA TTGAACAAAT GCTAAAATTT CGCGCCAGCC GTCACGAAGA 60
      mprA ggatagttcg tttacgccca ttgaacaaat gctaaaattt cgcgccagcc gtcacgaaga 60

pUT18C-mprA CTTTCCTTAT CAGGAAATTC TTTTGACTCG TCTTTGTATG CACATGCAAG GCAAGCTCCT 120
      mprA ctttccttat caggaaattc ttttgactcg tctttgtatg cacatgcaag gcaagctcct 120

pUT18C-mprA GGAAAACCGC AATAAGATGC TGAAGGCTCA AGGGATTAAC GAGACGTTGT TTATGGCGTT 180
      mprA ggaaaaccgc aataagatgc tgaaggctca agggattaac gagacgttgt ttatggcgtt 180

pUT18C-mprA GATTACGCTG GAGTCTCAGG AAAACCACAG TATTCAGCCG TCTGAATTAA GCTGCGCGCT 240
      mprA gattacgctg gagtctcagg aaaaccacag tattcagccg tctgaattaa gctgcgcgct 240

pUT18C-mprA GGNTTCATCT CGCACCAATG CGACACGCAT TGCAGATGAG CTGGAAAAAC GCGGCTGGAT 300
      mprA gggttcatct cgcaccaatg cgacacgcat tgcagatgag ctggaaaaac gcggctggat 300

pUT18C-mprA TGAGCGTCGT GAGAGCGATA ACGACCGCCG TTGCCTGCAT CTGCAATTAA CGGAGAAAGG 360
      mprA tgagcgtcgt gagagcgata acgaccgccg ttgcctgcat ctgcaattaa cggagaaagg 360

pUT18C-mprA CCAGGCATTT TTGCAAGAAG TGCTTCCGCC TCAGCATCAT TGTCTGCATC AACTCTGGTC 420
      mprA ccaggcattt ttgcaagaag tgcttccgcc tcagcatcat tgtctgcatc aactctggtc 420

pUT18C-mprA ATCACTTAGC ACAGCCGAAA AAGATCAACT TGAGCACATC ACTCGTAAAC TCCTGACGCG 480
      mprA atcacttagc acagccgaaa aagatcaact tgagcacatc actcgtaaac tcctgacgcg 480

pUT18C-mprA TCTTGATCAA ATGGAGCAGG AAGGCACTGT TCTTGAGGCG CTGCGCTAA 529
      mprA tcttgatcaa atggagcagg aaggcactgt tcttgaggcg ctgcgctaa 529
```

Figure 23: pUT18C-*mprA* sequencing result. The alignment between pUT18C-*mprA* gene sequencing result and published *mprA* gene.

```
pKT25-phoP GATGCGCGTA CTGGTTGTAG AGGANAATGC ATTATTACGC CACCACCTGA AGGTTCAGCT 60
     phoP gatgcgcgta ctggttgtag aggataatgc attattacgc caccacctga aggttcagct 60

pKT25-phoP CCAGGATTCA GGTCACCAGG TCGATGCCGC AGAAGATGCC AGGGAAGCTG ATTACTACCT 120
     phoP ccaggattca ggtcaccagg tcgatgccgc agaagatgcc agggaagctg attactacct 120

pKT25-phoP TAATGAACAC CTTCCGGATA TCGCTATTGT CGATTTAGGT CTGCCGGATG AAGACGGCCT 180
     phoP taatgaacac cttccggata tcgctattgt cgatttaggt ctgccggatg aagacggcct 180

pKT25-phoP TTCCTTAATA CGCCGCTGGC GCAGCAGTGA TGTTTCACTG CCGGTTCTGG TGTTAACCGC 240
     phoP ttccttaata cgccgctggc gcagcagtga tgtttcactg ccggttctgg tgttaaccgc 240

pKT25-phoP GCGCGAAGGC TGGCAGGATA AAGTCGAGGT TCTCAGCTCC GGGGCCGATG GCTACGTGAC 300
     phoP gcgcgaaggc tggcaggata aagtcgaggt tctcagctcc ggggccgatg actacgtgac 300

pKT25-phoP GAAGCCATTC CACATCGAAG AGGTAATGGC GCGTATGCAG GCGTTAATGC GCCGTAATAG 360
     phoP gaagccattc cacatcgaag aggtaatggc gcgtatgcag gcgttaatgc gccgtaatag 360

pKT25-phoP CGGTCTGGCC TCCCAGGTGA TCAACATCCC GCCGTTCCAG GTGGATCTCT CACGCCGGGA 420
     phoP cggtctggcc tcccaggtga tcaacatccc gccgttccag gtggatctct cacgccggga 420

pKT25-phoP ATTATCCGTC AATGAAGAGG TCATCAAACT CACGGCGTTC GAATACACCA TTATGGAAAC 480
     phoP attatccgtc aatgaagagg tcatcaaact cacggcgttc gaatacacca ttatggaaac 480

pKT25-phoP GCTTATCCGT AACAACGGTA AAGTGGTCAG CAAAGATTCG CTGATGCTTC AGCTGTATCC 540
     phoP gcttatccgt aacaacggta aagtggtcag caaagattcg ctgatgcttc agctgtatcc 540

pKT25-phoP GGATGCGGAA CTGCGGGAAA GTCATACCAT TGATGTTCTC ATGGGGCGTC TGCGGAAAAA 600
     phoP ggatgcggaa ctgcgggaaa gtcataccat tgatgttctc atggggcgtc tgcggaaaaa 600

pKT25-phoP AATACAGGCC CAGTATCCGC ACGATGTCAT TACCACCGTA CGCGGACAAG GATATCTTTT 660
     phoP aatacaggcc cagtatccgc acgatgtcat taccaccgta cgcggacaag gatatctttt 660

pKT25-phoP TGAATTGCGC 670
     phoP tgaattgcgc 670
```

Figure 24: pKT25-*phoP* sequencing result. The alignment between pKT25-*phoP* gene sequencing result and published *phoP* gene.

```
pKNT25-phoP GATGCGCGTA CTGGTTGTAG AGGATAATGC ATTATTACGC CACCACCTGA AGGTTCAGCT 60
      phoP gatgcgcgta ctggttgtag aggataatgc attattacgc caccacctga aggttcagct 60

pKNT25-phoP CCAGGATTCA GGTCACCAGG TCGATGCCGC AGAAGATGCC AGGGAAGCTG ATTACTACCT 120
      phoP ccaggattca ggtcaccagg tcgatgccgc agaagatgcc agggaagctg attactacct 120

pKNT25-phoP TAATGAACAC CTTCCGGATA TCGCTATTGT CGATTTAGGT CTGCCGGATG AAGACGGCCT 180
      phoP taatgaacac cttccggata tcgctattgt cgatttaggt ctgccggatg aagacggcct 180

pKNT25-phoP TTCCTTAATA CGCCGCTGGC GCAGCAGTGA TGTTTCACTG CCGGTTCTGG TGTTAACCGC 240
      phoP ttccttaata cgccgctggc gcagcagtga tgtttcactg ccggttctgg tgttaaccgc 240

pKNT25-phoP GCGCGAAGGC TGGCAGGATA AAGTCGAGGT TCTCAGCTCC GGGGCCGATG GCTACGTGAC 300
      phoP gcgcgaaggc tggcaggata aagtcgaggt tctcagctcc ggggccgatg actacgtgac 300

pKNT25-phoP GAAGCCATTC CACATCGAAG AGGTAATGGC GCGTATGCAG GCGTTAATGC GCCGTAATAG 360
      phoP gaagccattc cacatcgaag aggtaatggc gcgtatgcag gcgttaatgc gccgtaatag 360

pKNT25-phoP CGGTCTGGCC TCCCAGGTGA TCAACATCCC GCCGTTCCAG GTGGATCTCT CACGCCGGGA 420
      phoP cggtctggcc tcccaggtga tcaacatccc gccgttccag gtggatctct cacgccggga 420

pKNT25-phoP ATTATCCGTC AATGAAGAGG TCATCAAACT CACGGCGTTC GAATACACCA TTATGGAAAC 480
      phoP attatccgtc aatgaagagg tcatcaaact cacggcgttc gaatacacca ttatggaaac 480

pKNT25-phoP GCTTATCCGT AACAACGGTA AAGTGGTCAG CAAAGATTCG CTGATGCTTC AGCTGTATCC 540
      phoP gcttatccgt aacaacggta aagtggtcag caaagattcg ctgatgcttc agctgtatcc 540

pKNT25-phoP GGATGCGGAA CTGCGGGAAA GTCATACCAT TGATGTTCTC ATGGGGCGTC TGCGGAAAAA 600
      phoP ggatgcggaa ctgcgggaaa gtcataccat tgatgttctc atggggcgtc tgcggaaaaa 600

pKNT25-phoP AATACAGGCC CAGTATCCGC ACGATGTCAT TACCACCGTA CGCGGACAAG GATATCTTTT 660
      phoP aatacaggcc cagtatccgc acgatgtcat taccaccgta cgcggacaag gatatctttt 660

