INVESTIGATIONS

OF THE

PIT-ACCESSORY PROTEIN

INVESTIGATIONS OF THE FUNCTION OF THE PIT-ACCESSORY PROTEIN (PAP) IN *SINORHIZOBIUM MELILOTI*

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Lay Abstract

Microbes require phosphorus in the form of inorganic phosphate (Pi) as an essential nutrient, but it is often found in growth-limiting concentrations in the environment. Bacteria have developed a diverse set of Pi transport systems to scavenge and take up phosphate from the environment. In the soil bacterium, *Sinorhizobium meliloti,* one such Pi transport system is the Pap-Pit system. Pit is a membrane transporter for Pi and is associated with a cytosolic protein of unknown function known as Pap. Various mutations in both *pap* and *pit* have been constructed in an attempt to determine the function of Pap in Pi uptake via Pit. The *pap* gene appears to be required immediately upstream of *pit* in an operon for functional Pi transport. The *pap* and *pit* genes overlap by a single nucleotide and this may suggest a translational coupling mechanism that is required for functional Pi transport via Pap-Pit.

Abstract

Phosphate (PO_4^{-3} or Pi) is an essential molecule necessary for sustaining life and it plays important roles in nucleic acid and cell membrane integrity. However, phosphate is found in growth-limiting concentrations in most environments. Bacteria have developed a diverse set of transport systems to uptake and scavenge phosphate from their environment for use in cellular processes. In the soil bacterium, *Sinorhizobium meliloti,* one such Pi transport system is the Pap-Pit system. Pit is a membrane transporter for Pi and is associated with a cytosolic protein of unknown function known as Pap (Pit-accessory protein). Interestingly, the stop codon of *pap* overlaps with the start codon of *pit* by a single nucleotide. In previous work, the *pap* gene appeared to be required immediately upstream of *pit* in an operon for functional Pi transport. Thus, in a *pap* deletion mutant, when *pap* is present *in trans*, there is no Pi transport. This suggests a possible translational coupling mechanism between Pap and Pit, in which the translation of Pap is required for the translation of Pit. Here, an alkaline phosphatase (*phoA/lacZ*) and a β-glucuronidase (*gusA*) translational reporter were fused to Pit as a measure of its translation and to understand the role of translational coupling in the Pap-Pit system. Growth complementation experiments with a conditionally Pi transport deficient *S. meliloti* mutant carrying various mutations in both *pap* and *pit* have also been performed in an attempt to determine the function of Pap in Pi uptake. The results presented here provide evidence that *pap* and *pit* are translationally coupled, and this is necessary for functional Pi transport via Pap-Pit.

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List of Abbreviations and Symbols

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger RNA
P	Phosphorus
Pi	Inorganic Phosphate
Δ	Genetic deletion
Φ	Bacteriophage transduction
MFS	Major Facilitator Superfamily
a.a.	Amino acids
nt	Nucleotide
SD	Shine Dalgarno
RBS	Ribosome binding site

Declaration of Academic Achievement

I declare that the research contribution which follows is original work, completed and written by me, with editorial assistance from my supervisor Dr. Turlough M. Finan.

The following individuals are recognized for their contributions:

- In Chapter 2.5, "Previous work"
    - The deletion constructs of the *pap-pit* genes were generated by Dr. Rahat Zaheer and Hui Zhao.
    - The Pap missense mutations were originally constructed by Hui Zhao and Dr. Vahid Husseininaveh.
    - The background strain RmP3421 was constructed by Dr. George C. diCenzo as part of his publication entitled "PhoU allows rapid adaptation to high phosphate concentrations by modulating PstSCAB transport rate in *Sinorhizobium meliloti*" J Bacteriol JB-00143.
    - The RmP3926 and RmP3925 background strains used in this work were made by Daniel Hsieh by transducing the *Δpap-pit* and Δ*pit* deletions into RmP3421.

Chapter 1 - Introduction and Literature Review

1.1 Importance of Phosphorus and the Pho Regulon

Phosphorus, most commonly found as inorganic phosphate (Pi or PO_4^{3-}), is an essential element for life. It is required for various processes within the cell, such as the formation of DNA or ATP. However, Pi is commonly found in low concentrations in the environment and is often growth limiting for many organisms (Bieleski, 1973). Without a way to take up phosphate from the environment, the organism will not survive. Thus, species have adopted many different ways to acquire phosphate from the environment including using various phosphate transport systems (Hsieh and Wanner, 2010). These systems are often well conserved across species.

1.1.1 PhoR-PhoB and Regulation of Phosphate Transport

In bacteria, the phosphate (*pho*) regulon consists of genes whose expression is regulated by environmental phosphorus concentrations. In most bacterial species, many of the genes in the *pho* regulon are controlled by a two-component regulatory system, denoted as PhoR-PhoB in *Escherichia coli*, where PhoR is the histidine kinase sensor and PhoB is the response regulator (Makino *et al.*, 1989; Hsieh and Wanner, 2010). This two-component system allows the organism to sense and respond quickly to changes in environmental Pi concentrations. In low Pi conditions, PhoR is auto-phosphorylated resulting in the phosphorylation of PhoB (Makino *et al.*, 1989). PhoB is then able to bind to a specific DNA sequence known as the PHO box to activate or repress transcription of

genes involved in phosphate homeostasis (Yuan, Zaheer, Morton, *et al.*, 2006) . This allows the organism to respond by taking up Pi from the environment using a high affinity transport system under low phosphate conditions. In high Pi conditions, PhoB will not be phosphorylated and activation and repression of these phosphate homeostasis genes will not occur (Yuan, Zaheer, Morton, *et al.*, 2006). *Sinorhizobium meliloti* is a nitrogen-fixing symbiont that is able to form nodules on the roots of legumes, such as alfalfa and it has three different phosphate transport systems, PstSCAB, PhoCDET, and Pap-Pit (Cox *et al.*, 1989; Bardin *et al.*, 1996, 1998), that are regulated by the PhoR-PhoB two-component regulatory system.

1.1.2 PstSCAB Transport Systems

The phosphate specific transporter PstSCAB is a high affinity ABC (ATP Binding Cassette)-type transporter consisting of a substrate binding protein (PstS), two permease proteins (PstC and PstA) and an ATPase (PstB) (Cox *et al.*, 1989). Transcription of the *pstSCAB* genes is activated by PhoB under low Pi conditions and is one of the most conserved Pi transport systems across all species (Santos-Beneit, 2015). In *S. meliloti*, PstSCAB exists as operon *pstSCAB-phoUB* with *phoR* just upstream under a separate promoter. *phoR* and *phoB* form the two-component system discussed above and the function of the regulatory protein, PhoU, is unclear (Bardin *et al.*, 1996). It is thought that PhoU negatively regulates phosphate uptake via PstSCAB in response to increases in intracellular Pi concentrations (Steed and Wanner, 1993; diCenzo *et al.*, 2017). Deletion of

phoU results in constitutive phosphate uptake via PstSCAB and accumulation of polyphosphate, which, is lethal to the cell (Steed and Wanner, 1993; diCenzo *et al.*, 2017).

1.1.3 PhoCDET Transport System

PhoCDET is a high affinity ABC-type transporter of phosphonates but it can also transport Pi (Metcalf and Wanner, 1993; Bardin *et al.*, 1996; Voegele *et al.*, 1997). PhoB also activates *phoCDET* transcription under low Pi conditions (Yuan, Zaheer, Morton, *et al.*, 2006). PhoCDET of *S. meliloti* was previously known as *ndvF* (nodule development phenotype) since, in a *S. meliloti* 1021 background, mutations at this locus resulted in nodules containing few bacteria unable to fix nitrogen (Fix^- phenotype) (Charles *et al.*, 1991; Bardin *et al.*, 1996). PhoCDET or PhnCDET was identified as a phosphonate/phosphate transporter in *S. meliloti* and other species (Metcalf and Wanner, 1993; Voegele *et al.*, 1997). The Fix^- symbiotic phenotype was later found to be background specific as *S. meliloti* strain 1021 carries a frame-shift mutation in the *pstC* gene and correction of that mutation restores a Fix^+ phenotype to *phoCDET* mutants (Yuan, Zaheer, and Finan, 2006).

1.1.4 Inorganic Phosphate Transport (Pit) Systems

The final transporter, Pit (Pi transport), is a low affinity Pi transporter and is a part of the Major Facilitator Superfamily (MFS). MFS transporters couple transport to an electrochemical gradient using ions or solutes such as protons or sodium (Yan, 2015). Pit proteins are identified by the PHO4 domain (Pfam: PF01384) and contain an N-terminal

and a C-terminal PHO4 domain. Pit proteins have been studied in a variety of species including *E. coli*, *S. meliloti*, and eukaryotes such as *Saccharomyces cerevisiae*, and *Homo sapiens*. In humans, the Pit1 and Pit2 proteins have been studied due to their involvement in vascular calcification, and mineralization and osteoblast associated diseases (Jono *et al*., 2000, Bøttger & Pedersen, 2002).

Pit proteins can be functional on their own, as is the case with *E. coli*'s PitA (499a.a.). While not experimentally established (this work), some Pit proteins appear to require a Pit associated protein (Pap) in order to be functional, such as in *Bacteroides thetaiotaomicron* or *S. meliloti*. Interestingly, a phylogeny of PitA-like proteins and Pit proteins that are associated with Pap showed they form distinct clades (Hsieh, unpublished). PitA-like proteins are often larger in size than Pap-associated Pit proteins and have a conserved 100-120a.a. intracellular loop in the C-terminal PHO4 domain. This loop is not present in Pap-associated Pit proteins and may have some function that allows it to not require a Pap protein for Pi Transport. Among *pap-pit* orthologues, more than 95% appear as a co-transcribed operon, with *pap* upstream of *pit* in most cases (Hsieh, unpublished). Pit proteins in other species, such as the human Pit2, have also been shown to form homodimers (Bøttger and Pedersen, 2002; Yan, 2015).

1.2 Pap-Pit Transport Systems

1.2.1 *S. meliloti* Pap-Pit

S. meliloti strain Rm1021 *phoCDET* mutants form Fix^- nodules on alfalfa. However, it was observed that some plants inoculated with these mutants formed nitrogen fixing nodules and genetic analyses of bacteria from these Fix^+ nodules revealed that they contained second-site suppressor mutations and two classes of suppressor mutations (I and II) were identified (Oresnik *et al.*, 1994). Five independent suppressor mutations were designated *sfx-1* through *sfx-5* for suppressor of nitrogen fixation. It was found that *phoC* mutants grew poorly on minimal medium with inorganic Pi as the sole source of P and also *phoC* mutants had a decreased rate of phosphate uptake compared to wildtype. The *sfx-1* suppressor mutations restored wildtype growth and wildtype Pi transport to a *phoC* mutant (Bardin *et al.*, 1998). The ability of *sfx-1* to rescue the Pi-related and Fix-related phenotypes suggested that the suppressor mutation may be located in or near a gene involved in phosphate homeostasis and not in a gene directly related to nitrogen fixation.

The *sfx-1* locus was mapped to an approximately 2 kb region that was sequenced and analyzed (Bardin *et al.*, 1998). Two open reading frames were identified and designated *orfA* and *pit*. The *orfA* stop codon and *pit* start codon overlapped by a single nucleotide (Figure 1.1) (Bardin *et al.*, 1998). Pit was found to be similar in amino acid sequence to Pit proteins from other species, such as *E. coli* PitA, and thus was hypothesized to function as a phosphate transporter in *S. meliloti* (Bardin *et al.*, 1998). Sequencing of the region upstream of *orfA* in three independent class I suppressor mutant strains identified a deletion

of a single thymidine in a hepta-thymidine sequence in comparison to wildtype (Bardin *et al.*, 1998). Using transcriptional *lacZ* fusions to *orfA* and *pit*, it was found that the *sfx-1* mutation increased transcription of *orfA* by five times and *pit* by three times as much compared to wildtype (Bardin *et al.*, 1998). It was concluded that this increase in *orfA-pit* expression was responsible for the suppression of the *phoC* mutant phenotype to allow for sufficient growth and nitrogen fixation on alfalfa (Bardin *et al.*, 1998). *orfA* has since been renamed to *pap* for pit associated protein as it is thought to play a role in phosphate transport via Pit. Using similar methods, class II suppressor mutations were found to map to the phosphate regulatory genes *phoU* and *phoB* (Bardin and Finan, 1998).

Transcription of *pap-pit* (*orfA-pit*) was shown to be negatively regulated by PhoB in a phosphate dependent manner, and thus is repressed under low Pi conditions (Bardin and Finan, 1998). The identification of this third transporter in *S. meliloti* is of interest because it is thought to allow the organism to adapt and respond quickly to changes in the availability of Pi. As previously noted, transcription of the *pstSCAB* and *phoCDET* transport genes are activated by PhoB under low Pi conditions, while *pap-pit* transcription is repressed. Under high Pi conditions, *pstSCAB* and *phoCDET* transcription is not activated and *pap-pit* is not repressed by PhoB, so Pi uptake is thought to occur via Pap-Pit under conditions of excess Pi.

It has previously been shown that *pap* is required immediately upstream of *pit* for functional Pi transport via Pit (Hsieh, unpublished). Various recombinants were generated from recombination of pUCP30T plasmids with promoter-*pap-pit*, promoter-*pap*, promoter-*pit*, truncated *pap-pit* (no promoter), or promoter-*pap*-truncated *pit* into

RmP3924 (Δ*pap*) and RmP3925 (Δ*pit*) (Figures 1.2 and 1.3). When *pap* is present *in trans*, there is no Pi transport. Only when a wildtype *pap-pit* operon is restored upon recombination into the chromosome is growth and complementation observed. This suggested a possible translational coupling mechanism between Pap and Pit that is required for functional Pi transport.

1.2.2 *Bacteroides* and *Shewanella* Pap-Pit

When analyzing the protein structures of both PhoU and Pap, they appear to be similar. Two protein structures of Pap have been identified by X-ray diffraction from *Bacteroides thetaiotaomicron* (PDB: 3L39) and *Shewanella oneidensis* (PDB: 2OLT) (Figure 3). Pap resembles the structure of PhoU as both consist of 6 parallel α-helices (Liu *et al.*, 2005). *S. meliloti* Pap (214 aa) is similar in size to *Bacteroides* Pap (216 aa) and *Shewanella* Pap (226 aa). However, *Shewanella* Pit (424 aa) is larger than *Bacteroides* Pit (340 aa) and *Sinorhizobium* Pit (334 aa), possibly suggesting differences in the requirement of Pap for proper functioning of Pit. For comparison, *E. coli* PitA does not require a Pap protein for Pi transport, but it is 200 aa larger than *S. meliloti* Pit. The structural similarities between Pap and PhoU raise the question of whether their functions are also similar. For example, as proposed by diCenzo *et al*, in response to high intracellular Pi, PhoU senses an intracellular phosphate metabolite and inhibits or reduces Pi uptake via PstSCAB to maintain phosphate homeostasis within the cell in *S. meliloti* (diCenzo *et al.*, 2017). However, there is currently no evidence that this is the case for Pap.

1.3 Translational Coupling in Prokaryotic Operons

Translational coupling of a polycistronic operon refers to the requirement of the translation of the first gene in order to get translation of the following gene (Spanjaard and van Duin, 1989). Translational coupling involves both ribosome re-initiation and *de novo* initiation. Ribosome re-initiation occurs when translation of the upstream coding sequence is terminated and the ribosome dissociates, partially or completely, and then begins translation of the downstream gene (Spanjaard and van Duin, 1989). Ribosome *de novo* initiation involves the translation of the upstream gene that results in unfolding of the secondary structure of the mRNA that allows for a ribosome to initiate translation of the downstream gene (Oppenheim and Yanofsky, 1980). The efficiency of translation from a RBS can be affected by the strength of the Shine Dalgarno sequence and the secondary structure of the mRNA around the RBS (Spanjaard and van Duin, 1989). Some mutations can be polar, in which the transcription or translation of the downstream genes does not occur when there is a mutation in an upstream gene of an operon (Adhya and Gottesman, 1978). In some cases, this could be due to rho-dependent termination of transcription. This can occur when the RNA polymerase pauses at the site of the mutation and the rho factor catches up to it and causes the complex to terminate transcription prematurely so transcription of the rest of the operon will not occur. (Adhya and Gottesman, 1978).

Codon usage has been found to play an important role in the translation of bacterial proteins. Synonymous codon changes have been found to dramatically affect the production of some proteins (Nørholm *et al.*, 2012). It has been shown that these synonymous codon changes can influence mRNA stability and secondary structure, as well

as translation initiation and elongation, and protein folding (Nørholm *et al.*, 2012). Rare codon clusters have been identified in numerous genes, particularly in the 5' end of the mRNA and are thought to cause ribosomal pausing (Nørholm *et al.*, 2012). Regions of mRNA that are translated more slowly are often found downstream of protein domains and this is thought to allow for correct co-translation folding of the protein (Thanaraj and Argos, 1996). Membrane proteins in particular are found to have a 'U' bias, specifically associated with a higher number of hydrophobic amino acids and the hydrophilic amino acids serine and tyrosine (Prilusky and Bibi, 2009). The codons encoding these amino acids more often have a 'U' at the second position than you would expect by chance. Although the reason behind this 'U' bias is unclear, changing the codon to a synonymous one with fewer U's could prevent proper translation or protein folding (Prilusky and Bibi, 2009).

There are many examples of translational coupling previously described in the literature such as the DrrAB complex (an ABC transporter) that confers doxorubicin resistance in *Streptomyces peucetius* and also when cloned into *E. coli* (Pradhan *et al.*, 2009). Similar to *S. meliloti pap-pit*, the stop codon of *drrA* and start codon of *drrB* overlap (ATGA) and the genes are thought to be translationally coupled. A frameshift mutation in *drrA* resulted in almost no DrrB protein being produced, even when *drrA* was provided in trans on a plasmid (Pradhan *et al.*, 2009). Using RT-PCR, they were able to determine that transcription was not being terminated early in any of the constructs, which could result in no translation of *drrB* (i.e. by rho-dependent termination of transcription). They were also able to express *drrA* and *drrB in cis* with the insertion of various spacers between the two genes that maintained the RBS for *drrB*. However, none of the *in cis* constructs generated

in this work, aside from the wildtype *drrAB* sequence, conferred doxorubicin resistance. The authors proposed that *drrA* and *drrB* are translationally coupled and that this coupling is necessary to produce a functional protein complex (Pradhan *et al.*, 2009).

Co-translation of proteins has also been studied, which involves the coupling of translation with protein folding and complex assembly. For example, the bacterial luciferase complex is encoded as operon *luxCDABE* and includes the protein subunits LuxA and LuxB (Shieh *et al.*, 2015). The authors demonstrated that encoding *luxA* and *luxB* on distant chromosomal locations from each other was less efficient in the formation of LuxAB heterodimers than when they were encoded *in cis*. They also showed that LuxA interacts with nascent LuxB with the help of a chaperone as it is exiting the ribosome (Shieh *et al.*, 2015). The chaperone is proposed to prevent premature association of LuxAB and proper folding of LuxB. They propose that operon structure is important in bacteria as it helps to promote efficient, timely, non-stochastic protein complex assembly (Shieh *et al.*, 2015). Although we do not have evidence that *S. meliloti* Pap and Pit interact, if they did, translational coupling and co-translation could play an important role in ensuring a functional complex is formed.

1.4 Research Objectives and Hypothesis

The goal of this work is to further our understanding of the function of Pap in Pi transport via the Pit protein. Pap appears to be required immediately upstream of Pit for functional Pi transport and growth in minimal media (Hsieh, unpublished). Pap is hypothesized to be required for the translation of Pit because it is co-transcribed as a *pap-pit* operon in more than 95% of orthologues. Modulating the translation of Pit in *pap-pit* operons may be one function of Pap and may be required for functional Pi transport. A translational reporter to pit will be used to measure Pit expression, as we currently do not have a way to determine if Pit is being translated in the cell. This will help to determine if a lack of Pi transport or growth in *pap* mutants is due to Pit not being translated or if Pit is produced but is non-functional. Growth complementation experiments were also conducted in conditions in which growth should only occur if Pi transport via Pap-Pit is functional. Various constructs in which *pit* may or may not be translated were tested.

Chapter 2 - Materials and Methods

A list of strains and plasmids used in this work are outlined in Table 2.1.

2.1 Growth Media

2.1.1 LB Medium

LB was prepared using 1% (w/v) Bacto™ Tryptone, 0.5% (w/v) Bacto™ Yeast Extract, and 0.5% (w/v) NaCl dissolved in MilliQ water. LB agar was prepared with 1.5% (w/v) Difco agar. LB and LB agar were sterilized by autoclaving. LBmc was LB supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$.

Antibiotics were used as follows. For *E. coli*, gentamicin was supplied at 10 µg/mL, ampicillin at 100 µg/mL, and chloramphenicol at 20 µg/mL. For *S. meliloti*, streptomycin was supplied at 200 µg/mL, spectinomycin at 200 µg/mL, gentamycin at 60 µg/mL, and neomycin at 200 µg/mL. When required, isopropyl β-D-1-thiogalactopyranoside (IPTG) was included at a final concentration of 1 mM. *E. coli* was grown at 37°C and *S. meliloti* was grown at 30°C. Half the concentrations of antibiotics were used when growing cultures in liquid media.

2.1.2 MOPS-Buffered Medium

MOPS-buffered minimal medium was prepared using 40 mM MOPS, 20 mM KOH, 8.6 mM NaCl, 18.7 mM NH_4Cl, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.3 µg/mL biotin, 10 ng/mL $CoCl_2$ (stock concentration 0.01 mg/mL (1000×), 1 mL added per 1 L of media), 38

μM $FeCl_2$ (stock concentration 100 mM, 380 μL added per 1 L of media), 1 μg/mL thiamine-HCl. The first six ingredients were mixed together as a 10× stock, filter sterilized, and stored at -20°C. The remaining reagents were filter sterilized prior to adding to the media. Glucose was added at a concentration of 20 mM as a carbon source. Phosphate was added in the form of 2 mM K_2HPO_4.

2.2 Cloning Methods

2.2.1 Polymerase Chain Reaction (PCR)

Oligonucleotides for PCR were supplied by Integrated DNA Technologies Inc. (IDT; Coralville, Iowa, USA) and were resuspended in ddH_2O to 100 μM for storage at -20°C. A working stock concentration of 10μM was used. DNA was amplified using the 2× PCR Bestaq™ MasterMix (Applied Biological Materials, Vancouver, Canada) following the manufacturer's instructions unless otherwise noted. The final concentration of primers and dNTPs used in the PCR reaction was 0.4 μM and 20-50 ng of DNA template was used in the reaction. For colony PCR a single colony was resuspended in 20 μL of sterile ddH_2O and 1 μL of the mixture was used in the PCR reaction. Melting temperatures (T_m) were determined using the UC Berkeley Oligo Calculator (http://mcb.berkeley.edu/labs/krantz/tools/oligocalc.html). Some or all of the PCR reaction was run on a 0.8% Agarose gel at 100V for 1 hour. PCR products were purified using the EZ-10 Spin Column DNA Cleanup Miniprep Kit (Bio Basic, Toronto, Canada) or gel extracted using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany).

2.2.2 Overlap Extension PCR

Overlap Extension PCR was used to splice together two fragments or to introduce point mutations or small deletions into the sequence (Figure 2.2). Four primers were designed with similar melting temperatures with two of the primers carrying regions of homology to each other at the overlap site, typically about 20 nucleotides. This region of homology also contained any mutations that would be introduced. Two independent PCR reactions were carried out as described above to amplify the two desired fragments to be spliced. After the DNA was cleaned, equimolar amounts of DNA (0.1-1 pmol) were mixed together in a PCR reaction that lacked all primers and 15 cycles of the reaction were carried out. The annealing temperature of the reaction was calculated based on the region of overlap homology between the two DNA fragments. Finally, 5 µL of the PCR mix from the previous 15 cycle step was added to a 50 µL PCR reaction with the end primers to amplify the entire spliced together DNA fragment. This reaction proceeded as normal for 30 cycles using the annealing temperatures of the end primers. The DNA was run on a gel and then purified using one the kits described above.