pKNT25-phoP TGAATTGCGC CGG 673
      phoP tgaattgcgc taa 673
```

Figure 25: pKNT25-*phoP* sequencing result. The alignment between pKNT25-*phoP* gene sequencing result and published *phoP* gene.

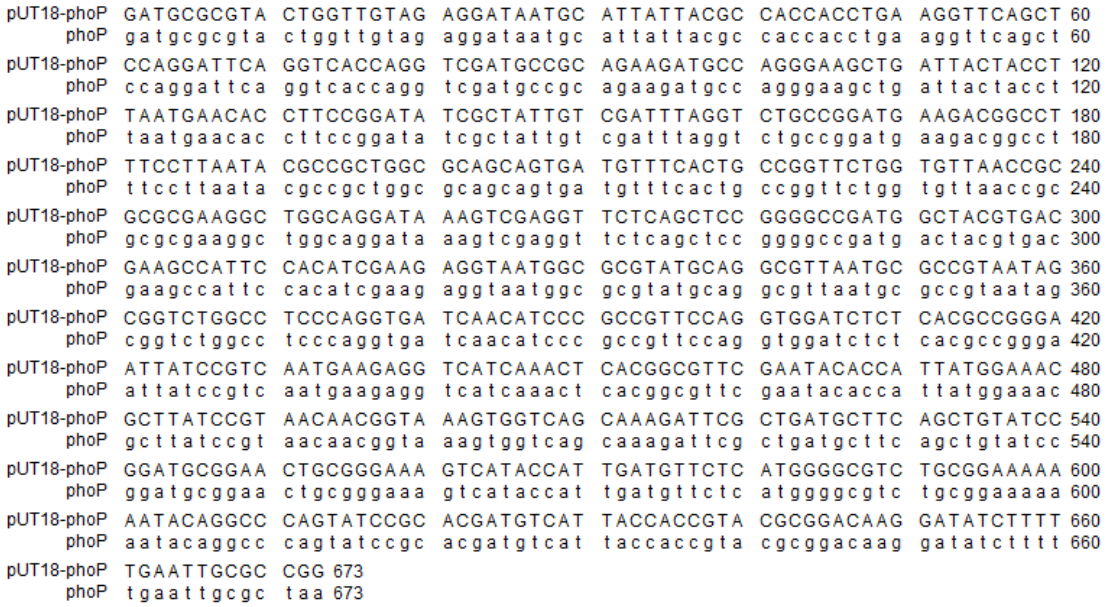

Figure 26: pUT18-*phoP* sequencing result. The alignment between pUT18-*phoP* gene sequencing result and published *phoP* gene.

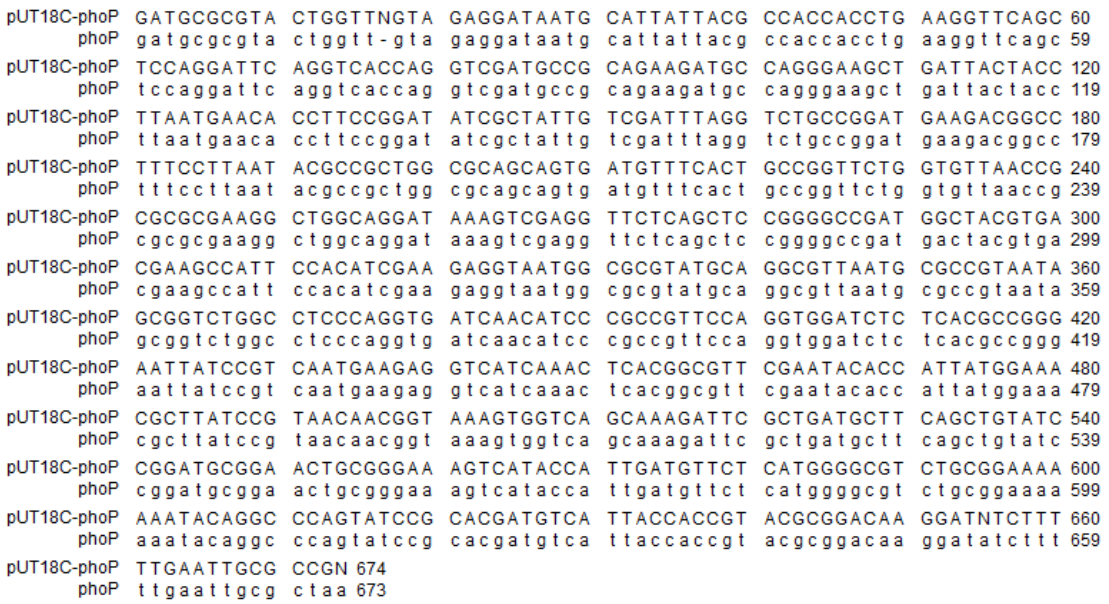

Figure 27: pUT18C-*phoP* sequencing result. The alignment between pUT18C-*phoP* gene sequencing result and published *phoP* gene.

Two-hybrid Screening and β-galactosidase Enzymatic Activities Assay

After comparing the sequencing results and verifying that the target DNAs were successfully ligated on plasmid DNAs, all new built plasmids with target DNAs were transformed into reporter stain DHM1. Plasmids with either T25 or T18 fragments included target DNAs were transformed together in DHM1, then inoculated

on plates with X-Gal in order to test the protein-protein interactions between the transcriptional regulator genes. Positive results showed a colony color change, from white to blue. The lacZ marker gene expression in Escherichia coli is activated due to the interaction between proteins. X-Gal acts as a substrate of β-galactosidase and allows the colonies to undergo the color change.

Negative controls were made by inserting an empty plasmid (T25 or T18 fragments) and a plasmid with DNAs inserted carries another antibiotic resistance into the reporter strain DHM1. With only one transcriptional regulator gene, there's no proteins interacted. Positive controls were made by the combination of pKT-Zip and pUT-Zip.

By comparing results with negative controls, pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, and pKNT25-*slyA* pUT18-*slyA* yielded positive results with the blue color of colonies on X-Gal screening plates after a 40 hours incubation at 30°C. As shown in figure 28. Other combinations yielded negative result, meaning no protein-protein interaction occurred. As shown in figure 29-figure 33.

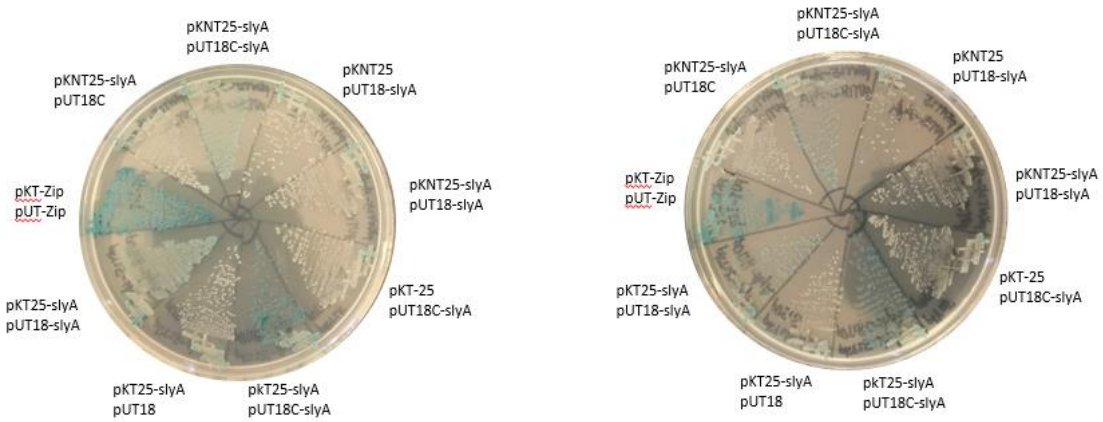

Figure 28: The X-gal screening assay of *slyA* combinations. The two-hybrid combination of *slyA* and itself (pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, pKNT25-*slyA* pUT18-*slyA*, pKNT25-*slyA* pUT18C-*slyA*) were streaked with their negative controls (pKT25-*slyA* pUT18, pKT25 pUT18C-*slyA*, pKNT25 pUT18-*slyA*, pKNT25-*slyA* pUT18C) and a positive control (pKT-Zip pUT-Zip). pKT-Zip pUT-Zip, pKT25-*slyA* pUT18C-*slyA*, pKNT25-*slyA* pUT18-*slyA* and pKNT25-*slyA* pUT18C-*slyA* formed blue color colonies.

Figure 29: The X-gal screening assay of *mprA* combinations. The two-hybrid combination of *mprA* and itself (pKT25-*mprA* pUT18-*mprA*, pKT25-*mprA* pUT18C-*mprA*, pKNT25-*mprA* pUT18-*mprA*, pKNT25-*mprA* pUT18C-*mprA*) were streaked with their negative controls (pKT25-*mprA* pUT18, pKT25 pUT18C-*mprA*, pKNT25 pUT18-*mprA*, pKNT25-*mprA* pUT18C) and a positive control (pKT-Zip pUT-Zip). pKT-Zip pUT-Zip formed blue color colonies.

Figure 30: The X-gal screening assay of *phoP* combinations. The two-hybrid combination of *phoP* and itself (pKT25-*phoP* pUT18-*phoP*, pKT25-*phoP* pUT18C-*phoP*, pKNT25-*phoP* pUT18-*phoP*, pKNT25-*phoP* pUT18C-*phoP*) were streaked with their negative controls (pKT25-*phoP* pUT18, pKT25-*phoP* pUT18C, pKNT25-*phoP* pUT18, pKNT25-*phoP* pUT18C) and a positive control (pKT-Zip pUT-Zip). pKT-Zip pUT-Zip formed blue color colonies.

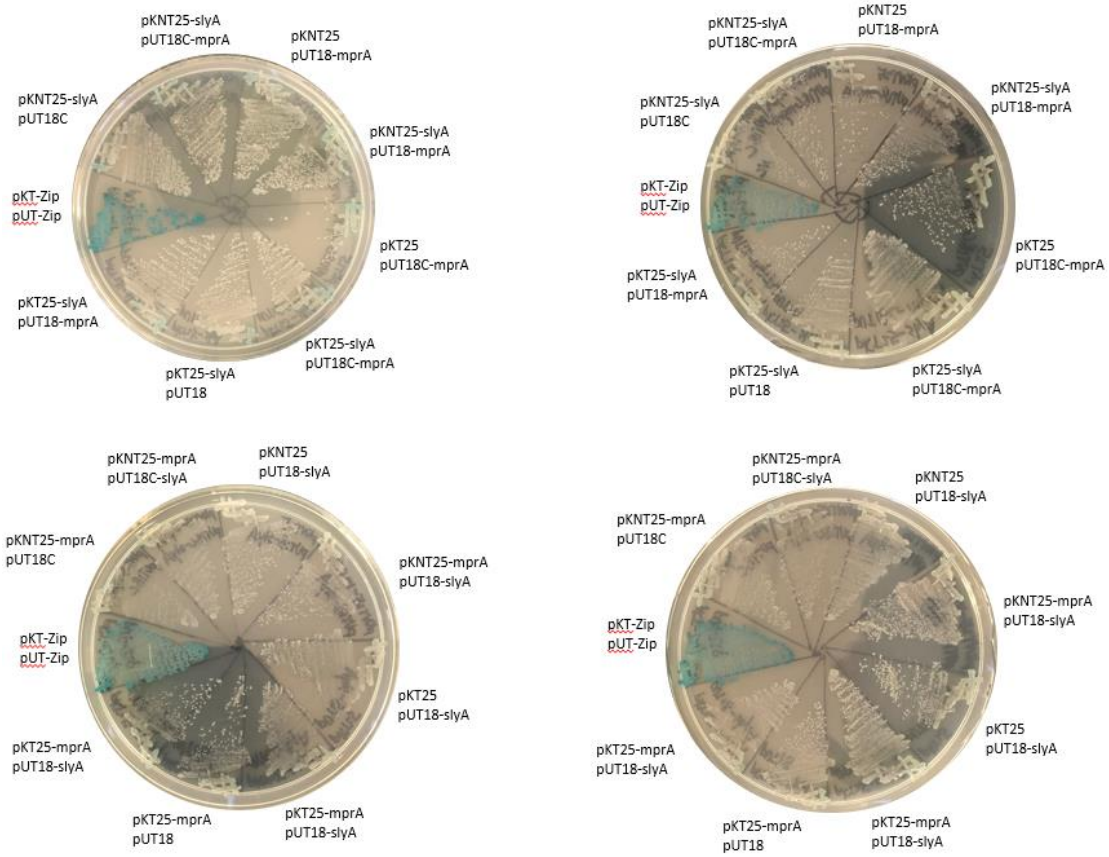