2.2.3 Traditional Cloning

Plasmid DNA was extracted from *E. coli* DH5α overnight cultures using the EZ-10 Spin Column Plasmid DNA Miniprep Kit (Bio Basic, Toronto, Canada) as per the manufacturer's instructions. Elution buffer (2 mM Tris-HCl, pH 8) was used in most cases with ddH_2O being used to elute the DNA when the plasmid needed to be sequenced.

Plasmid DNA concentrations were determined by UV spectrophotometry at absorbances of 260/280 nm (BioTek^{Tm} Cytation 3 96-well plate Spectrophotometer).

Restriction enzymes for DNA digestion were obtained from New England Biolabs (NEB, Ipswich, Massachusetts, USA). Digestion reactions were designed according to the manufacturer's instruction with DNA not exceeding 30% of the reaction volume. The DNA was then cleaned using one of the DNA purification kits described above.

A 10 µL ligation reaction was prepared using a 1:3 or 1:4 vector:insert ratio with 100 ng of vector used and T4 DNA ligase from New England Biolabs (NEB, Ipswich, Massachusetts, USA). The ligation mixture contained 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, and 10 mM DTT. Ligation reactions were incubated at room temperature (~20^{o}C) for 20-30 minutes prior to adding the DNA to a tube of 200 µL chemically competent *E. coli* DH5α on ice. The DNA and competent cell mixture were incubated on ice for an additional 20-30 minutes. Subsequently the cells were heat shocked at 42^{o}C for 45 seconds, recovered on ice for 2 minutes, and 800 µL of LB was added to the tube. The cells were then spun at 37^{o}C for 1-2 hours before plating on selective media. One hundred microlitres of cells were plated on the selective media, while the remaining cells were spun down and the supernatant removed to leave ~100 µL of cells which were then resuspended and plated on the selective media. Plates were incubated overnight at 37^{o}C.

Several transformants were inoculated in LB with appropriate antibiotics and incubated overnight and the plasmid DNA was isolated using a DNA purification kit. Restriction digests of the plasmid and PCR were used to identify transformants carrying the correct plasmid. The purified plasmid was then sequenced by Sanger sequencing at the

McMaster MOBIX Sequencing Facility (McMaster University, Hamilton, Canada) to confirm the cloned fragment was correct.

2.3 Transduction and Conjugation

General transduction was performed as previously described (Finan *et al.*, 1984). An overnight culture of the donor was diluted in LBmc to an OD_{600nm} of 0.05 and grown to an OD_{600nm} of 0.4. At this point, 100 µL of RmP110 phage ϕM12 was added to the culture and grown overnight. Two hundred microlitres of chloroform was added to the culture to kill any remaining cells and the lysate was pipetted out and stored at 4°C. Five hundred microlitres of a 1 in 25 dilution of the lysate was mixed with 500 µL of the recipient culture and incubated at 30°C for 20 minutes. The 10^0 dilution was plated on selective media without calcium to prevent repeated phage infection.

Conjugations were conducted as previously described (Milunovic *et al.*, 2014). In brief, 1 mL each of culture of the *E. coli* strain carrying the plasmid that was to be conjugated, the helper strain MT616 (pRK600), and the recipient *S. meliloti* strain were spun down and resuspended in 0.85% NaCl. Twenty-five microlitres of each were mixed and the mixture was placed in the center of a LBmc plate and incubated at 30°C overnight. The following day the mating spot was resuspended in 0.85% NaCl and diluted to 10^{-7}. One hundred microlitres of the 10^0 to 10^{-3} dilutions were plated on selective media and the 10^{-5} to 10^{-7} dilutions were plated on media to select for the original recipient. Exconjugants were streak purified twice on selective media.

2.4 Detection of Pap using Polyacrylamide Gel Electrophoresis and Western Blot

2.4.1 *S. meliloti* Cell Lysis

Single colonies were inoculated in LBmc with the appropriate antibiotics and grown overnight. The cells were spun down and washed in 0.85% NaCl and diluted to an OD_{600nm} of 1 in 1 mL of 0.85% NaCl. The cells were then spun down again, and the supernatant was removed. The cells were resuspended in 75 µL 1× phosphate-buffered saline (PBS; 10 mM NaH_2PO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.15 mM phenylmethylsulfonyl fluoride (PMSF)) and mixed with 25 µL 4× protein loading dye (30 mM Tris-HCL pH 6.8, 8% SDS, 400 mM DTT, 20% glycerol, 0.005% bromophenol blue). The samples were boiled for 10 min at 95°C, vortexed for 15-30 seconds, and spun down at 16000×g for 15 minutes.

2.4.2 Polyacrylamide Gel Electrophoresis

For denaturing SDS polyacrylamide gel electrophoresis (SDS-PAGE), a 12% separating gel (Tris-HCl pH 8.8) and 4% stacking gel (Tris-HCl pH 6.8) were prepared using a 30% Acylamide/Bis solution (37.5:1) from Bio-Rad Laboratories Inc. The solidified gels were assembled in the Bio-Rad Mini-PROTEAN system and the tank was filled with 1× running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Ten microlitres of the supernatant from each sample were loaded into each well of the gel. The gel was run at 175 V for 45-60 minutes.

2.4.3 Western Blot of Pap

The gel was removed from the glass plates and equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol) for 20-60 minutes. A polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Ltd.) was soaked in 80% methanol for 1 minute followed by soaking in transfer buffer for 5-10 minutes. Two pieces of 5 mM blotting paper were also soaked in the transfer buffer for 5-10 minutes. One piece of blotting paper was placed onto the Trans-blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, Ltd.), followed by the PVDF membrane placed on top of the blotting paper, then the gel, and finally the second piece of blotting paper. The transfer apparatus was run at 10 V for 75 minutes to transfer the proteins from the gel to the PVDF membrane.

The membrane was soaked in blocking buffer (5% skim milk in ddH_20) for 2 hours shaking at room temperature. The membrane was then washed with 25 mL Tris-buffered saline with Tween-20 (TBST; 10 mM Tris-HCl pH 8, 15 mM NaCl, 0.5% Tween-20) for 10 minutes with shaking at room temperature. This wash step was performed three times in total. Next, the membrane was incubated with 150 mL primary rabbit-anti-Pap antibody solution (1:5000 dilution; 1.5% skim milk, 10 mM Tris-HCl pH 8, 15 mM NaCl, 0.5% Tween-20) overnight at 4^{o}C. The next day, the membrane was washed with 25 mL TBST three times as before. The membrane was then treated with 100 mL secondary goat-anti-rabbit (IgG-AP conjugate) antibody solution (1:3000 dilution; 1.5% skim milk, 10 mM Tris-HCl pH 8, 15 mM NaCl, 0.5% Tween-20) for 2 hours shaking at room temperature. The membrane was washed with TBST three times as before and then equilibrated for 5 minutes in 25 mL alkaline phosphatase buffer (100 mM Tris-HCl pH 9, 100 mM NaCl, 5

383 mM $MgCl_2$). The membrane was developed with 25 mL alkaline phosphatase colour
384 developing buffer (alkaline phosphatase buffer, 0.33 mg/mL nitro blue tetrazolium chloride
385 (NBT), 0.17 mg/mL 5-bromo-4-chloro-3-inoyl phosphate (BCIP)) in the dark until the
386 desired colour was reached. Ten millilitres of alkaline phosphatase stop solution (20 mM
387 Tris-HCl pH 8, 5 mM EDTA) was added to the membrane and poured off immediately.
388 The membrane was then soaked in 25 mL of the alkaline phosphatase stop solution for a
389 minute. The solution was poured off and the membrane was allowed to dry.
390
391 **2.5 Previous Work**
392
393 **2.5.1 Construction of a phosphate uptake mutant**
394 In this work we made use of a previously constructed *S. meliloti* RmP110 derivative
395 RmP3421 whose genotype is *lacI*$_q$, P_{lac}::*pstSCAB-phoUB,* P_{lac}::*phoR* and *phnC*::Sp^R
396 (diCenzo *et al.*, 2017). RmP3421 was used in various experiments to test the effects of
397 mutation or deletion of *pap-pit*. We note that expression of *pstSCAB-phoUB* and *phoR* in
398 RmP3421 is induced by including IPTG in the culture media. Thus, in a RmP3421 Δ*pap-*
399 *pit* mutant, growth requires the expression of the *pstSCAB* transport system to allow Pi
400 transport. To construct RmP3421 derivatives carrying various *pap-pit* mutant alleles (e.g.
401 RmP3421 Δ*pap-pit*), a phenylalanine auxotroph mutation (*phe-232*::Gm^R) was transduced
402 from RmG340 into RmP3421, resulting in RmP3923. Transductants were selected on M9-
403 glucose with 1 mM phenylalanine. The phenylalanine auxotrophy (*phe-232*::Gm^R) likely
404 results from a *pheA::*Gm^R insertion and *pheA* is approximately 40 kb downstream of *pap-*

pit. Unmarked deletions of *pap* (RmP1628), *pit* (RmP1629), or *pap-pit* (RmP3151), generated by Dr. Rahat Zaheer and Hui Zhao, could be transduced into RmP3923 by selecting for phenylalanine prototrophs on M9-glucose without phenylalanine but with IPTG to create RmP3924 (Δ*pap*), RmP3925 (Δ*pit*), and RmP3926 (Δ*pap-pit*). All three strains were sequenced using Sanger sequencing to confirm the deletions. These strains, generated by Daniel Hsieh, were used as the primary background for this work in studying the function of Pap-Pit. I used these background strains to integrate various *pap-pit* constructs to determine if they allowed for functional Pi uptake and growth in a conditionally Pi uptake deficient mutant.

2.5.2 Point mutations in *pap*

Several point mutations in *pap* were generated by Dr. Vahid Husseininaveh using overlap extension PCR (Figure 2.2). The resulting final products were cloned into pUCP30T via XbaI and HindIII. Transformants were selected for on LBGm[10] and screened by restriction digest and sequencing to confirm the point mutation was present. The resulting plasmids were conjugated into various Δ*pap-pit* backgrounds and their growth was tested in minimal media. I also utilized the same method to generate new point mutations in *pap* and to clone the *sfx1* promoter to drive transcription of *pap-pit*. The pap^{P75A} plasmid (pTH2906) also served as the basis for several cloning experiments.

2.5.3 Generating an alkaline phosphatase translational reporter to *pit*

The wildtype *pap-pit* sequence was amplified and cloned into pLE01 (pBluescript II SK (+), Amp^R, carrying a recombinant alkaline phosphatase-β-galactosidase fusion (*phoA*/*lacZ*α)) via PstI, creating a C-terminal translation fusion of *pit* with a 6 amino acid linker region to *phoA*/*lacZ* (Figure 2.3) (Islam *et al.*, 2010). Subsequently, 500 bp of sequence from downstream of *pit* in *S. meliloti* was cloned downstream of the *phoA*/*lacZ* fusion via HindIII. From this plasmid, the *pit~phoA*/*lacZ* fusion was subcloned into pTH2824 via StuI and BspEI, resulting in a *pap-pit~phoA*/*lacZ* fusion in pUCP30T (pTH3198). pTH3198, generated by Daniel Hsieh, was then sequenced by Sanger sequencing to confirm the translational fusion to *pit*. This plasmid was then conjugated into RmP3926 and integrated into the chromosome at the Δ*pap-pit* locus (Figure 2.4). Future *pap-pit~phoA/lacZ* translational fusions generated in this work were made from this plasmid.