Figure 31: The X-gal screening assay of *mprA* and *slyA* combinations. The two-hybrid combination of *mprA* and *slyA* (pKT25-*mprA* pUT18-*slyA*, pKT25-*mprA* pUT18C-*slyA*, pKNT25-*mprA* pUT18-*slyA*, pKNT25-*mprA* pUT18C-*slyA*, pKT25-*slyA* pUT18-*mprA*, pKT25-*slyA* pUT18C-*mprA*, pKNT25-*slyA* pUT18-*mprA*, pKNT25-*slyA* pUT18C-*mprA*) were streaked with their negative controls (pKT25-*mprA* pUT18, pKT25 pUT18C-*slyA*, pKNT25 pUT18-*slyA*, pKNT25-*mprA* pUT18C,pKT25-*slyA* pUT18, pKT25 pUT18C-*mprA*, pKNT25 pUT18-*mprA*, and pKNT25-*slyA* pUT18C) and a positive control(pKT-Zip pUT-Zip). pKT-Zip pUT-Zip formed blue color colonies.

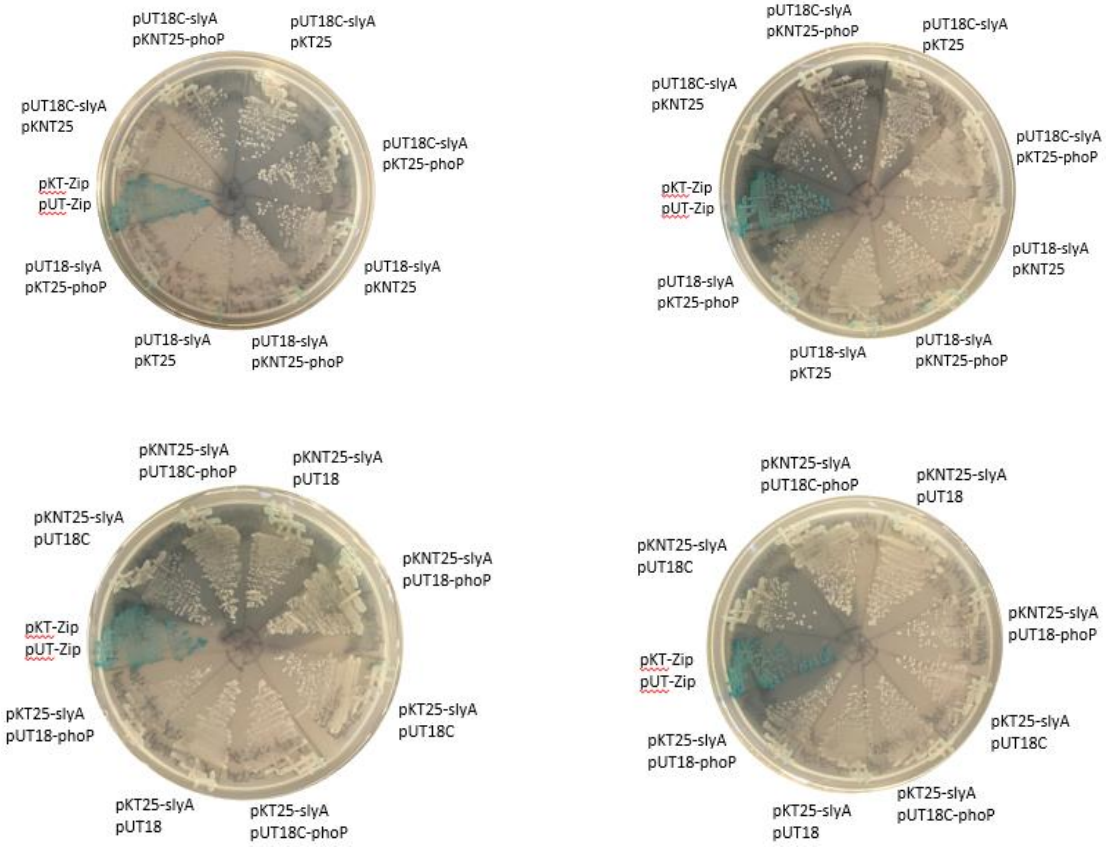

Figure 32: The X-gal screening assay of *phoP* and *slyA* combinations. The two-hybrid combination of *phoP* and *slyA* (pUT18-*slyA* pKT25-*phoP*, pUT18-*slyA* pKNT25-*phoP*, pUT18C-*slyA* pKT25-*phoP*, pUT18C-*slyA* pKNT25-*phoP*, pKT25- *slyA* pUT18- *phoP*, pKT25-*slyA* pUT18C-*phoP*, pKNT25-*slyA* pUT18-*phoP*, and pKNT25-*slyA* pUT18C-*phoP*,) were streaked with their negative controls (pUT18-*slyA* pKT25, pUT18-*slyA* pKNT25, pUT18C-*slyA* pKT25, pUT18C-*slyA* pKNT25, pKT25-*slyA* pUT18, pKT25-*slyA* pUT18C, pKNT25-*slyA* pUT18, and pKNT25-*slyA* pUT18C) and a positive control (pKT-Zip pUT-Zip). pKT-Zip pUT-Zip formed blue color colonies.

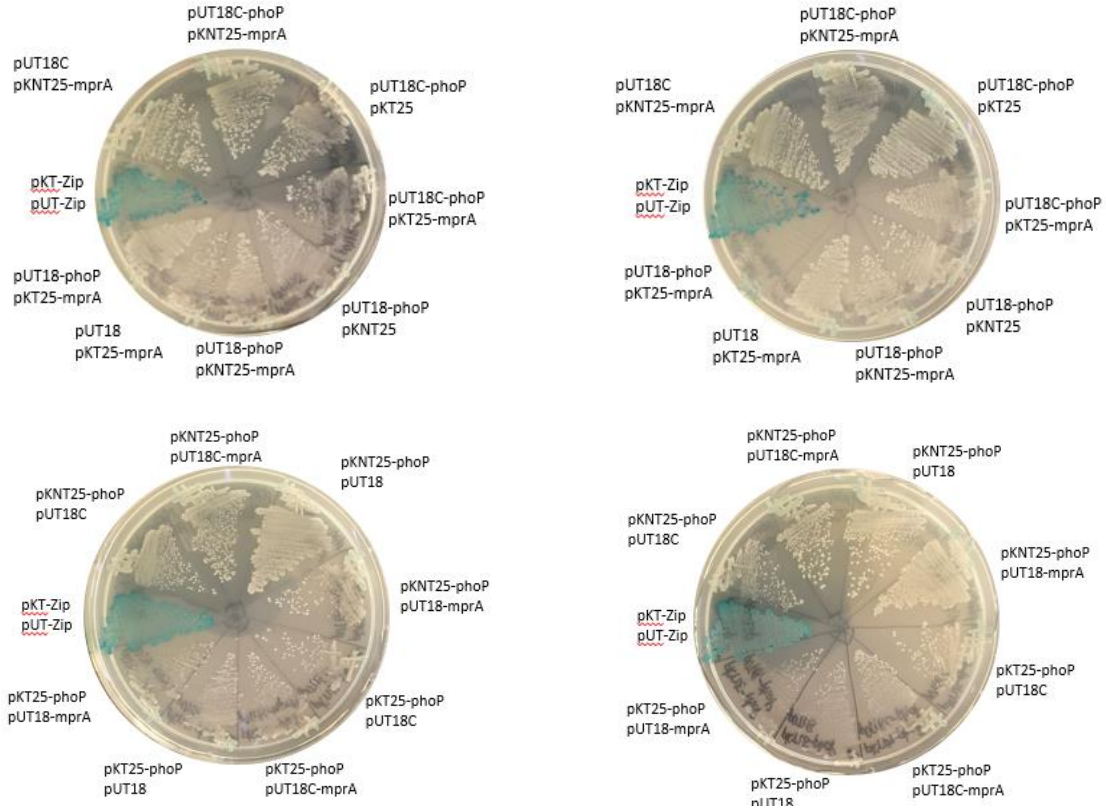

Figure 33: The X-gal screening assay of *phoP* and *mprA* combinations. The two-hybrid combination of *phoP* and *mprA* (pUT18-*phoP* pKT25-*mprA*, pUT18-*phoP* pKNT25-*mprA*, pUT18C-*phoP* pKT25-*mprA*, pUT18C-*phoP* pKNT25-*mprA*, pKT25-*phoP* pUT18-*mprA*, pKT25-*phoP* pUT18C-*mprA*, pKNT25-*phoP* pUT18-*mprA*, and pKNT25-*phoP* pUT18C-*mprA*,) were streaked with their negative controls (pUT18 pKT25-*mprA*, pUT18-*phoP* pKNT25, pUT18C-*phoP* pKT25, pUT18C pKNT25-*mprA*, pKT25-*phoP* pUT18, pKT25-*phoP* pUT18C, pKNT25-*phoP* pUT18, and pKNT25-*phoP* pUT18C) and a positive control (pKT-Zip pUT-Zip). pKT-Zip pUT-Zip formed blue color colonies

Results confirmed that blue colonies with the combination of pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, and pKNT25-*slyA* pUT18-*slyA* on X-Gal plates had activated adenylate cyclase, lacZ gene was expressed, so blue colonies were yielded when β-galactosidase was encoded. As shown in figure 34 and figure 35.

Colonies from plates were used to inoculate into LB broth. After the 24-hour re-inoculation, cultures were used on a β-galactosidase enzymatic activities assay. By comparing results of β-galactosidase enzymatic activities between different

combinations and their negative controls, a significant difference (at least 2-time difference) of β-galactosidase enzymatic activities were observed between the combination of pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, and pKNT25-*slyA* pUT18-*slyA* and its negative control. No significant differences on β-galactosidase enzymatic activities detected between SlyA and MprA, SlyA and PhoP, MprA and PhoP, also SlyA with itself and MprA with itself. As shown in figure 45.

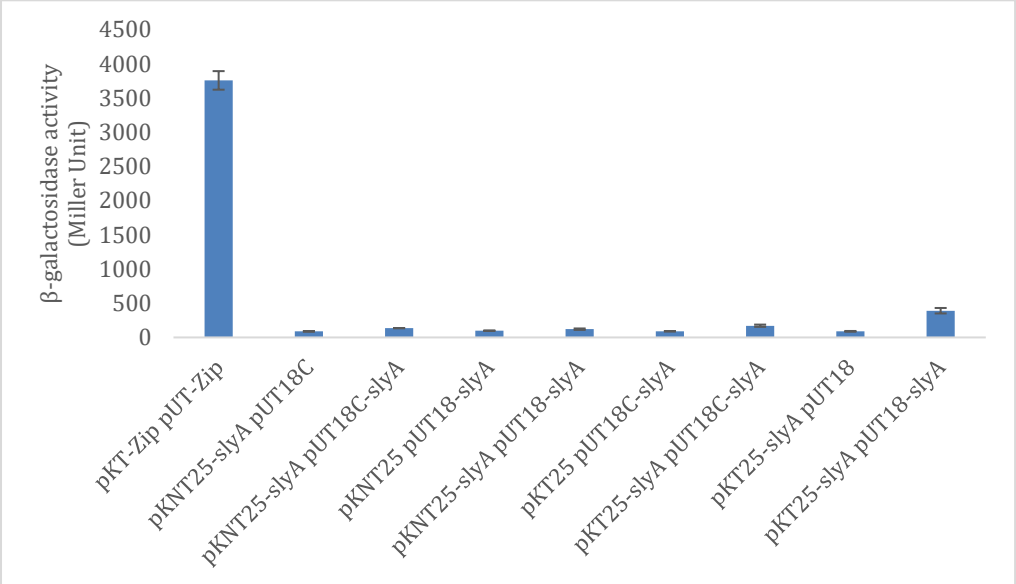

Figure 34: β-galactosidase enzymatic activities assay of SlyA. The β-galactosidase activities of SlyA and itself were detected and compared with negative controls and the positive control. pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, and pKNT25-*slyA* pUT18-*slyA* had stronger β-galactosidase activities comparison with their negative controls.

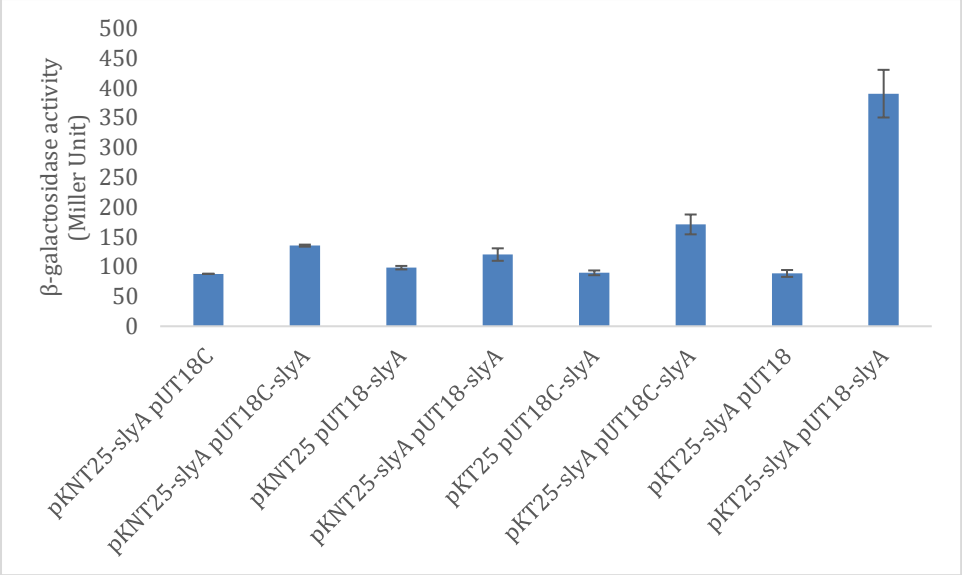

Figure 35: β-galactosidase enzymatic activities assay of SlyA without positive control. The β-galactosidase activities of SlyA and itself were detected and compared with negative controls. pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, and pKNT25-*slyA* pUT18-*slyA* had stronger β-galactosidase activities comparison with their negative controls.

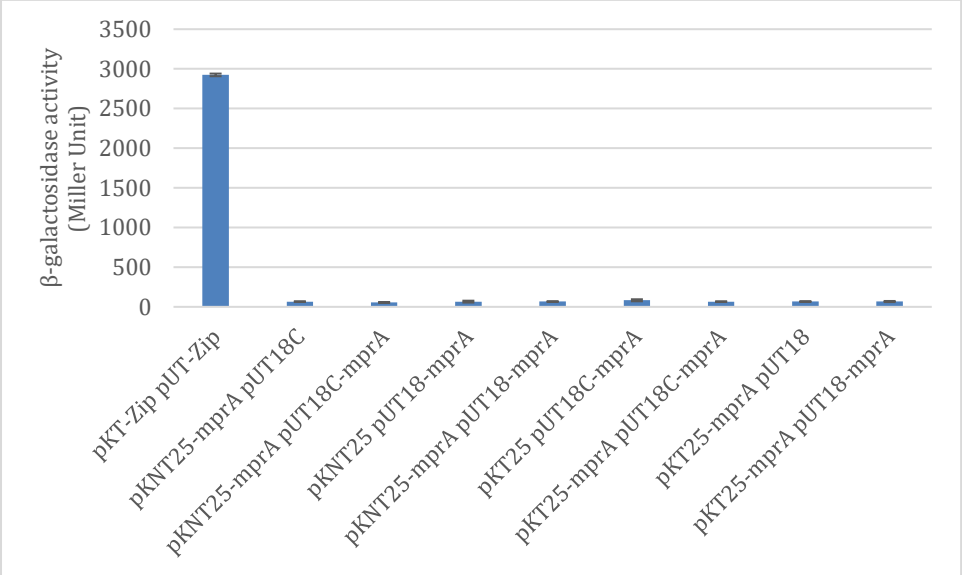

Figure 36: β-galactosidase enzymatic activities assay of MprA. The β-galactosidase activities of MprA were detected and compared with negative controls and the positive control. MprA didn't have a significant difference on β-galactosidase activities.