2.6 Measuring the growth of *S. meliloti* phosphate uptake mutants

Growth experiments for *S. meliloti* were conducted in MOPS-buffered minimal medium. All growth experiments were conducted in the RmP3926 (Δ*pap-pit*) background. Single colonies were inoculated in 3 mL LBmc with appropriate antibiotics and 1 mM IPTG and grown overnight at 30°C. The IPTG induces the expression of the PstSCAB transport system to allow for growth in LBmc. The following day, the cells were harvested and washed twice in 1 mL 0.85% NaCl and resuspended in 1 mL 0.85% NaCl. The cells were subcultured into 5 mL MOPS-buffered media (without IPTG) to an OD_{600nm} of 0.1

and grown at 30°C for 16-18 hours. This subculturing step depletes the cells of the PstSCAB transport system, as well as PhoR. The cells were harvested and washed once in 1 mL of 0.85% NaCl and resuspended in 1 mL 0.85% NaCl. The OD_{600nm} of the cells was measured and they were diluted to an OD_{600nm} of 0.5 in 0.85% NaCl. Fifteen microlitres of cells were added to 135 µL of MOPS-buffered medium with and without IPTG in triplicate to a 96-well plate (Corning In., Durham, NC, USA), giving 6 wells total per sample. Fifteen microlitres of 0.85% NaCl was used in place of the cells as a blank for the OD_{600nm}. The lid was taped to the plate to prevent evaporation of the samples and the plate was placed in the BioTek^{Tm} Cytation 3 plate reader. The plate was incubated at 30°C for 24-48 hours with continuous double-orbital shaking (1 mm radius, 807 cycles per minute) and the OD_{600nm} was read every 15 minutes. The average OD_{600nm} of the 0.85% NaCl blank wells was subtracted from the OD_{600nm} of the sample wells. The average of the three replicates after subtracting the blank was plotted in excel as time versus the $\log(OD_{600nm})$. Most constructs were tested in at least two independent growth experiments to confirm the growth phenotypes.

2.7 Using a translational reporter to measure Pit translation

Two translational reporters, alkaline phosphatase (*phoA*/*lacZ*) and β-glucuronidase (*gusA*), were used in an attempt to measure the level of Pit translation in the cell. For both the alkaline phosphatase assay and the β-glucuronidase assay the following cell harvesting protocol was used. Single colonies were inoculated in LBmc with IPTG and antibiotics and grown overnight at 30°C. The cells were harvested, washed twice in 1 mL 0.85% NaCl,

and resuspended in 1 mL 0.85% NaCl. The cells were subcultured in MOPS-buffered medium without IPTG to an OD_{600nm} of 0.1 and grown at 30°C for 16-18 hours. This subculturing step was only required for strains in the RmP3926 background in order to deplete the expressed PstSCAB from the cells. The cells were harvested again and washed once with 1 mL of 0.85% NaCl and resuspended in 1 mL of 0.85% NaCl. In most cases, the cells did not need to be diluted prior to the assay.

For both assays, 20 µL of cells were added to six wells of a 96-well plate (Corning In., Durham, NC, USA). In addition, 0.85% NaCl was used in six of the wells as a blank. Eighty microlitres of the appropriate reaction buffer (described below) were added to three of the wells. The other three wells were the controls described in the respective sections below. One hundred microlitres of stop solution was added when a well had turned yellow and the time of the reaction was recorded (time from the addition of the reaction buffer to the addition of the stop solution). The plate was observed for up to two hours and stop solution was added to any of the wells that had not turned yellow prior to the two hours. The $OD_{410nm/405nm}$ and OD_{600nm} of the wells were read. The Miller Units (MU) of each sample were calculated using the formula $MU = (1000 * OD_{410nm/405nm}) / (OD_{600nm} * time)$, where the OD values are the corrected values after subtracting the appropriate controls and the time is the time in minutes between adding the start buffer to adding the stop buffer.

2.7.1 Cloning the *phoA*/*lacZ* translational fusion downstream of pap^{P75A}-*pit*

From the previously made plasmid pTH3198 (*pap-pit~phoA*/*lacZ*), the *pit~phoA*/*lacZ* fusion was digested out and subcloned into pTH2906 via AgeI and HindIII

restriction sites to create pTH3246. The 500 bp of downstream *pit* sequence was not included in the new plasmid.

2.7.2 Construction of the *gusA* translational fusion to *pit*

The *gusA* gene was amplified from pTH1522 using the primers GusA-F and GusA-R. The PCR fragment was purified, digested, and subcloned into pTH3198 via PstI and HindIII restriction sites. Transformants were selected for on LBGm[10]. This allowed for the replacement of the *phoA/lacZ* translational fusion with the *gusA* translational fusion, resulting in pTH3252 (Figure 2.3). A 7 amino acid linker region connected *pit* and *gusA*. pTH3253 (*pap*^{P75A}*-pit~gusA*) was also generated using the same method. Other constructs were subcloned into pTH3252 using various restriction sites in the *pap-pit* coding sequence or the promoter region (eg. AgeI, XhoI, XbaI) to generate additional *pit~gusA* translational fusions.

2.7.3 Detection of the *pit~phoA/lacZ* translational reporter in *S. meliloti*

An assay to detect the *phoA/lacZ* translational fusion to *pit* was performed following the cell harvesting protocol in section 2.7. The reaction buffer for this assay was the alkaline phosphatase start buffer (2 mM p-nitrophenyl phosphate (pNPP), 2 mM $MgCl_2$, 100 mM Tris-HCl pH 8). The stop buffer was 0.5 M NaOH. One hundred microlitres of stop solution was added to the three negative control wells of each sample. The samples were incubated at 30°C for up to two hours, initially checking the plate every few minutes. At the end of the two hours, 80 µL of alkaline phosphatase buffer was added to the negative control wells

and the OD_{600nm} and OD_{410nm} were read for each well. The OD_{600nm} and OD_{410nm} of the 0.85% NaCl blank was subtracted from the OD_{600nm} and OD_{410nm} of each of the samples and negative controls. The triplicate average alkaline phosphatase activity (Miller units) of the negative controls was subtracted from the activity of its respective test samples.

2.7.4 Detection of the *pit~gusA* translational reporter in *S. meliloti*

An assay to detect the *gusA* translational fusion to *pit* was performed following the cell harvesting protocol in section 2.7. The reaction buffer was the β-glucuronidase buffer (0.5 mg/mL 4-nitrophenyl β-D-glucopyranoside (PNPG), 50 mM sodium phosphate buffer pH 7, 1 mM EDTA pH 7, 50 mM DTT, 0.0125% SDS). Eighty microlitres of the β-glucuronidase buffer lacking the substrate PNPG was added to the three negative control wells (containing cells) of each sample. These wells did not change colour and act as a blank for the OD_{405nm}. The samples were incubated at room temperature for up to two hours, initially checking the plate every few minutes. The stop buffer was 1 M Na_2CO_3. At the end of the two hours, 100 µL of stop buffer was added to the negative control wells and the OD_{600nm} and OD_{405nm} were read for each well. The triplicate average OD_{410nm} and OD_{600nm} of the negative controls was subtracted from its respective test samples.

2.8 Modification of the *pap-pit* nucleotide sequence while maintaining the same protein sequence

The region between the XhoI restriction site of *pap* and the AgeI restriction site in *pit* was changed manually to maintain the native amino acid sequence while changing the

nucleotide sequence (Figure 2.5). The codon usage of each gene was also maintained. A double-stranded gene block was ordered from IDT (Coralville, Iowa, USA) containing this sequence with additional nucleotides on either end of the restriction site. The gene block was initially cloned into the pJET1.2/blunt vector with selection on LBAmp^{100}. This plasmid was purified, the gene block was cut out from the vector using the XhoI and AgeI restriction sites and cloned into pTH2824. Transformants were selected for on LBGm^{10}. Diagnostic restriction digest and sequencing confirmed the plasmid was correct. The plasmid was recombined into RmP3926 via single homologous recombination in the *pap* promoter region.

2.9 Cloning of the *sfx1* promoter upstream of *pap*

To create the plasmids carrying the *sfx1* promoter upstream of *S. meliloti pap-pit* with and without the alkaline phosphatase reporter, we used pTH276, which includes a 4.8 kb fragment of *pap-pit* with the *sfx1* promoter (Bardin *et al.*, 1998). The *sfx1* promoter was digested from plasmid pTH276 using a 2.5 kb BamHI and XhoI restriction fragment and cloned into pTH2824 to generate pTH3248, and into pTH2906 to generate pTH3249, and into pTH3198 to generate pTH3250, and into pTH3246 to generate pTH3251.

To create pTH3256 (*sfx1* (Δ*pap*)-*pit*), overlap extension PCR was used with pTH2340 ((Δ*pap*)-*pit*) serving as the template for the PCR. The first primer pair used was PromSfx1F and PromSfx1R and the second pair was PitSfx1F and PitSfx1R. PromSfx1R and PitSfx1F included a region of complementarity to each other which also contained the single 'T' deletion to generate the *sfx1* promoter. After the extension PCR, the PCR product

was digested and cloned into pTH2824 via XbaI and AgeI to generate *sfx1* (Δ*pap*)-*pit* in pUCP30T (pTH3256). Transformants were selected for on LBGm[10]. Diagnostic restriction digest with EcoRI and sequencing identified the correct clones.

In cloning the *sfx1* promoter upstream of *Bacteroides pap-pit*, overlap extension PCR was used to generate the single T deletion in the *pap* promoter region. One PCR product was amplified from pTH3187 using PromF and PromSfx1R and another PCR product was amplified from pTH3187 using PitSfx1F and Rev-BT. The single 'T' deletion of the *sfx1* promoter was included in the overlap between primers PromSfx1R and PitSfx1F. After the overlap extension PCR, the final product was digested and cloned into pUCP30T via EcoRI and BamHI. Transformants were selected for on LBGm[10]. Diagnostic restriction digest with EcoRV, which cuts inside the cloned fragment, and sequencing identified the correct clones.

2.10 Homologous recombination of *pap* mutants into a Δ*pit* strain

Several of the *pap* point mutants were conjugated into RmP3926 (Δ*pap-pit*) and RmP3925 (Δ*pit*). Colony PCR was performed using the primers Pit299F and PitSfx1R on the exconjugants. The resulting PCR product was purified using the Bio Basic PCR Cleanup Kit (Bio Basic, Toronto, Canada) and sequenced using one or both of the primers for the colony PCR. This was to confirm that the point mutation was upstream of *pit* and that the wildtype *pap-pit* had not been restored upon conjugation into RmP3925 (Figure 2.6).

579 **2.11 Modification of the *pap-pit* overlapping region and fusing of *pap* and *pit***

580 Overlap extension PCR was used to generate six different constructs in which the
581 nucleotide sequence between *pap* and *pit* was altered (Figure 2.7). The first PCR primer set
582 used Pap-Up with a construct specific 'reverse' primer and the second primer set used Pit-
583 Down with a construct specific 'forward' primer. The following construct specific primers
584 were used: pTH3335, StopRBS-F/R; pTH3336, NoStopNoSpaceF/R; pTH3337,
585 StopNoSpaceF/R; pTH3338, NoStopWSpaceF/R; pTH3339, NoStopNoSpaceNoMetF/R;
586 pTH3340, NoStopWSpaceNoMetF/R. Four constructs removed the stop codon of *pap*
587 resulting in the fusion of the *pap* protein sequence with *pit*. The other two constructs
588 maintained the *pap* stop codon. pTH3335 had a 13 nt insertion between the *pap* stop codon
589 and the *pit* start codon. This insertion consisted of the last 13 nt of the *pap* coding sequence
590 (including the stop codon) which also included the predicted RBS for *pit*. pTH3336 had no
591 *pap* stop codon and the last amino acid of *pap* was immediately upstream of the start codon
592 for *pit*. pTH3337 had an insertion of an 'A' immediately after the *pap* stop codon to remove
593 the one nucleotide overlap between the two genes. pTH3338 had the *pap* stop codon
594 removed and a 4 amino acid GGSG linker between *pap* and *pit*. pTH3339 had the *pap* stop
595 codon and *pit* start codon removed. pTH3340 had the *pap* stop codon and *pit* start codon
596 removed and connected again by the GGSG linker.
597
598 **2.12 Deletion of the *pap* start codon**
599 Overlap extension PCR was used to change the *pap* start codon to a leucine residue
600 (ATG→CTC; pap^{M1L}) to prevent translation of the Pap protein. This construct should still

allow for the transcription of *pap-pit* to mRNA. The first primer set used was Pap-Up and NoPapStartR and the second set used was Pit-Down and NoPapStartF. After extension PCR, the final product was digested and cloned into pUCP30T via XbaI and HindIII. Transformants were selected for on $LBGm^{10}Xgal^{40}$ and screened using diagnostic digestion and sequencing with M13-Rev. This plasmid was integrated into the chromosome of RmP3926 and RmP3925 to determine if translation of *pap* is required for *pit* function. A Western blot was also performed to confirm that no Pap protein was being produced in RmP3926.