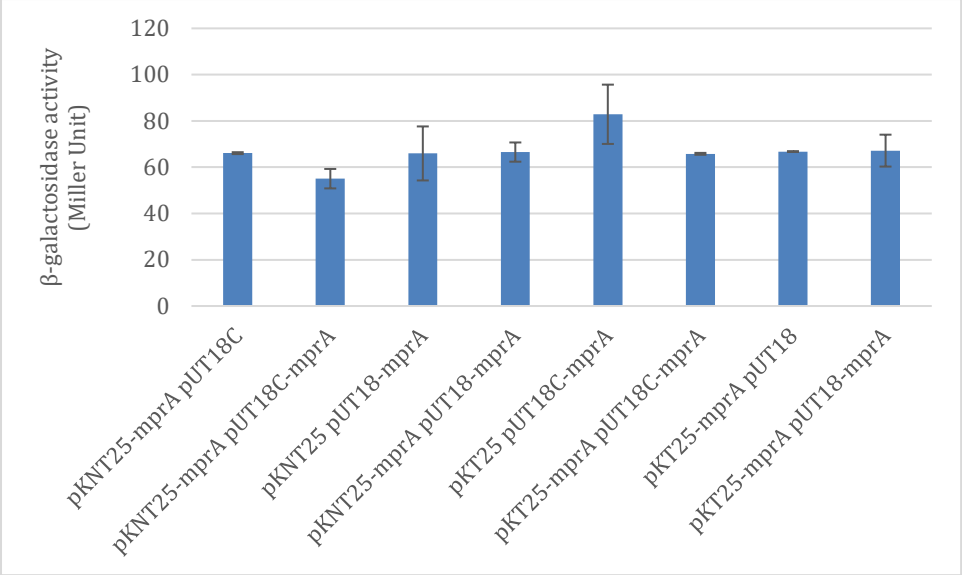

Figure 37: β-galactosidase enzymatic activities assay of MprA without positive control. The β-galactosidase activities of MprA were detected and compared with negative controls and the positive control. MprA didn't have a significant difference on β-galactosidase activities.

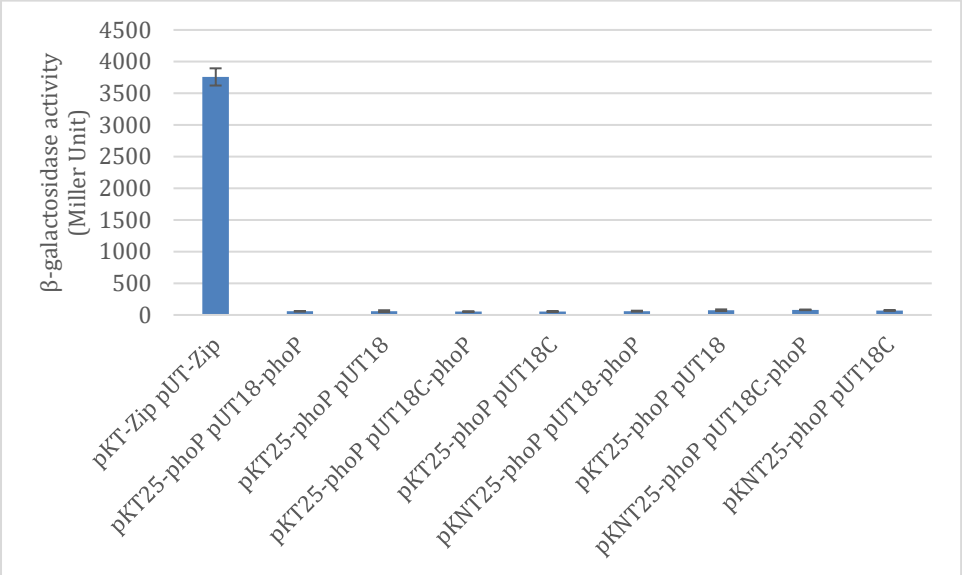

Figure 38: β-galactosidase enzymatic activities assay of PhoP. The β-galactosidase activities of PhoP were detected and compared with negative controls and the positive control. PhoP didn't have a significant difference on β-galactosidase activities.

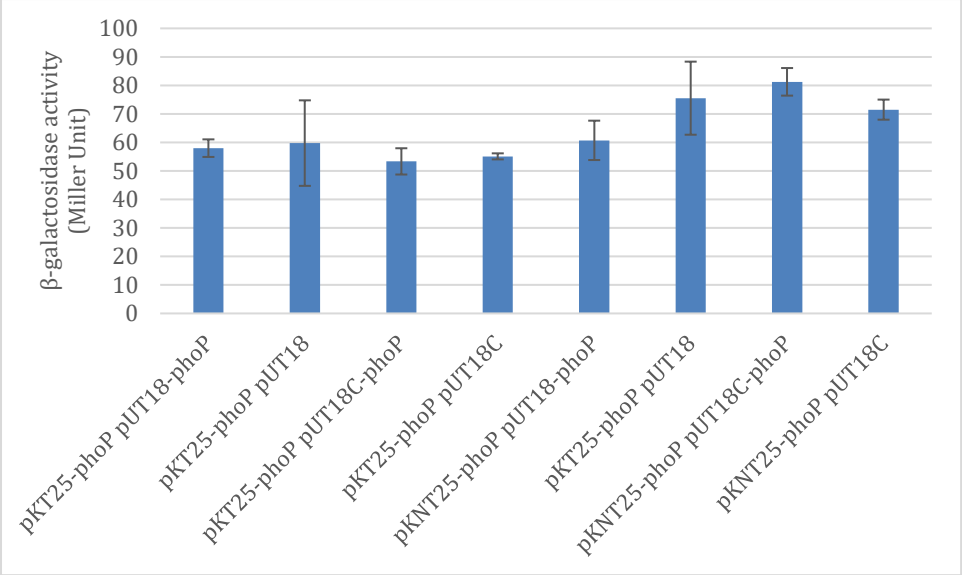

Figure 39: β-galactosidase enzymatic activities assay of PhoP without positive control. The β-galactosidase activities of PhoP were detected and compared with negative controls and the positive control. PhoP didn't have a significant difference on β-galactosidase activities.

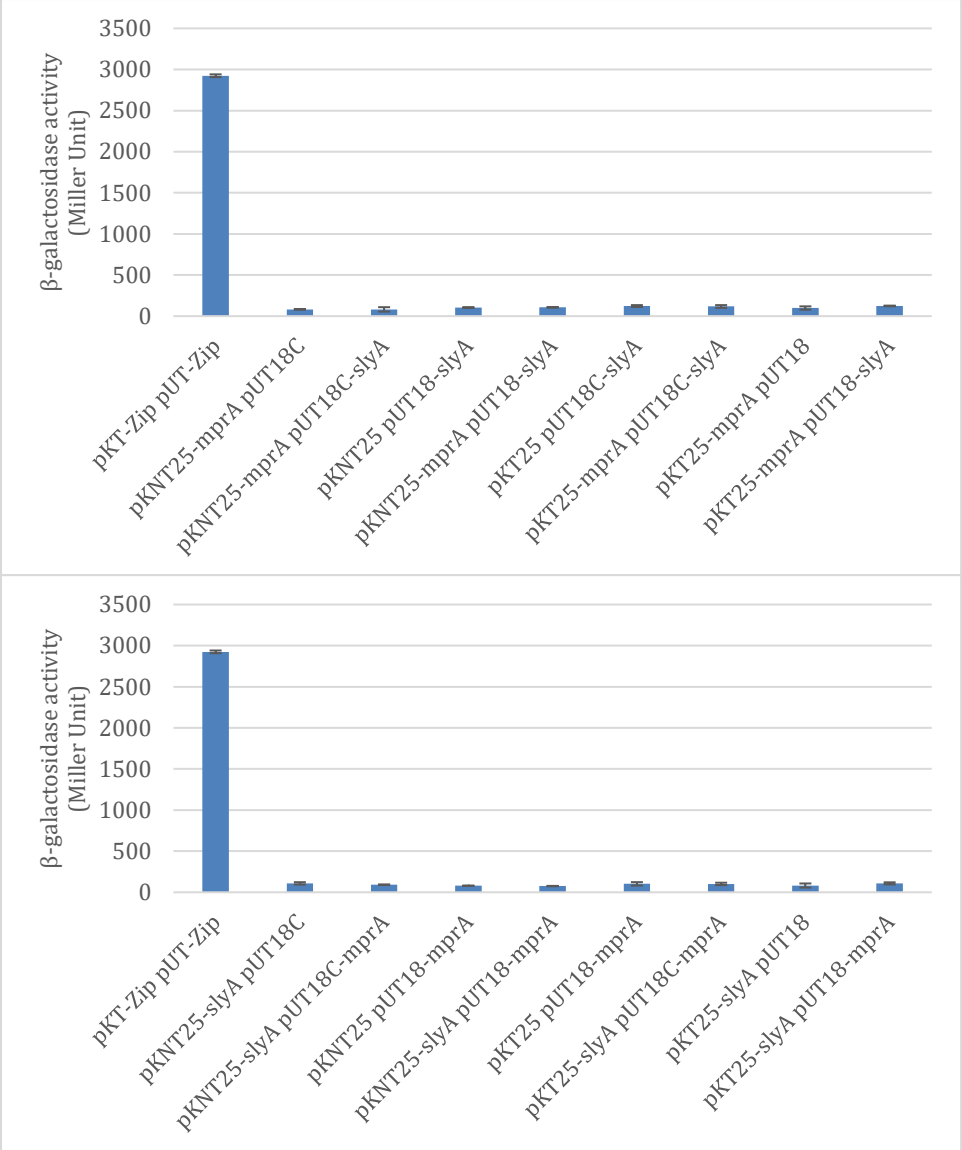

Figure 40: β-galactosidase enzymatic activities assay of SlyA and MprA. The β-galactosidase activities of SlyA and MprA were detected and compared with negative controls and the positive control. SlyA and MprA didn't have a significant difference on β-galactosidase activities.

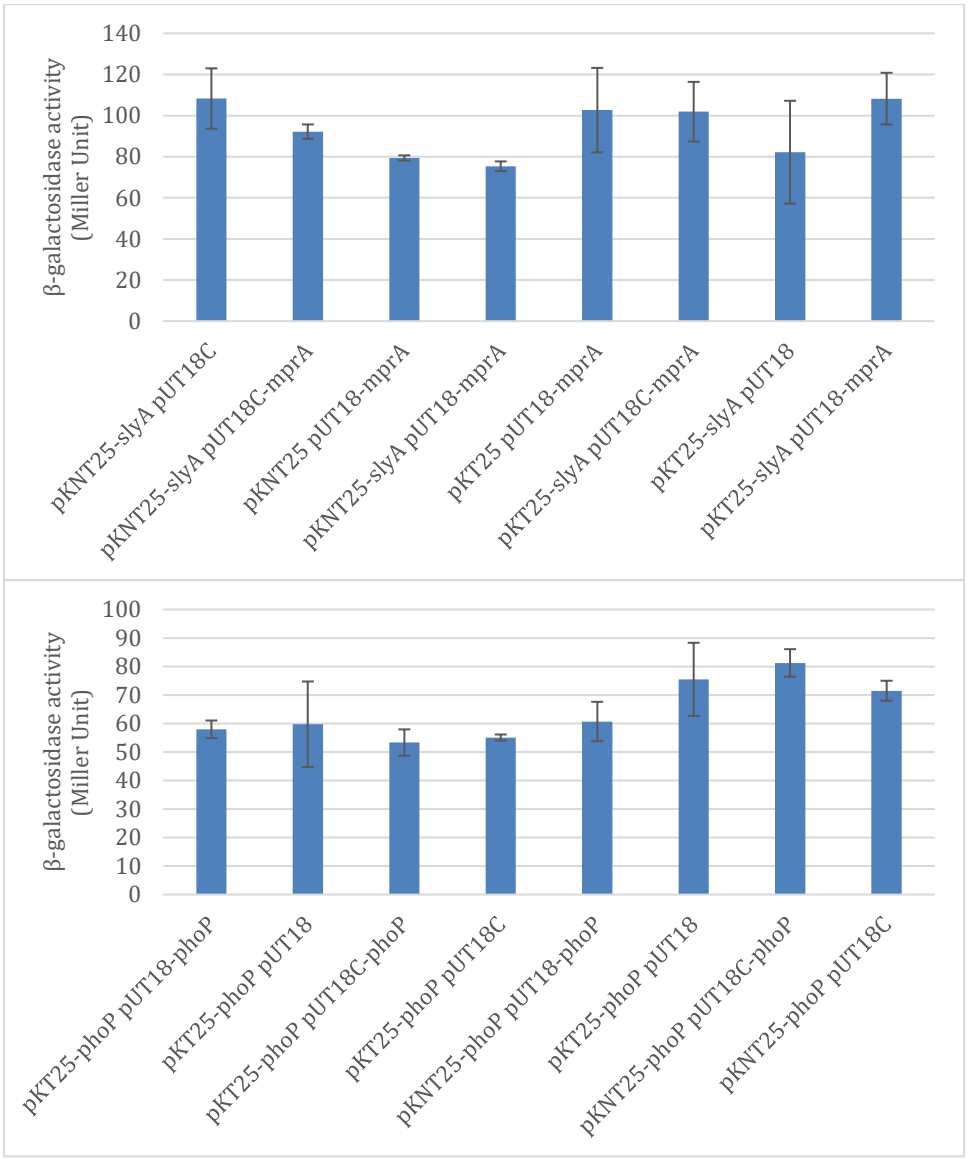

Figure 41: β-galactosidase enzymatic activities assay of SlyA and MprA without positive control. The β-galactosidase activities of SlyA and MprA were detected and compared with negative controls and the positive control. SlyA and MprA didn't have a significant difference on β-galactosidase activities.

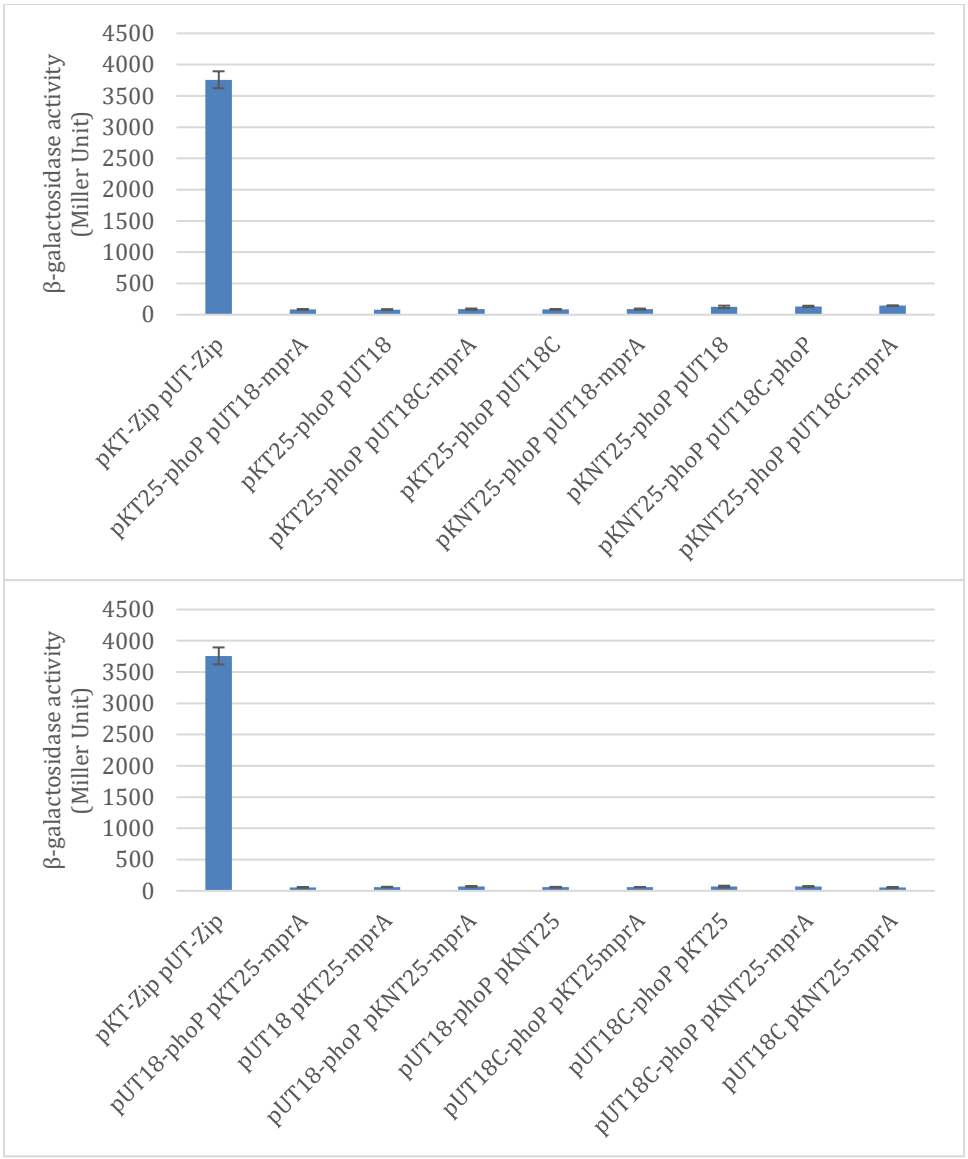

Figure 42: β-galactosidase enzymatic activities assay of PhoP and MprA. The β-galactosidase activities of PhoP and MprA were detected and compared with negative controls and the positive control. PhoP and MprA didn't have a significant difference on β-galactosidase activities.

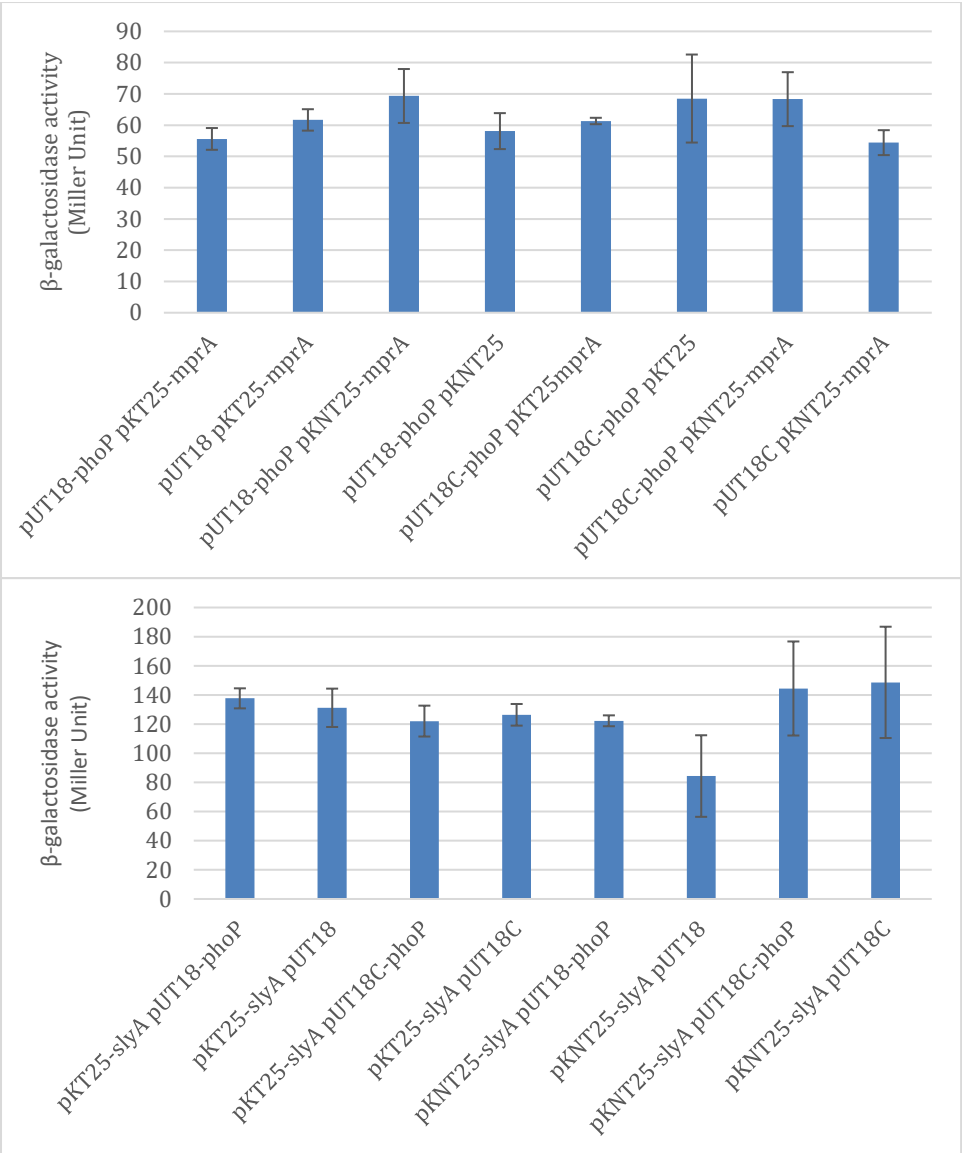

Figure 43: β-galactosidase enzymatic activities assay of PhoP and MprA without positive control. The β-galactosidase activities of PhoP and MprA were detected and compared with negative controls and the positive control. PhoP and MprA didn't have a significant difference on β-galactosidase activities.

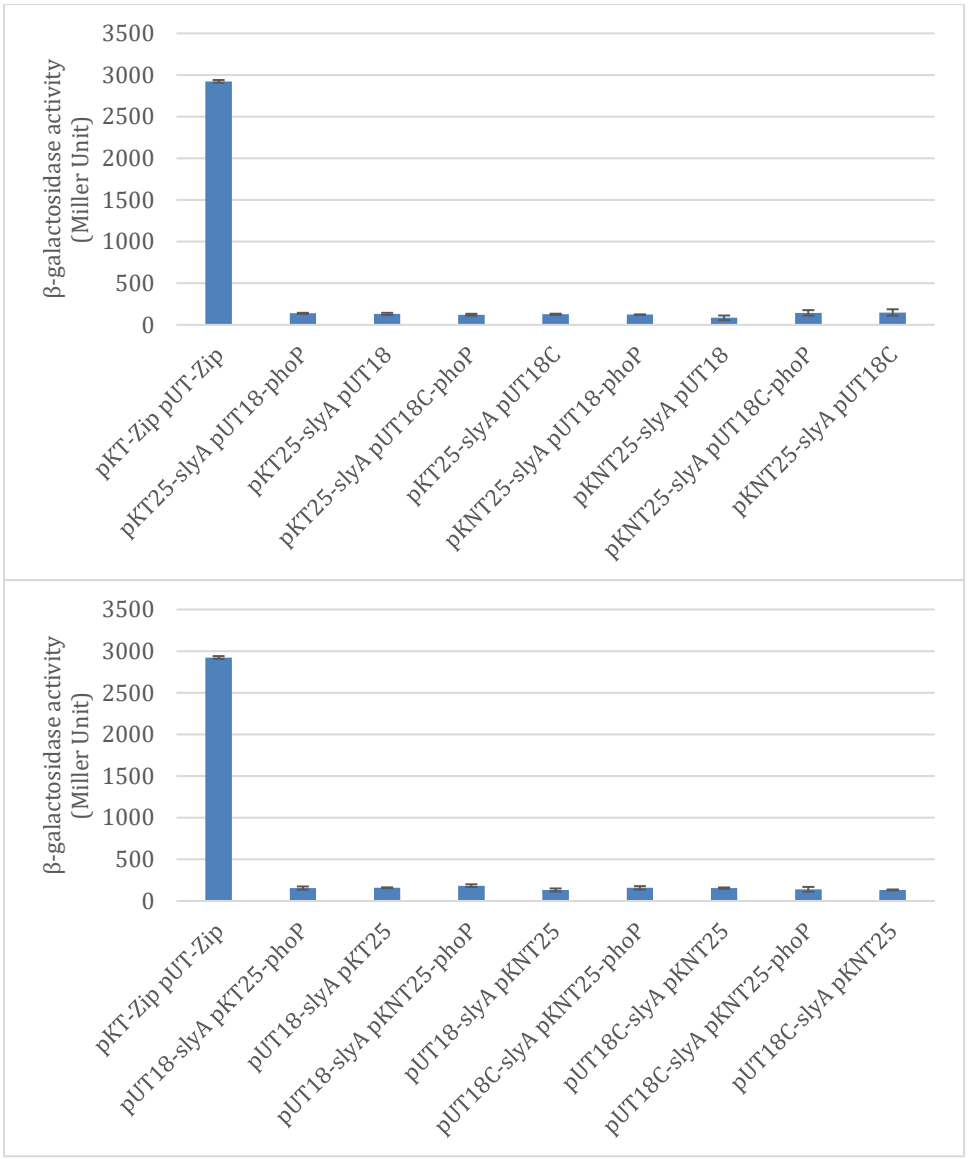

Figure 44: β-galactosidase enzymatic activities assay of PhoP and SlyA. The β-galactosidase activities of PhoP and SlyA were detected and compared with negative controls and the positive control. PhoP and SlyA didn't have a significant difference on β-galactosidase activities.

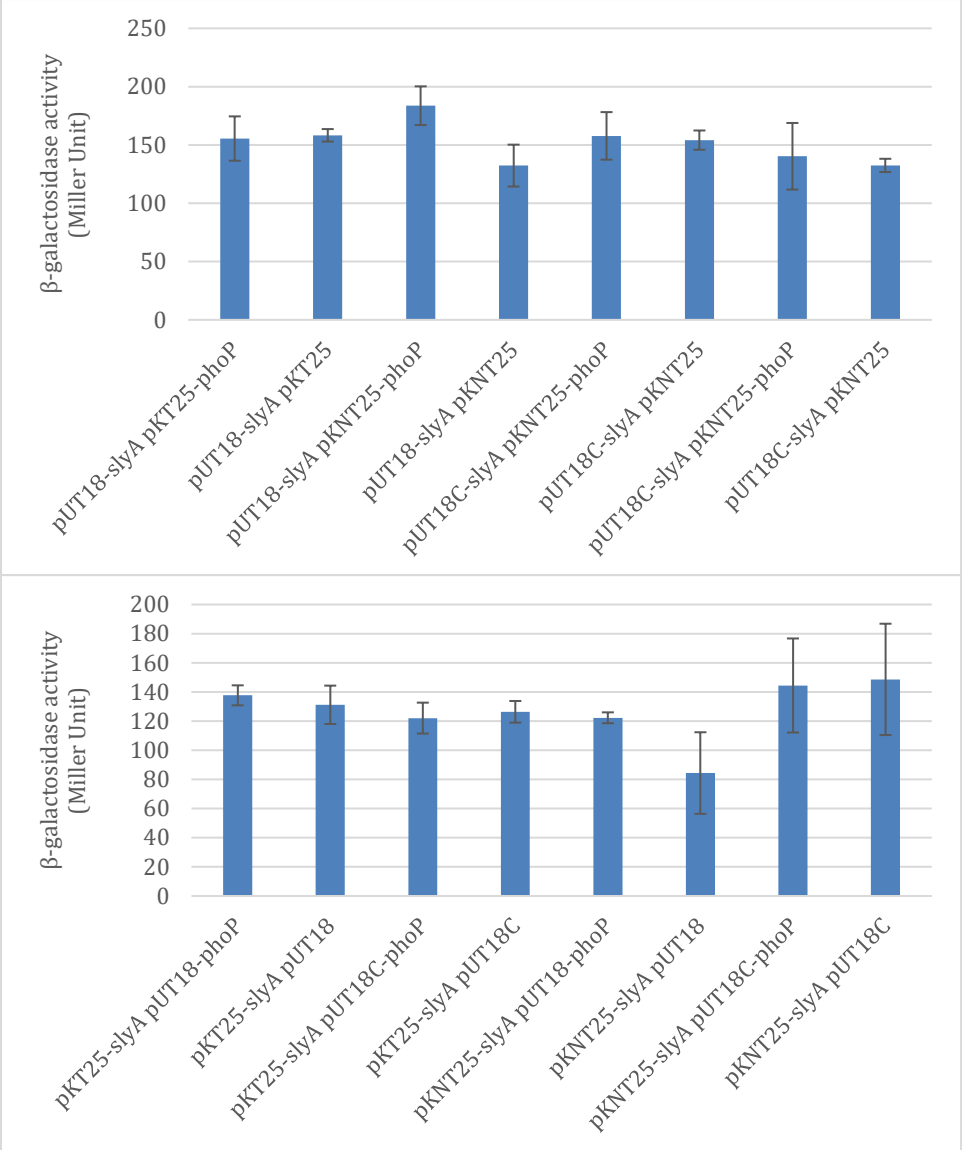

Figure 45: β-galactosidase enzymatic activities assay of PhoP and SlyA without positive control. The β-galactosidase activities of PhoP and SlyA were detected and compared with negative controls and the positive control. PhoP and SlyA didn't have a significant difference on β-galactosidase activities.

For detecting β-galactosidase enzymatic activities of SlyA base on the significant β-galactosidase activities of pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, and pKNT25-*slyA* pUT18-*slyA* combination, another set of β-galactosidase enzymatic activity assay with the SlyA and itself were ran in four different times (4 hours, 12 hours, 24 hours, and 48 hours) to research the different β-galactosidase enzyme activities in different given times. This is because β-

galactosidase enzyme activities may perform differently depending on the bacteria growth. After a 4-hour incubation, the combination of pKT25-*slyA* pUT18-*slyA* showed some significant β-galactosidase enzyme activities of more than 2-time differences by the comparison of its negative controls. No significant β-galactosidase enzyme activities differences were observed in other combinations. As shown in figure 46.

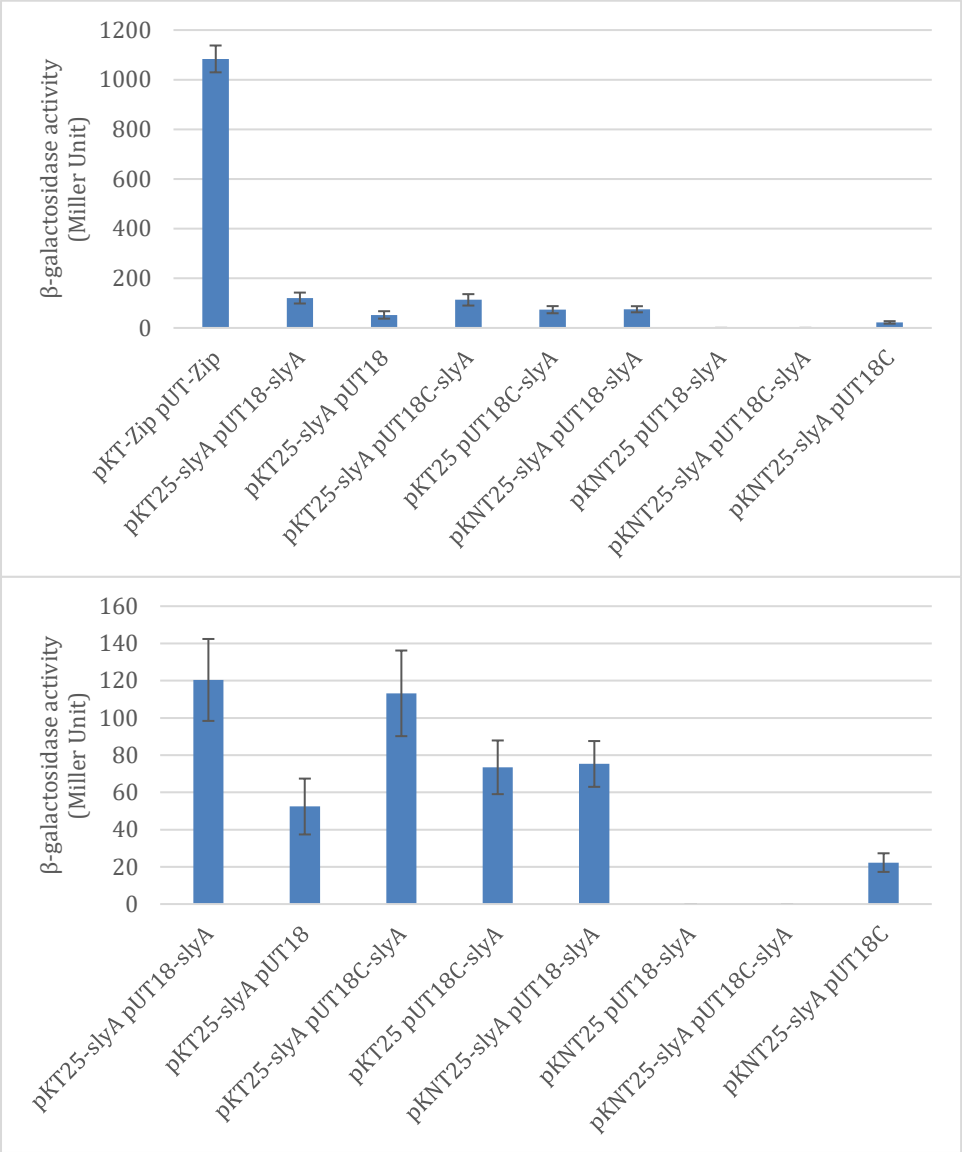

Figure 46: β-galactosidase enzymatic activities assay of SlyA with and without positive control with 4-hour incubation. The β-galactosidase activities of SlyA and itself were detected at 4-hour and compared with negative controls. pKT25-*slyA* pUT18-*slyA* and pKT25-*slyA* pUT18C-*slyA*, had stronger β-galactosidase activities comparison with their negative controls.

After 12-hour incubation, a 4-time differences of β-galactosidase enzyme activities were detected between pKT25-*slyA* pUT18-*slyA* and its negative control. The β-galactosidase enzyme activities of pKT25-*slyA* pUT18C-*slyA* was 11-time stronger than the native control. By compare the pKNT25-*slyA* pUT18-*slyA* and its negative control, a 2-time difference was observed. pKNT25-*slyA* pUT18C-*slyA* had a 3-time stronger β-galactosidase enzyme activity by compare with its negative controls. As shown in figure 47.

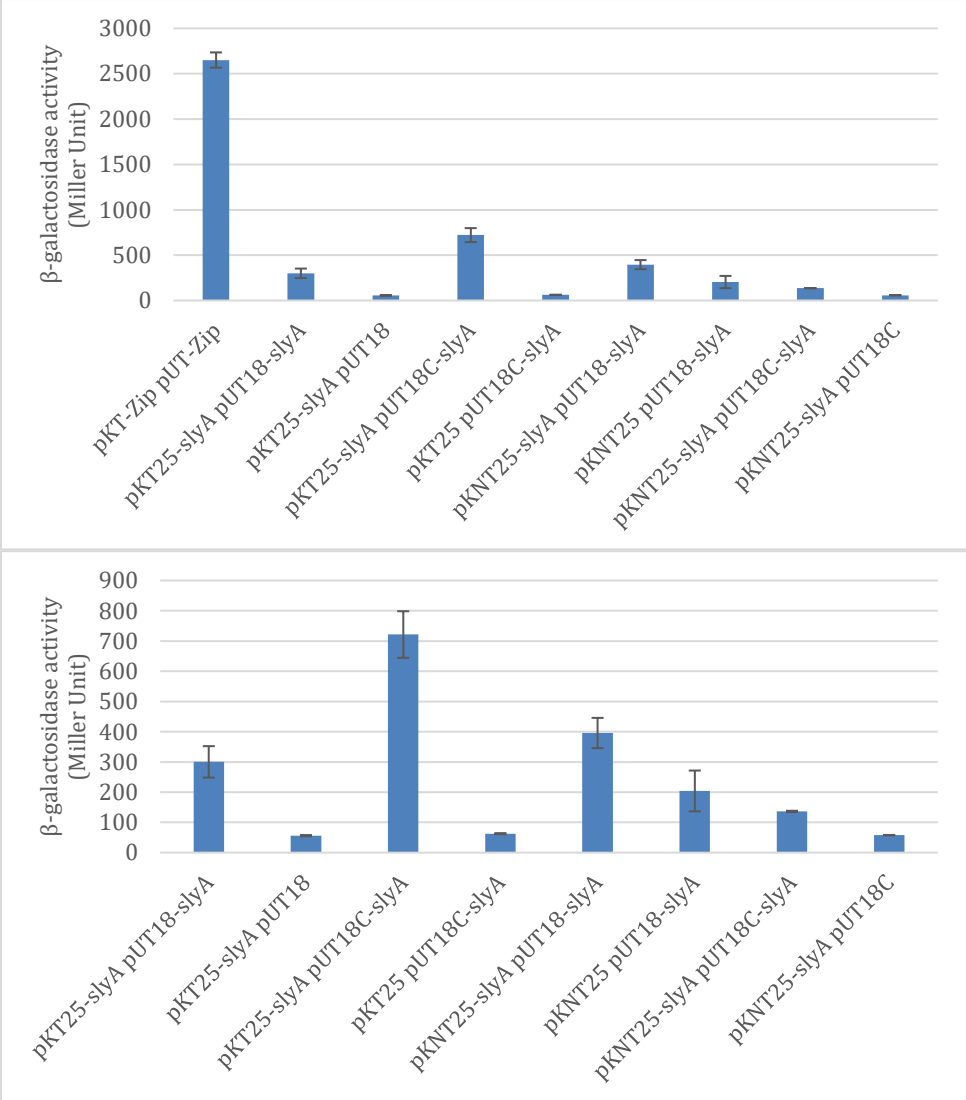

Figure 47: β-galactosidase enzymatic activities assay of SlyA with and without positive control with 12-hour incubation. The β-galactosidase activities of SlyA and itself were detected at 12-hour and compared with negative controls. pKT25-*slyA* pUT18-*slyA* had 4 times stronger β-galactosidase activities comparison with their negative controls. pKT25-*slyA* pUT18C-*slyA* had 11 times stronger β-galactosidase activities comparison with their negative controls.

After 24-hour incubation, a 5-time differences of β-galactosidase enzyme activities were detected between pKT25-*slyA* pUT18-*slyA* and its negative control. The β-galactosidase enzyme activities of pKT25-*slyA* pUT18C-*slyA* was 11-time stronger than the native control. By compare the pKNT25-*slyA* pUT18-*slyA* and its negative control, a 2-time difference was observed. pKNT25-*slyA* pUT18C-*slyA* had a

3-time stronger β-galactosidase enzyme activity by compare with its negative controls. As shown in figure 48.

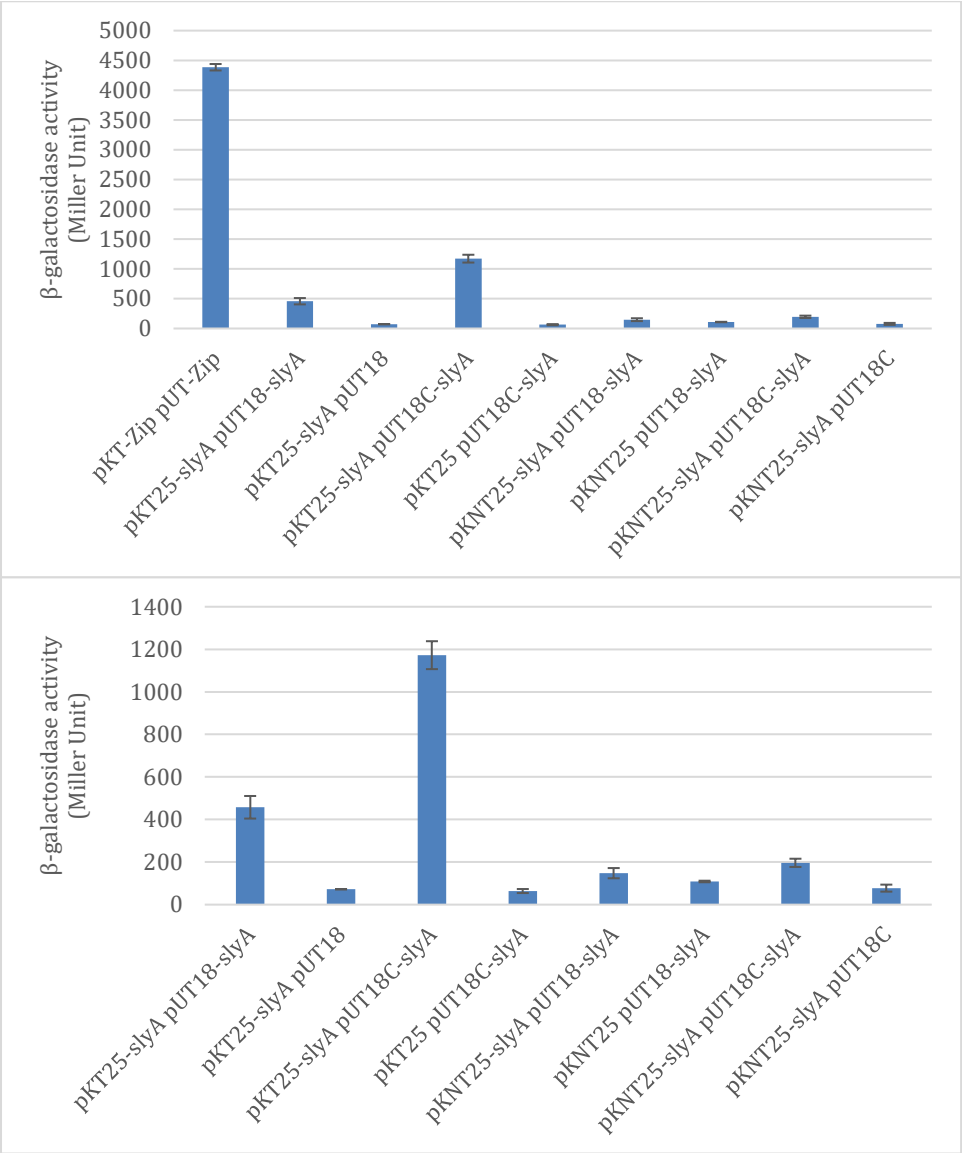

Figure 48: β-galactosidase enzymatic activities assay of SlyA with and without positive control with 24-hour incubation. The β-galactosidase activities of SlyA and itself were detected at 24-hour and compared with negative controls. pKT25-*slyA* pUT18-*slyA* had 5 times stronger β-galactosidase activities comparison with their negative controls. pKT25-*slyA* pUT18C-*slyA* had 11 times stronger β-galactosidase activities comparison with their negative controls. pKNT25-*slyA* pUT18-*slyA* had 2 times stronger β-galactosidase activities comparison with their negative controls. pKNT25-*slyA* pUT18C-*slyA* had 3 times stronger β-galactosidase activities comparison with their negative controls.

After 48-hour incubation, a 5-time differences of β-galactosidase enzyme activities were detected between pKT25-*slyA* pUT18-*slyA* and its negative control.

The β-galactosidase enzyme activities of pKT25-*slyA* pUT18C-*slyA* was 11-time stronger than the native control. By compare the pKNT25-*slyA* pUT18-*slyA* and its negative control, a 2-time difference was observed. pKNT25-*slyA* pUT18C-*slyA* had a 3-time stronger β-galactosidase enzyme activity by compare with its negative controls. As shown in figure 49.

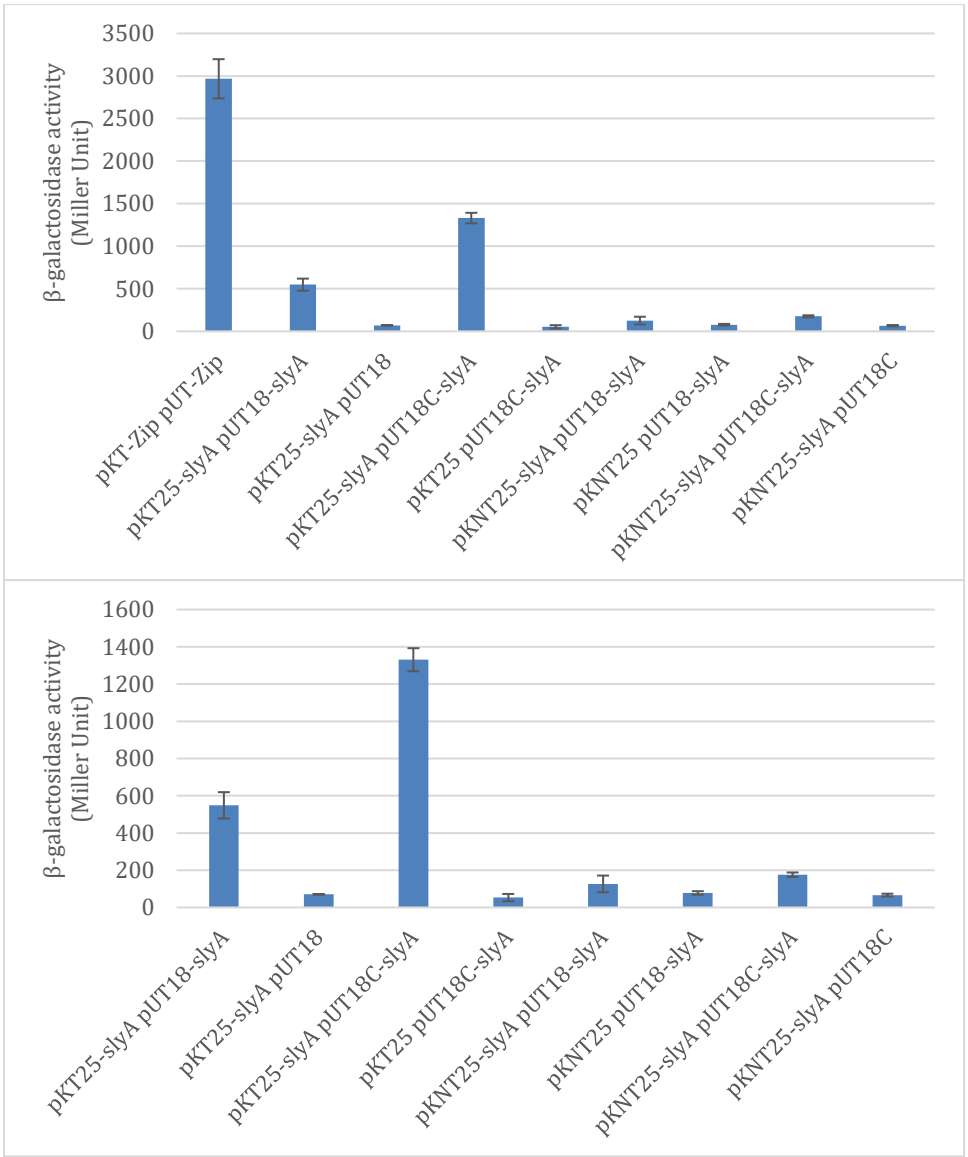

Figure 49: β-galactosidase enzymatic activities assay of SlyA with and without positive control with 48-hour incubation. The β-galactosidase activities of SlyA and itself were detected at 48-hour and compared with negative controls. pKT25-*slyA* pUT18-*slyA* had 5 times stronger β-galactosidase activities comparison with their negative controls. pKT25-*slyA* pUT18C-*slyA* had 11 times stronger β-galactosidase activities comparison with their negative controls. pKNT25-*slyA* pUT18-*slyA* had 2 times stronger β-galactosidase activities comparison with their negative controls.