Chapter 3 - Results

To determine the effects of mutations in the *pap* or *pit* gene on Pi-transport, we made use of a strain in which the *phoCDET* transporter was inactivated (*phoC*::ΩSp^r) and in which *pstSCAB-phoUphoB* and *phoR* were transcribed from P_{lac} promoters in an IPTG-dependent manner. In this background, deletion of *pap-pit* (Δ*pap-pit*) generated mutant cells that could only grow in media containing IPTG (i.e. transported Pi via the PstSCAB system). Moreover, in this background, recombination of *pap* or *pit* mutant constructs contained on the suicide vector pUCP30T into the Δ*pap-pit* locus allowed us to examine the effect of mutations on Pi transport using growth in a glucose minimal medium with 2 mM Pi (high Pi) as a proxy for Pi transport. As is evident below, recombination of the wildtype *pap-pit* genes resulted in growth similar to a wildtype strain. Recombination of constructs at the Δ*pap-pit* locus occurred via the *pap-pit* promoter region that is present in the Δ*pap-pit* locus and the introduced plasmid derivatives.

3.1 Overexpression of *S. meliloti pap-pit* using the *sfx1* promoter

Previous experiments demonstrated that the *sfx1* mutation (single thymidine deletion in the *pap-pit* promoter) increased transcription of the *pap-pit* operon (Bardin *et al.*, 1998). The *sfx1* promoter was used to drive expression of pap^{P75A} and Δ*pap* mutants in an attempt to restore wildtype-like Pi transport and growth of an *S. meliloti* Δ*pap-pit* strain.

3.1.1 The *sfx1* promoter restores wildtype growth of a *pap*^{P75A}*-pit* mutant

Previously, the *pap*^{P75A} mutation was shown to result in reduced Pi transport and growth in various *S. meliloti* backgrounds. It was proposed that increasing the transcription of *pap*^{P75A}*-pit* in *S. meliloti* may restore a wildtype-like level of growth by increasing the amount of Pap and Pit protein present in the cell. The *sfx1* promoter cloned upstream of *pap*^{P75A}*-pit* (pTH3249) was integrated into RmP3926 (Δ*pap-pit*). The *pap*^{P75A}*-pit* construct under the wildtype promoter (pTH2906) allowed for some growth in RmP3926 but it did not grow as well as wildtype (Figure 3.1). However, under the *sfx1* promoter, growth in MOPS-P2 was restored to a wildtype level by pTH3249 (Figure 3.2). This suggested that increasing transcription of *pap*^{P75A}*-pit* allowed for increased Pi transport resulting in better growth.

3.1.2 Expression of a Δ*pap* mutant under the *sfx1* promoter did not allow for growth

Increased transcription of *pit* in a Δ*pap* construct using the *sfx1* promoter was attempted to determine if excess Pit protein would allow for Pi transport and growth of an *S. meliloti* Δ*pap-pit* mutant in minimal medium in the absence of Pap. The *sfx1* promoter was cloned upstream of a Δ*pap* mutant to drive expression of *pit* (pTH3256). The growth of RmP3926 (Δ*pap-pit*) with pTH3256 was compared to RmP3926 with a Δ*pap* mutant under the wildtype *pap* promoter (pTH2340). Neither construct was able to restore the growth of RmP3926 in MOPS-P2 (Figure 3.3).

3.1.3 Expression of the *pap*^{P75A} mutant under the *sfx1* promoter results in increased Pap protein

The amount of Pap protein in the cell was detected by western blotting to determine if increasing the transcription of *pap*^{P75A}*-pit* using the *sfx1* promoter resulted in more Pap protein being produced. The total protein was isolated from *S. meliloti* RmP3926 with one of pTH2824 (*pap-pit*), pTH2906 (*pap*^{P75A}*-pit*), pTH3248 (*sfx1 pap-pit*), pTH3249 (*sfx1 pap*^{P75A}*-pit*), pTH3250 (*sfx1 pap-pit~phoA/lacZ*), pTH3251 (*sfx1 pap*^{P75A}*-pit~phoA/lacZ*). The Pap protein was detected using an anti-Pap primary antibody and an IgG alkaline phosphatase conjugate secondary antibody. A band for wildtype Pap was observed to be present at around 23 kDa (Figure 3.4). However, pTH2906 (*pap*^{P75A}*-pit*) showed only a very faint band at that size. Under the *sfx1* promoter, the wildtype Pap (pTH3248 and pTH3250) band appeared brighter. For pTH3249 (*sfx1 pap*^{P75A}*-pit*) and pTH3251 (*sfx1 pap*^{P75A}*-pit~phoA/lacZ*) under the *sfx1* promoter, a bright band similar in size to wildtype Pap was observed. This suggests that the transcription and/or translation of *pap* for both wildtype *pap* and *pap*^{P75A} increased when expressed from the *sfx1* promoter.

3.2 *S. meliloti* strains with translational fusions to *pit* did not show impaired growth

The growth of *S. meliloti* strains carrying either an alkaline phosphatase or β-glucuronidase translational reporter to *pit* was measured in minimal medium with excess phosphate (MOPS-P2), to determine if the translational reporter had an effect on Pi transport via Pit, resulting in differences in growth. Previous C-terminal translational

fusions to Pit (His10, Strep II, Rho1D4, FLAG) were also created but either inhibited growth or were unable to be detected (Hsieh, unpublished).

3.2.1 The alkaline phosphatase translational reporter to *pit* did not affect growth of *S. meliloti* wildtype and *pap*^{P75A}*-pit* mutant strains

Several *S. meliloti* strains with and without *pit~phoA*/*lacZ* translational reporters were tested to determine if the translational reporter to Pit had an effect on growth. RmP3926 (Δ*pap-pit*) with pTH3198 (*pap-pit~phoA*/*lacZ*) grew the same as RmP3926 with pTH2824 (*pap-pit*) (Figure 3.5). Similarly, RmP3926 with pTH3246 (*pap*^{P75A}*-pit~phoA*/*lacZ*) grew the same as RmP3926 with pTH2906 (*pap*^{P75A}*-pit*). Strains carrying the *sfx1* promoter instead of the wildtype promoter were also tested. RmP4192 (RmP3926 PhoX-) with one of pTH3248 (*sfx1 pap-pit*), pTH3249 (*sfx1 pap*^{P75A}*-pit*), pTH3250 (*sfx1 pap-pit~phoA*/*lacZ*), or pTH3251 (*sfx1 pap*^{P75A}*-pit~phoA*/*lacZ*) all grew the same as RmP4192 with wildtype *pap-pit* (pTH2824) (Figure 3.6). This suggested that the alkaline phosphatase translational fusion had no effect on the function of Pap-Pit in Pi transport as growth was the same regardless of whether or not the strain carried the *pit~phoA*/*lacZ* translational reporter.

3.2.2 The β-glucuronidase translational reporter to *pit* did not impair growth of *S. meliloti pap*^{P75A} and Δ*pap* mutants

As an alternate reporter and protein fusion construct to support the results with the alkaline phosphatase reporter, we investigated whether fusing GusA (β-glucuronidase) to

694 the C-terminus of Pit affected Pi-transport as measured by growth of RmP3926 (Δ*pap-pit*)
695 in glucose minimal medium with 2 mM Pi. Growth of RmP3926 carrying either pTH2824
696 (*pap-pit*) or pTH3252 (*pap-pit~gusA*) was found to occur similar to the wildtype strain
697 RmP110 and we therefore concluded that the Pit~GusA fusion was functional for Pi
698 transport (Figure 3.7). RmP3926 with pTH3253 (pap^{P75A}-*pit~gusA*) grew slightly better
699 than RmP3926 with pTH2906 (pap^{P75A}-*pit*). RmP3926 with pTH3295 ((Δ*pap*)-*pit~gusA*)
700 grew slightly better than RmP3926 with pTH3173 ((Δ*pap*)-*pit*) (Figure 3.8). The same was
701 also true for RmP3926 (Δ*pap-pit*) with pTH3256 (*sfx1* (Δ*pap*)-*pit~gusA*), which grew
702 slightly better than pTH3262 (*sfx1* (Δ*pap*)-*pit*). However, none of the four Δ*pap* strains
703 grew very well at all when compared to wildtype. RmP3926 with pTH3297 (*sfx1* pap^{P75A}-
704 *pit~gusA*) grew the same as RmP3926 with pTH3249 (*sfx1* pap^{P75A}-*pit*) which both grew
705 like wildtype (Figure 3.9). These results suggested that the β-glucuronidase translational
706 reporter may actually improve the Pi transport capabilities of Pit, resulting in slightly
707 improved growth.
708
709 **3.3 Using a translational reporter to measure Pit expression**
710 Initially an alkaline phosphatase reporter was used as a measure of *pit* translation.
711 However, the activity of the reporter was very low so small differences between strains
712 were hard to detect. The presence of other alkaline phosphatases in *S. meliloti* also provided
713 some background level of activity, even when *phoX* was deleted from RmP3926 (Δ*pap-pit*)
714 (RmP4192). In section 3.3.1 below, I present results obtained using a Pit~PhoA protein

fusion and in section 3.3.2 below, results employing an alternate Pit~GusA reporter are presented.

3.3.1 *S. meliloti pap*^{P75A} strains with an alkaline phosphatase reporter showed reduced Pit translation compared to wildtype

The level of Pit translation was measured in an *S. meliloti* Δ*pap-pit* mutant with wildtype *pap-pit* or *pap*^{P75A}*-pit* constructs integrated into the chromosome to determine if the *pap*^{P75A} mutation had an effect on the translation of Pit. Expression from the wildtype *pap* promoter and the *sfx1* promoter were tested. Four different constructs were tested in both a RmP3926 (Δ*pap-pit*, *phoX*$^{+}$) and RmP4192 (*phoX*$^{-}$) background: pTH2824 (*pap-pit*), pTH2906 (*pap*^{P75A}*-pit*), pTH3198 (*pap-pit~phoA*/*lacZ*), and pTH3246 (*pap*^{P75A}*-pit~phoA*/*lacZ*) (Figure 3.10). The level of alkaline phosphatase activity was then measured. Overall, the level of alkaline phosphatase activity in the RmP4192 (*phoX*$^{-}$) background was lower than in the RmP3926 (*phoX*$^{+}$) background, as expected (Figure 3.10-B). A small decrease in alkaline phosphatase activity was observed in *pap*^{P75A}*-pit~phoA*/*lacZ* compared to *pap-pit~phoA*/*lacZ* in the RmP4192 (*phoX*$^{-}$) background, suggesting less *pit* translation in the *pap*^{P75A} mutant (Figure 3.10-A). However, there were only slight differences between strains that had a *pit~phoA/lacZ* reporter and those that did not. The background level of alkaline phosphatase activity of RmP3926 and RmP4192 was also rather high. Some strains that had a *pit~phoA*/*lacZ* translational fusion even had a lower level of alkaline phosphatase activity compared to those that did not have a *pit~phoA*/*lacZ* translational fusion.

In the RmP4192 (Δ*pap-pit*, *phoX*$^{-}$) background, strains carrying a *sfx1* promoter and a *pit~phoA*/*lacZ* translational fusion (pTH3250 (*sfx1 pap-pit~phoA*/*lacZ*) and pTH3251 (*sfx1 pap*^{P75A}*-pit~phoA/lacZ*)) had a higher level of alkaline phosphatase activity than those that did not have a *pit~phoA*/*lacZ* translational fusion (pTH3248 (*sfx1 pap-pit*) and pTH3249 (*sfx1 pap*^{P75A}*-pit*)) (Figure 3.11). Due to the high background levels of alkaline phosphatase activity in *S. meliloti* and the low overall activity of the alkaline phosphatase reporter to Pit, we decided to test another translational reporter to confirm these results and the findings are presented below.