pKNT25-*slyA* pUT18C-*slyA* had 3 times stronger β-galactosidase activities comparison with their negative controls.

CHAPTER 4

DISCUSSION

Gene Isolation

Three genes (*slyA*, *mprA*, and *phoP*) were used and successfully amplified through the PCR reaction. Four plasmids (pKT25, pKNT25, pUT18, and pUT18C) were isolated by using phenol alkaline plasmid isolation. After purifying PCR products, a successful digestion was made by using KpnI on both PCR products and isolated plasmid DNAs. After digested products get purified, insert DNAs were successfully ligated with target vector DNAs, positive results were confirmed by ID-PCR and ID-Digestion. Results of the sequencing check was the last confirmation when both ID-PCR and ID-Digestion were positive and matched their expected size. While running gel with digestion products, some bands showed strong background connected with their bands, which was a strong signal of incomplete digestion. Incomplete digestion happens commonly in the process of digestion and it normally causes by over or less amount of enzyme in the digestion system, or by unknown compounds that amplified due to contaminations.

It was noticed that some false-positive results were observed on the gel by running ID-PCR results. While PCR was in progress, if PCR products weren't purified, or with an inefficient purification, some compounds may affect DNA samples, causing DNA degradation, and contaminating DNA samples. DNA degradation is critical during the PCR reaction because it strongly affect the DNA amplifications. Also, contaminations are possibly to lower the speed of DNA amplifications or affect the specificity due to the interaction between primers and

DNAs. Those contaminations may produce a lot of artifactual PCR products. When running the gel by using such PCR products, false-positive results were observed.

Two-hybrid screening and β-galactosidase Enzymatic Activities Assay Improvements

While different two-hybrid combinations (with both T25 and T18 fragments) were streaked on X-Gal plates with their negative controls (only one target DNA inserted) and positive control pKT-Zip pUT-Zip. After a 40-hour inoculation in a 30^{o}C incubator, expected color changes were observed. Positive control on every plate showed an obvious color change and formed blue color colonies. There was no color change observed between the gene combination of *slyA* with *mprA*, *slyA* with *phoP*, *mprA* with *phoP*, *mprA* with *mprA*, and *phoP* with *phoP*. Color change was overserved with the combination between *slyA* and itself.

X-Gal is a substrate of β-galactosidase. If adenylate cyclase was activated, lacZ gene was expressed, blue colonies were yielded when β-galactosidase was encoded. When color change was observed, meaning there were protein-protein interaction detected between two transcriptional regulators. Protein-protein interaction is a significant signal and it allows organisms respond with different environment to adapt in to those changes by using a stimulus-response mechanism. When environmental stimulus was detected, histidine kinase sends this signal of stimulus to its regulator to active expressions from target genes.

False positive results were observed due to an over inoculation. This is possibly because of the His+ transformants. Colonies with His+ transformants contains hybrid proteins without plasmids encoding. These hybrid proteins sometimes interact with their target proteins if colonies were over inoculated, which forms another type of protein-protein interaction causing the color change of colonies.