3.3.2 S. *meliloti* Δ*pap* and *pap*^{P75A} strains with a β-glucuronidase reporter showed reduced Pit translation compared to wildtype

S. meliloti RmP3926 (Δ*pap-pit*) with plasmids carrying a β-glucuronidase translational reporter in place of the alkaline phosphatase reporter were tested to see if the *pit~gusA* reporter would more reliably report on the level of Pit translation in various *pap* mutants. RmP3926 with plasmids that did not contain a *pit~gusA* translational fusion showed very low levels of β-glucuronidase activity, typically less than 2 MU (Miller Units). RmP3926 with pTH3252 (*pap-pit~gusA*) had an activity that was much higher, typically around 110 MU (Figure 3.12). In comparison, RmP3926 (Δ*pap-pit*) with pTH3253 (*pap*^{P75A}*-pit~gusA*) had an activity of approximately one third of pTH3252 at approximately 40MU. RmP3926 with pTH3295 ((Δ*pap*)-*pit~gusA*) had an activity that was only slightly above background levels at around 6 MU (Figure 3.13). RmP3926 with pTH3262 (*sfx1* (Δ*pap*)-*pit~gusA*) had an activity that was higher than pTH3295 at

approximately 20 MU but still only one fifth that of wildtype. These values suggested that deletion of *pap* resulted in only a low level of pit translation.

Strains carrying the *sfx1* promoter had an overall higher activity level than those under the wildtype *pap* promoter. RmP3926 (Δ*pap-pit*) with pTH3296 (*sfx1 pap-pit~gusA*) or pTH3297 (*sfx1 pap^{P75A}-pit~gusA*) had a much higher level of β-glucuronidase activity than pTH3252 (*pap-pit~gusA*) at approximately 330 MU (Figure 3.14). This result suggested that transcription and translation of *pap-pit* were increased by the *sfx1* promoter.

3.4 Modification of the *pap-pit* nucleotide sequence, while maintaining the amino acid sequence, resulted in reduced growth of *S. meliloti*

To determine if the mRNA sequence has an effect on the formation of a functional Pi transporter, a modified nucleotide sequence of *pap-pit* was constructed and the growth of an *S. meliloti* Δ*pap-pit* strain was measured. An approximately 900 bp fragment of *pap-pit* was modified to change the nucleotide sequence but maintain the same amino acid sequence. Codon usage within each gene was also maintained when designing the new sequence. The engineered sequence was subcloned into pTH2824 to create pTH3325 and the ability to allow RmP3926 (Δ*pap-pit*) to grow was tested. Integration of pTH3325 (modified *pap-pit*) into RmP3926 resulted in no growth in MOPS-P2 (Figure 3.15). This suggested that the nucleotide sequence of *pap* may play an important role in the translation and function of Pit.

After testing pTH3325 in RmP3926 (Δ*pap-pit*) it was noticed that the native RBS for *pit* had been changed by a single nucleotide. In order to determine if this had an effect

on the growth of RmP3926 with pTH3325 the RBS was changed backed to its native sequence. This resulted in the RBS being changed from GAAAA in pTH3325 to GAGAA in pTH3334 and this new plasmid was integrated into RmP3926. Some growth was restored by pTH3334 in RmP3926, but it still did not grow like wildtype (Figure 3.15). This suggested that changing the predicted RBS for *pit* was responsible for some of the observed decreased growth.

Detection of Pap by western blot in both the modified *pap-pit* strain (pTH3325) and the strain with the modified sequence and native RBS (pTH3334) showed the appropriate band for Pap at approximately 23 kDa (Figure 3.16). However, the band for pTH3325 was darker than wildtype Pap and the band for pTH3334 was lighter than wildtype. This may suggest that the stability of the Pap protein or the rate of translation of *pap* was affected in these strains. This result may also help to address the differences in growth between the sequence-modified *pap-pit* constructs and wildtype.

3.5 Growth of *S. meliloti pap* mutants with wildtype *pap* under a separate promoter

Plasmids carrying point mutations in *pap* were integrated into RmP3926 (Δ*pap-pit*) and RmP3925 (Δ*pit*) to determine if a wildtype Pap (present in RmP3925) could complement a *pap-pit* construct carrying a point mutation in *pap*. Integration into RmP3925 resulted in two possibilities: one in which a wildtype *pap-pit* operon is restored and another where the mutant *pap* is in an operon with *pit* with wildtype *pap* under a separate promoter. Colony PCR amplification of this region using Pit299F and PitSfx1R followed by Sanger

sequencing confirmed which *pap* was upstream of *pit* in the RmP3925 integrants. Growth of the integrants in both RmP3926 and RmP3925 was measured.

When pTH2906 (*pap*^{P75A}*-pit*) was integrated into RmP3925, growth in MOPS-P2 was observed when either wildtype *pap* or *pap*^{P75A} was upstream of *pit* (Figure 3.17). This suggested that the wildtype *pap* did not need to be present in the operon with *pit* for wildtype Pi transport if the mutant *pap* still allowed for the transcription and translation of *pit*. Alternatively, when pTH3044 (*pap*^{P75fs}*-pit*) was integrated into RmP3925 complementation was not observed when the *pap* carrying the frameshift mutation was upstream of *pit* (Figure 3.18). The frameshift mutation results in a premature stop codon being introduced into *pap* resulting in a 157 amino acid protein. This suggests that *pit* is not being translated in this construct so the wildtype Pap cannot complement due to the lack of Pit protein produced.

Detection of *pap* by western blot of pTH3044 in both RmP3926 and RmP3925 was performed. In RmP3926, no band for Pap (23kDa) was observed (Figure 3.16). In RmP3925, a band that was fainter than wildtype Pap was observed. This indicates that no wildtype Pap is present in the RmP3926 strain as expected due to the frameshift mutation resulting in a truncated Pap protein. In RmP3925, the band for Pap is likely coming from the wildtype under the separate promoter downstream of *pap*^{P75fs}*-pit*.

3.6 Modification of the *pap-pit* overlapping region and fusion of the two genes resulted in reduced growth in *S. meliloti*

Six constructs were developed using overlap extension PCR which changed the overlap between the *pap* and *pit* genes to determine if this would affect the Pi transport and growth of an *S. meliloti* Δ*pap-pit* mutant upon recombination of the constructs into the chromosome. Four constructs fused the two genes into a single protein by removing the *pap* stop codon to determine if a Pap-Pit protein fusion would allow for functional Pi transport and growth. These constructs were cloned into pUCP30T which was then recombined into RmP3926 at the Δ*pap-pit* locus. Growth was observed for RmP3926 with the two constructs containing a *pap* stop codon (pTH3335 and pTH3337) (Figure 3.19). In the remaining four constructs in which the two genes were fused to create one protein, no growth was observed (pTH3336, pTH3338, pTH3339, and pTH3340). For pTH3337 where the *pap* stop codon and *pit* start codon no longer overlap (one nucleotide shift to give TGAATG), growth similar to RmP3926 with wildtype *pap-pit* was observed. For pTH3335 where there was a 13 nt insertion between the *pap* stop codon and the *pit* start codon generated by repeating the last 13 nt of *pap*, some growth was observed but it was less than with wildtype *pap-pit*. Detection of Pap by western blot only showed a band for pTH3335 and pTH3337 and not for pTH3336, pTH3338, pTH3339, or pTH3340 (Figure 3.20). This confirms that the Pap and Pit proteins were likely fused by the removal of the *pap* stop codon in those strains that did not give a band at 23 kDa.

3.7 Removal of the *pap* start codon resulted in no Pap protein and reduced Pit translation

Overlap extension PCR was used to change the *pap* start codon to a leucine residue (*pap*^{M1L}) to stop translation of the Pap protein. Recombination of *pap*^{M1L}*-pit* into a Δ*pit* mutant was performed in an attempt to complement the mutation in *pap* and restore Pi transport and growth. Recombination of this plasmid (pTH3342) into RmP3926 (Δ*pap-pit*) resulted in no growth in MOPS-P2 (Figure 3.21). Recombination into RmP3925 (Δ*pit*) resulted in two growth phenotypes in MOPS-P2. Two of the three colonies tested showed no growth, while the third showed wildtype growth. This is likely due to the recombination event in which a wildtype *pap-pit* operon is restored in the colony that grew well. In the other two colonies that grew poorly, a *pap*^{M1L}*-pit* operon is likely present with wildtype *pap* downstream. In this scenario, the wildtype *pap* does not appear to allow growth of the *pap*^{M1L} mutant, and this could be due to a lack of translational coupling between *pap* and *pit*. Detection of Pap by Western blot of pTH3342 in RmP3926 resulted in no band at the appropriate size (23kDa) (Figure 3.16). However, pTH3342 in RmP3925 gave a band similar to wildtype Pap. This is likely due to translation of the wildtype Pap under the separate promoter downstream of *pap*^{M1L}*-pit*.

3.8 *Bacteroides pap-pit* allowed for growth of *S. meliloti* Δ*pap-pit* only when expressed from the *sfx1* promoter

Heterologous expression of *Bacteroides pap-pit* was performed to determine if this orthologue could allow for Pi transport and growth in an *S. meliloti* Δ*pap-pit* mutant. The

crystal structure of *Bacteroides* Pap has been determined and this could help to provide evidence that *S. meliloti* Pap and *Bacteroides* Pap have the same structure and function. *Bacteroides pap-pit* was cloned into pUCP30T for recombination into the *S. meliloti* Δ*pap-pit* site in RmP3926 to test for the ability of *Bacteroides pap-pit* to transport Pi in this background. Both the wildtype and *sfx1 S. meliloti* promoters were used to drive the expression of *Bacteroides pap-pit*. Under the wildtype *S. meliloti* promoter, no growth of RmP3926 with *Bacteroides pap-pit* (pTH3187) was observed (Figure 3.22). However, with *Bacteroides pap-pit* under the *sfx1* promoter (pTH3266), three different growth phenotypes were observed for independent colonies isolated from the same mating. The colonies either grew poorly (4 colonies), moderately (4 colonies), or like wildtype *S. meliloti pap-pit* (2 colonies).

To address which is the correct phenotype two colonies from each phenotype group had the *pap-pit* region amplified by colony PCR using a primer upstream of *pap* (PromF) and a primer at the end of *pit* (BtPitR). This PCR product was then purified and sequenced using the same primers used for the PCR. All six colonies sequenced were confirmed to have the *sfx1* promoter upstream of *Bacteroides pap-pit*. No obvious mutations were observed in the sequences of these colonies. A transduction of the *pit*::Tn5 transposon from RmP635 into the 10 colonies of RmP3926 with pTH3266 integrated was also performed to confirm that the plasmid had integrated into the Δ*pap-pit* site in RmP3926. This should result in the strain gaining neomycin resistance and losing the gentamicin resistance if the plasmid integrated correctly (gentamicin and neomycin resistance markers are linked). Interestingly, the colonies that grew poorly showed no linkage between the neomycin and

gentamicin resistance (i.e. they maintained both resistance markers). This could suggest that the plasmid integrated elsewhere in the *S. meliloti* genome, possibly due to some homology between the *Bacteroides pap-pit* and the genome and that the *Bacteroides pap-pit* is not functional in that location. This leaves either the moderate growth phenotype or the wildtype-like growth as the correct phenotype.

Attempts to determine whether those colonies that grew like wildtype were double integrants (carrying two copies of the plasmid instead of the intended one) via PCR were unsuccessful. Transduction from the recombinants into a fresh RmP3926 background gave the same growth phenotypes, making second site mutations in the genome not likely responsible for the differences in growth. A second mating was performed, and 10 more colonies were tested for their growth phenotype. From this mating, no colonies showed the wildtype-like growth, but instead only the moderate growth or poor growth phenotypes. Those with poor growth again showed that the gentamicin and neomycin resistance markers were not linked upon transduction of the *pit*::Tn5 transposon. This result suggests that the moderate growth phenotype is probably more likely to be the correct phenotype as it was also the most common among the 20 colonies tested. This suggests that increasing the transcription of *Bacteroides pap-pit* using the *S. meliloti sfx1* promoter allows for some Pi transport and growth.

Chapter 4 - Discussion

In this work, I employed growth experiments, translational reporter protein fusions to Pit, and detection of Pap protein by Western blot to investigate the role of *pap-pit* in Pi transport via Pit. The overall goal was to gain insight into the function of Pap in Pi transport via Pit.

4.1 Mutant *pap* decreases Pit Translation

Alkaline phosphatase (*phoA*/*lacZ*) and β-glucuronidase (*gusA*) were fused in frame to the C-terminus of Pit in order to detect expression of the Pit protein (Figure 2.3). We currently do not have an alternate way to determine if *pit* is being translated in the cell (other than Pi uptake or growth) as attempts to detect Pit using C-terminal protein tags have been unsuccessful. These methods include other tags such as a 10× His tag, Rho1D4 tag, FLAG tag, Strep II tag and we do not have an antibody to Pit (Hsieh, unpublished). The FLAG tag impaired the function of Pit and was unable to grow in minimal medium with Pi as the only phosphorus source (Hsieh, unpublished). The other tags did not impair the growth of the strains but went undetected after several attempts (Hsieh, unpublished). The *S. meliloti pap-pit* genes are transcribed at a low level in the cell, making detection of Pit even more difficult.