Colonies from plates were inoculated for 24 hours in 37 ºC for the β-galactosidase enzymatic activities assay. Results confirmed that the protein-protein interactions only happen between SlyA and itself, which pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, and pKNT25-*slyA* pUT18-*slyA* had increased β-galactosidase activities comparison to their negative controls. For more details on how SlyA had protein-protein interactions with itself, another set of β-galactosidase enzymatic activities assay were prepared with different inoculation times (4 hours, 12 hours, 24 hours, and 48 hours). When SlyA combines with itself, β-galactosidase activities have increased significantly. After the 4-hour inoculation, the positive control combination pKT-Zip pUT-Zip showed a significant increasing value of β-galactosidase activities. Combinations of pKT25-*slyA* pUT18-*slyA*, pKT25-*slyA* pUT18C-*slyA*, and pKNT25-*slyA* pUT18-*slyA* results also showed that β-galactosidase activities have increased comparison to their negative control combinations. β-galactosidase activities after 24-hour incubation were stabilized and combinations of SlyA have detected significant changes by comparison with their negative controls, meaning the protein interactions were detected.

Positive results were observed from the β-galactosidase enzymatic activities assay on *slyA* combinations, but different combination formed different β-galactosidase enzymatic activities. The reason causes these differences is due to the different position that *slyA* ligated on four different plasmids (pKT25, pKNT25, pUT18, and pUT18C). Genetic fusions occur on either N-termini or C-termini to allow protein interactions. On plasmid pKT25, the *slyA* was ligated at the 5’ end of the complementary fragment T25. When *slyA* was ligated on the plasmid pKNT25, the *slyA* was inserted onto the 3’ end of fragment T25. Similar to plasmid pKT25, *slyA* was ligated on the 5’ end of fragment T18 that located on the plasmid pUT18C,

and 3’ end of fragment T18 on plasmid pUT18C was the position where *slyA* ligated on. Adenylate cyclase domain includes T25 catalytic site and T18 calmodulin binding site. That is, when *slyA* ligated on plasmids with different positions, different β-galactosidase enzymatic activities are detected. Different combinations confirmed that β-galactosidase enzymatic activities were occurred due to protein interactions.

It was observed that there were no β-galactosidase enzymatic activities detected on the combination of pKNT25-*slyA* pUT18C-*slyA* and its negative control after the 4-hour inoculation. The probable reason causes this result is the concentration of culture wasn’t enough by only 4-hour of incubation.

Methodology Improvements

The Bacterial Adenylate Cyclase Two-Hybrid (BACTH) System method were used to study the protein-protein interactions. By comparison with other methods used on the study of protein interactions, Bacterial Adenylate Cyclase Two-Hybrid (BACTH) System doesn’t required sophisticated equipment, which is accessible to most microbiology labs. Also, this method is scalable, meaning the screening of the protein interactions can be detected among many proteins. Results from the BACTH system allows a similar quality by comparison the data that generated by the alternative approach of co-affinity purification followed by mass spectrometry. The disadvantage by using this method is commonly observed with high number of false positive and false negative identification. False identifications are observed when unnatural protein concentrations produced by the overexpression of the fusion protein. False identifications are also occurring when fused parts from hybrid protein inhibit certain interaction causing the inaccurate results. The most common reason to cause

the false negative result is when two interacting proteins found to be non-interacting when they are not localized to the nucleus.