The alkaline phosphatase translational reporter to *pit* gave unreliable results when tested in the RmP3926 (Δ*pap-pit*) background (Figure 3.10). This was likely due to the presence of several alkaline phosphatase-like proteins in the *S. meliloti* genome (Zaheer *et al.*, 2009). To improve this data, the most prominent alkaline phosphatase, PhoX, was

eliminated from RmP3926 by transferring a *phoX*::Tn5 insertion mutation into RmP3926 to generate RmP4192. The RmP4192 Δ*pap-pit phoX*- background improved the quality of the data by reducing the levels of background activity, but the alkaline phosphatase values were very low overall, even for those with a *pit~phoA/lacZ* reporter (all under 10 MU) (Figure 3.10). This is likely because *pap-pit* is expressed at low levels in the cell (Bardin *et al.*, 1998). However, in the RmP4192 background, pap^{P75A}*-pit~phoA/lacZ* showed a reduced AP activity in comparison to wildtype *pap-pit~phoA/lacZ*. This result suggests that the pap^{P75A} mutation decreases the transcription or translation of *pit* but should be taken with caution due to the lack of reliability of the *pit~phoA/lacZ* reporter.

Similar results were obtained using the *pit~gusA* translational reporter. Detection with the β-glucuronidase (*gusA* from *E. coli*) reporter gave much lower background levels of activity (less than 2 MU) relative to the activity levels detected in cells carrying a *pit~gusA* reporter. The low background for β-glucuronidase is consistent with the absence of an annotated β-glucuronidase gene in *S. meliloti*. RmP3926 (Δ*pap-pit*) with pap^{P75A}*-pit~gusA* showed reduced levels of β-glucuronidase activity in comparison to *pap-pit~gusA*, indicating reduced Pit protein in the cell (Figure 3.12). RmP3926 with (Δ*pap*)-*pit~gusA* showed almost no activity in comparison to *pap-pit~gusA*, suggesting that no or very little Pit is present in this cell (Figure 3.13). Combined, these results show that mutating *pap* results in a reduction in Pit protein in the cell and suggests that in the absence of *pap* there is little *pit* transcription or translation.

These results may indicate that maintenance of protein stoichiometry could be a possible role of Pap. Protein stoichiometry is the ratio of one protein to another. For

example, one Pit protein could be produced for every one Pap protein. This has been proposed for the *nasST* operon in *Azotobacter vinelandii* which is involved in nitrate sensing and regulation of gene expression of nitrate reductase operons (Wang *et al.*, 2014). Translational coupling between *nasS* and *nasT* ensures stoichiometric amounts of NasS and NasT are produced (Wang *et al.*, 2014). This prevents the activation of translation of the nitrate reductase operons (Wang *et al.*, 2014). Pap could play a role in sensing intracellular Pi concentrations and interact with Pit to allow Pi uptake to occur. In this scenario, proportional amounts of each protein could be important for this regulation of Pi uptake to occur. Future experiments could be directed at identifying if Pap interacts with any phosphate intermediates within the cell. There is also some evidence that Pap may interact with another protein, or possibly form a homo-oligomer (Hsieh, unpublished), similar to what has been observed for PhoU (diCenzo *et al.*, 2017).

4.2 Overexpression of *pap*^{P75A}*- pit* using the *sfx1* promoter allows for increased Pit translation and growth

The *sfx1* mutation was identified in suppressor mutants isolated from the root nodules of plants inoculated with Rm1021 *phoCDET* mutants (Bardin *et al.*, 1998). Rm1021 also has a frameshift mutation in the *pstC* gene involved in Pi transport via the PstSCAB transporter. These mutations rendered both the PhoCDET and PstSCAB both non-functional in Pi transport. However, the *sfx1* suppressor mutation increased the transcription of *pap-pit* in these cells to a level that allowed for nitrogen fixation and Pi uptake via Pap-Pit to occur (Bardin *et al.*, 1998). Using a *lacZ* transcriptional reporter, the

971 *sfx1* promoter was shown to increase the transcription of *S. meliloti pap* by five times and
972 *pit* by three times (Bardin *et al.*, 1998).

973 In this work, the *sfx1* promoter was cloned upstream of *S. meliloti* pap^{P75A}*-pit* in an
974 attempt to increase transcription. In the RmP3926 (Δ*pap-pit*) background (and RmP4192,
975 Δ*pap-pit phoX*::Tn5), the pap^{P75A}*-pit* construct showed a reduced rate of growth in MOPS-
976 P2 when expressed from the wildtype *S. meliloti pap* promoter (Figure 3.1). However, when
977 pap^{P75A}*-pit* was expressed from the *sfx1* promoter, growth in MOPS-P2 was similar to that
978 of the wildtype RmP110 (Figure 3.2). Expression of a (Δ*pap*)-*pit* construct under wildtype
979 and *sfx1* promoters did not allow growth in MOPS-P2 media (Figure 3.3). This result
980 suggests that the increased transcription by the *sfx1* promoter allows increased Pi uptake
981 via Pit even with a mutated Pap (pap^{P75A}). Presumably the Pap^{P75A} mutant protein retains a
982 low level of activity and increased levels of Pap^{P75A} protein yields sufficient active protein
983 to allow enough Pi uptake to support wildtype-like growth. Overexpression of Pit by itself
984 in a Δ*pap* mutant did not allow sufficient Pi uptake for wildtype growth but whether Pit
985 protein was translated from the (Δ*pap*)-*pit* construct is unclear.

986 A western blot of pap^{P75A}*-pit* under the wildtype and *sfx1* promoters revealed that
987 increased Pap protein was present in the cells where pap^{P75A}*-pit* was expressed from the
988 *sfx1* promoter (Figure 3.4). This result suggests that both transcription and translation of
989 *pap* is increased by the *sfx1* promoter. However, as we do not have antibody to Pit, we were
990 unable to detect Pit via western blot. Both an alkaline phosphatase reporter (*phoA/lacZ*)
991 and a β-glucuronidase (*gusA*) translational reporter were used in an attempt to measure the
992 amount of Pit in the cell. Both reporters suggested that the level of Pit translation in the

993 *pap*^{P75A}*-pit* mutant under the *sfx1* promoter was similar to *pap-pit* under the wildtype
994 promoter (Figures 3.11 and 3.14). This result supports the observation that both of these
995 strains grow similarly in MOPS-P2.
996
997 **4.3 Mutant Pap can be complemented by wildtype Pap under a separate promoter**
998 It was previously shown that when *pap* and *pit* are expressed under separate
999 promoters in a *phoCDET* mutant with *pstSCAB* under an IPTG inducible promoter, no
1000 growth in minimal medium with Pi as the only source of phosphorus is observed (Hsieh,
1001 unpublished). It was hypothesized that wildtype Pap may be able to complement a *pap-pit*
1002 operon where *pap* is mutated in various ways. Upon recombination of *pap*^{P75A}*-pit* into
1003 RmP3925 (Δ*pit*), growth similar to wildtype *pap-pit* was observed for all the colonies tested
1004 (Figure 3.17). Two single cross-over homologous recombinations between a non-
1005 replicating plasmid carrying *pap*^{P75A}*-pit* and the chromosomal *pap*-Δ*pit* region could occur.
1006 One recombinant could carry the *pap*^{P75A}*-pit* operon with wildtype *pap* downstream and
1007 the other one would have a wildtype *pap-pit* operon with *pap*^{P75A} downstream (Figure 2.6).
1008 The recombinant colonies were sequenced to determine which orientation they had.
1009 Wildtype growth was observed for both recombinants. This result indicated that the
1010 wildtype *pap* downstream was able to allow enough Pi uptake via Pit to give wildtype
1011 growth and that they did not have to be expressed in the same operon to be functional. The
1012 *pap*^{P75A} mutation would likely not have polar effects on the transcription or translation of
1013 *pit*. However, detection of Pit with a *gusA* translational reporter showed a reduced level of
1014 Pit translation in the *pap*^{P75A}*-pit* mutant (Figure 3.12), indicating some possible polar or

other regulatory effects on *pit* expression. We know that some Pit must be produced in this construct as Pi uptake levels have been measured previously (Hsieh, unpublished) and that this construct was able to grow moderately well in the absence of a wildtype Pap (Figure 3.1).

A *pap* mutant was generated where the start codon was changed to a leucine residue (*pap*^{M1L}*-pit*) and this construct was conjugated into RmP3925 (Δ*pit*), resulting in similar recombinants as above with *pap*^{P75A}*-pit* (Figure 3.21). In these recombinants, only those with the wildtype *pap-pit* operon restored were able to grow in minimal medium. Colonies with a *pap*^{M1L}*-pit* operon were not able to grow, suggesting that Pit was not being made in these cells. No Pap was detected by western blotting when this construct was recombined into RmP3926 (Δ*pap-pit*), confirming the requirement of the ATG codon for Pap translation (Figure 3.16). Pap at similar levels to the wildtype was detected when *pap*^{M1L}*-pit* was recombined into RmP3925 (Δ*pit*), indicating that wildtype Pap was present in this construct. In the RmP3925 recombinants with a *pap*^{M1L}*-pit* operon, if *pap* and *pit* were translationally coupled, then the translation of *pit* would not occur because *pap*^{M1L} was not translated. Alternatively, transcription of the *pap*^{M1L}*-pit* construct maybe terminated in *pap* via a rho-dependent termination system and thus, no *pit* transcription would occur.

Another *pap* mutant construct, *pap*^{P75fs}*-pit*, was also conjugated into RmP3925 (Δ*pit*) to give similar recombinants as in the previous two examples (Figure 3.18). In this case, only those with a wildtype *pap-pit* operon restored were able to grow in MOPS-P2. The *pap*^{P75fs} mutation results in the translation of a truncated Pap protein (158 a.a.), leaving a 171 bp gap until the start of the *pit* sequence. This result is similar to the one observed

for the *drrAB* operon that confers doxorubicin resistance in *Streptomyces peucetius* (Pradhan *et al.*, 2009). A frameshift mutation in *drrA* resulted in no DrrB production, even when *drrA* was supplied in trans on a plasmid (Pradhan *et al.*, 2009). In our system, pap^{P75fs}-*pit* could not be complemented by wildtype *pap* in trans and this could be due to premature termination of transcription of pap^{P75fs}-*pit* by rho, or by the requirement of translational coupling between *pap* and *pit* to unwind the mRNA and expose the SD sequence of *pit*.

Looking at these three constructs, it appears as though the translation of Pap is required for the translation of a functional Pit. However, it appears that a mutant *pap* that still allows for the transcription and translation of *pit* is able to be complemented by a wildtype Pap. Only the first construct where you would expect translation to occur normally through the entire *pap* gives functional Pi uptake via Pit. The other two constructs in which translation of *pap* likely does not occur at all, or is terminated by a premature stop codon, do not appear to give functional Pi uptake via Pit, even in the presence of a wildtype Pap. This could be due to polar effects on the transcription or translation of *pit*. In previous work, when *pap* and *pit* were expressed from separate promoters in the chromosome, I would hypothesize that Pit is not being translated (or maybe even transcribed) and that is why functional Pi uptake was not observed (Figure 1.3) (Hsieh, unpublished). Pit may also be unstable in the absence of Pap and may not be able to be transcribed or translated on its own. If the function of Pap was to act as a molecular chaperone for Pit, then Pit would fold incorrectly and may form protein aggregates (Baneyx and Mujacic, 2004).