Previous research suggests that two-hybrid system works for the study of both homodimers and heterodimers (26). In two-hybrid system, two complementary fragments (T25 and T18) need to join in order to initiate cAMP synthesis. By separating two fragments, or combining the same fragment, the cAMP cannot be produced. The fusion of polypeptides with the fragments causes a functional complementation. This only occurs when the fragments are in pairs and are able to initiate cAMP production. cAMP binds to the CAP, then cAMP/CAP complex regulator of gene transcription occurs in Escherichia coli.

Previous study suggests that the transcriptional regulator SlyA can form a homodimer (27). In the study, the DSS cross-linking occurred to form the SlyA homodimer. The similarity of the results from this study to the two-hybrid screening and β-galactosidase enzymatic activities assay, show that the protein interactions in SlyA occurred; which supports the statement that SlyA forms a homodimer.

According to previous experiment, both PhoP and MprA have been found to form homodimers (30) (31). This experiment contradicts to those previous studies since, in this case, PhoP and MprA were not found to form homodimers. In protein interactions, false-negative results were observed when low level of interactions are present. However, Phop and MprA did not result in homodimers for this experiment. This difference is most likely a result of a false-negative outcome. Although the BATCH system is an efficient method for detecting protein-protein interactions, false-negatives can still occur. These false-negative results can occur for several reasons. The first reason revolves around the expression of lactose operons (29). Both β-galactosidases and plating on the LB+X-GAL+KM+AMP plates are an indirect

measurement of the interactions that occur between the proteins. Low protein-protein interactions cannot be accurately measured using these techniques (29). If there were low levels of protein-protein interactions, then the color change within the samples may not be apparent.

Another reason that caused false-negative results was due to the lack of the mating and transformation (28). In the process of transformation, if there weren't enough cell numbers for testing two-hybrid combinations, false-negative may be presented.

Third, when the domain-specific misfolding occurs in some constructs based on two-hybrid combinations. Some protein interactions can still be detected, but nonfunctional for other interactions. This may cause false-negative results (28).

Another possible reason false-negative results were observed may be because of disturbances in the proteins during cell reproduction (28). Non-interacting proteins may come in contact with an interacting protein causing false-negative results. This is due to the weak, or limited, protein reactions caused by non-interacting proteins encountering interacting proteins. Interacting proteins must meet with a protein of its same kind in order to produce positive results.

CHAPTER 5

CONCLUSION

Two-hybrid screening results showed there were protein interactions between SlyA and itself. β-galactosidase enzymatic activities assay results have also confirmed that SlyA interacted with itself by significant increases of β-galactosidase enzymatic activities in two-hybrid combinations comparison with their negative controls. SlyA SlyA is able to form homodimers. However, no protein interactions were detected between SlyA and MprA, SlyA and PhoP, MprA and PhoP, and MprA, PhoP with itself. Therefore, MprA and PhoP couldn't form homodimers, and no heterodimers were form in between three transcriptional regulators. Based on Burbulis and Shirley's research (33), some proteins only interacted with each other in a specific way and orientation.

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