4.4 Partial complementation by *Bacteroides pap-pit* in *S. meliloti* under the *sfx1* promoter

Bacteroides pap-pit was expressed from the *S. meliloti* chromosome via the *S. meliloti pap* promoter. Expression under the wildtype promoter resulted in no growth in MOPS-P2. However, expression of *Bacteroides pap-pit* from the *S. meliloti sfx1* promoter resulted in a moderate level of growth (Figure 3.22). Increased expression of *Bacteroides pap-pit* presumably allowed for enough Pi uptake to occur to support this moderate level of growth. It is not clear why the increase in transcription allows for this change in growth phenotype. *Bacteroides pap-pit* has variations in GC content and codon usage when compared to *S. meliloti pap-pit*. One possibility is that the *Bacteroides Pap-Pit* has reduced Pi transport capabilities in *S. meliloti* and that the increased number of Pap and Pit proteins in the cell allows for enough Pi uptake to support some growth. Another possibility is that the increased level of transcription under the *sfx1* promoter allows for more translation of *Bacteroides pap-pit*. Under the wildtype promoter, maybe translation is not occurring at all or very little, but expression under the *sfx1* promoter allows for the translation of some *Bacteroides pap-pit*.

4.5 Model of translational coupling of the *S. meliloti pap-pit* operon

Several *pap-pit* gene fusion constructs were designed to test whether fusion of the two genes into a single protein would allow for functional Pi uptake. None of the four constructs fusing the two gene allowed for growth of RmP3926 in MOPS-P2 (Figure 3.19). A flexible GGSG linker was used in two of the constructs. This linker is commonly used

for fusion of two proteins that interact. It could be that Pap and Pit don't interact at all or that Pap may dissociate from Pit at times (e.g. if shuttling Pi throughout the cell). It could also be that this linker was not long enough for both of the proteins to interact or that they could not fold correctly when fused together. Other types of linkers could be tested in the future to identify one successful in fusing the proteins that allows for Pi uptake. Insertion of an 'A' immediately following the *pap* stop codon (TGA **A**TG), which resulted in removing the overlap between *pap* and *pit*, gave wildtype-like growth in MOPS-P2. Insertion of a larger 13 nt sequence, essentially repeating the last 13 nt of *pap* after the stop codon, including the proposed RBS for *pit*, resulted in moderate growth in MOPS-P2. Shifting the coding sequences by one nucleotide does not appear to have an effect on Pi uptake via Pit, but a small 13 nt insertion between the *pap* stop codon and the *pit* start codon did impair Pi transport in some way. This result indicates that the overlap between the two genes is important for functional Pi uptake as changing it too much impairs growth in MOPS-P2.

Modification of the *pap-pit* nucleotide sequence resulted in reduced growth in MOPS-P2 (Figure 3.15). This construct maintained the same amino acid sequence and codon usage of each gene (Figure 2.5). This result supports the idea that mRNA stability and structure can play important roles in the translation and folding of a protein (Nørholm *et al.*, 2012). This sequence likely results in a modified mRNA secondary structure that may make unwinding of the *pap-pit* mRNA more difficult. This may prevent exposure of the SD sequence to the nearby ribosomes. Changing the RBS site in the modified sequence back to the native RBS resulted in improved growth in MOPS-P2 but was still not as good

as wildtype (Figure 3.15). This could suggest that the native RBS is stronger than the one in the modified sequence and allows for some translation of Pit. It is possible that these constructs have changed the positions of rare codons that have been shown to slow the rate of translation and allow for proper protein folding, particularly in the 5' end of membrane proteins (Nørholm *et al.*, 2012).

Interestingly, detection of Pap by western blotting appeared to show that the sequence-modified *pap-pit* genes expressed more Pap protein than the wildtype *pap-pit* sequence. However, when the RBS of the sequence modified *pap-pit* genes was changed back to the native sequence, the amount of Pap protein decreased to less than wildtype. Possible reasons for this result include that the mRNA may be more or less stable in the cell in these constructs, or transcription or translation may be less efficient. The native RBS may cause more ribosomal pausing resulting in less efficient translation of Pap. This seems as though it may contradict the growth phenotypes in minimal medium as the strain with less Pap in the cell grows better. It is possible that the rate of translation in the construct that does not grow well, is too fast to allow for proper translation and folding of Pit, especially if Pap and Pit are co-translated (i.e. folded simultaneously during translation). Clearly, the mRNA sequence plays an important role in producing a functional Pap-Pit sequence.

I propose that *pap* and *pit* are in fact translationally coupled and that this is necessary to produce a functional protein complex. The mRNA sequence seems to play an important role in the production of a functional complex. The translation of Pap may allow the unwinding of the mRNA secondary structure at the intergenic region, exposing the Pit

SD sequence, and allowing for ribosome re-initiation or *de novo* initiation of translation. In the absence of *pap* immediately upstream of *pit*, translation, or possibly even transcription, is not able to occur. Therefore, mutant Pap can be complemented by wildtype Pap under a separate promoter only when the mutant Pap does not significantly impact the transcription or translation of Pit, such as in the pap^{P75A}-*pit* mutant. The inability of wildtype Pap to complement a frameshift Pap mutant also supports the idea that the unwinding of mRNA around the *pit* RBS during Pap translation is important in order to get translation of Pit (assuming transcription is not terminated prematurely by Rho). Further work analyzing the transcription (e.g. by RT-PCR) and translation of Pit in strains with a mutant Pap is required to better understand the role of translational coupling in Pi transport via Pit.

4.6 Relevance and Application

Understanding phosphate transport mechanisms in bacteria is of central importance as phosphorus is an essential nutrient for cells and is often a limiting nutrient for growth in soil environments. Moreover, following carbon and nitrogen, a source of phosphorus is the third most important macronutrient whose supply is critical for microbial cell growth. While soil microorganisms access multiple sources of carbon, the prominent source of phosphorus for most soil microorganisms is inorganic phosphate. Accordingly, how microorganisms acquire and regulate the transport of inorganic phosphate into their cells is of fundamental importance. In a recent survey of over 1000 bacteria, it was observed that over a third carried *pap*-*pit*-like genes and hence the study and understanding of how the Pap-Pit system functions has broad importance (Hsieh, unpublished).

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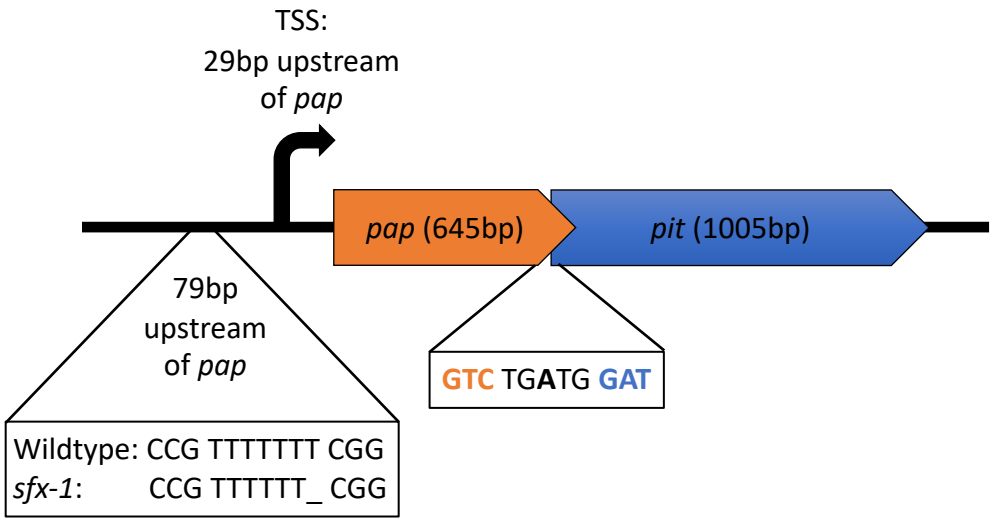


Figure 1.1. Diagram of the *S. meliloti pap-pit* operon. The transcriptional start site (TSS) is located 29 bp upstream of the *pap* coding sequence. *pap* is 645 bp in length and forms a 215 a.a. protein, while *pit* is 1005 bp and forms a 334 a.a. protein. The *pap* stop codon and *pit* start codon overlap by a single nucleotide (A). Located 79 bp upstream of *pap* is a hepta-thymidine sequence that is present in wildtype. Suppressors of nitrogen fixation were isolated from alfalfa root nodules in *pstC* and *phoCDET* mutants and found to contain a single T deletion in this hepta-thymidine sequence. This suppressor mutation (*sfx1*) was found to increase transcription of *pap-pit* and allow for Pi transport and nitrogen fixation with alfalfa.

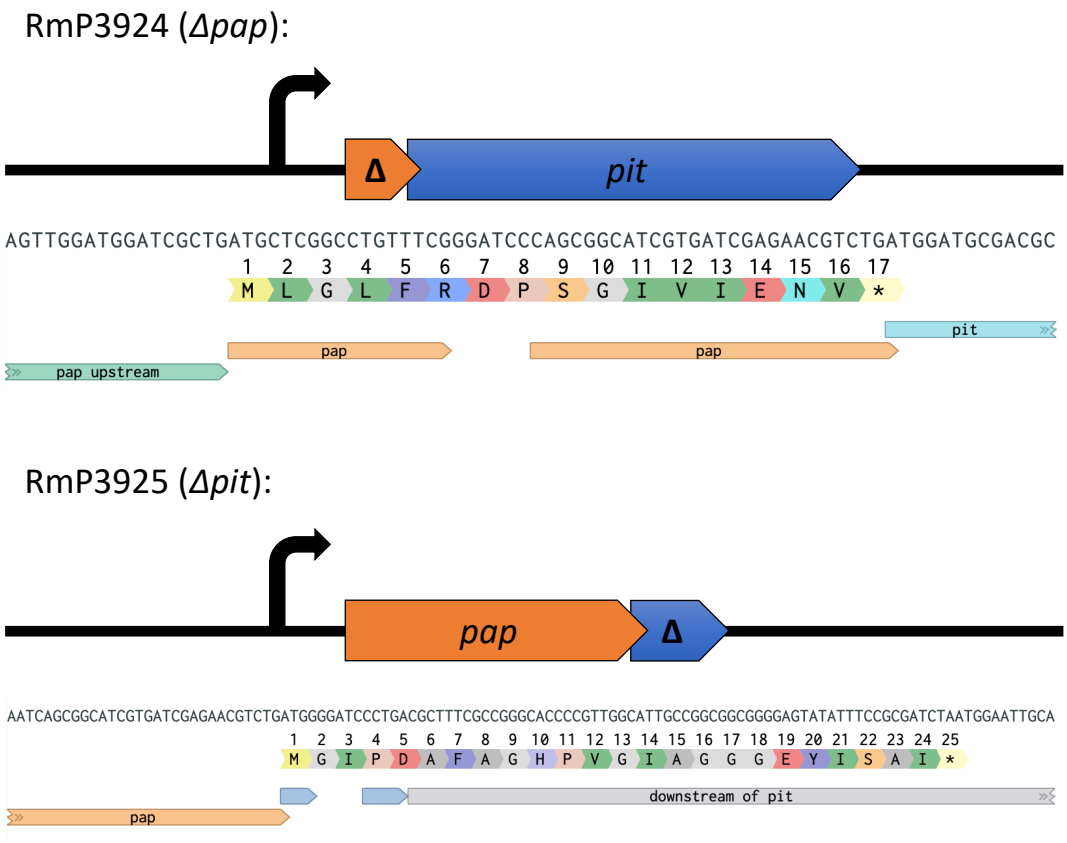


Figure 1.2. Diagram of *S. meliloti* RmP3924 (*Δpap*) and RmP3925 (*Δpit*) constructs. Recombination of pTH2340 into RmP110 occurred by *sacB*-mediated double crossover homologous recombination resulting in a deletion of *pap* from K7 to I206 and a 17 a.a. protein being produced (RmP1628). The single nucleotide overlap between *pap* and *pit* is maintained and the wildtype promoter sequence is present immediately upstream of *pap*. Similarly, recombination of pTH2341 into RmP110 occurred by *sacB*-mediated double crossover homologous recombination resulting in a deletion of *pit* from D2 to A334 and a 25 a.a. protein being produced due to the presence of a native TAA stop codon downstream (RmP1629). These deletions were transduced into RmP3421 (P_{lac}::*pstSCAB-phoUB*, P_{lac}::*phoR*, *lacIq*, *phoC*::ΩSp^R) using a *phe232*::Gm^R phenylalanine auxotroph marker located ~40 Kb downstream of *pap-pit*, resulting in RmP3924 (RmP3421 Δ*pap*) and RmP3925 (RmP3421 Δ*pit*).
*This work was done by Dr. Rahat Zaheer and Daniel Hsieh.

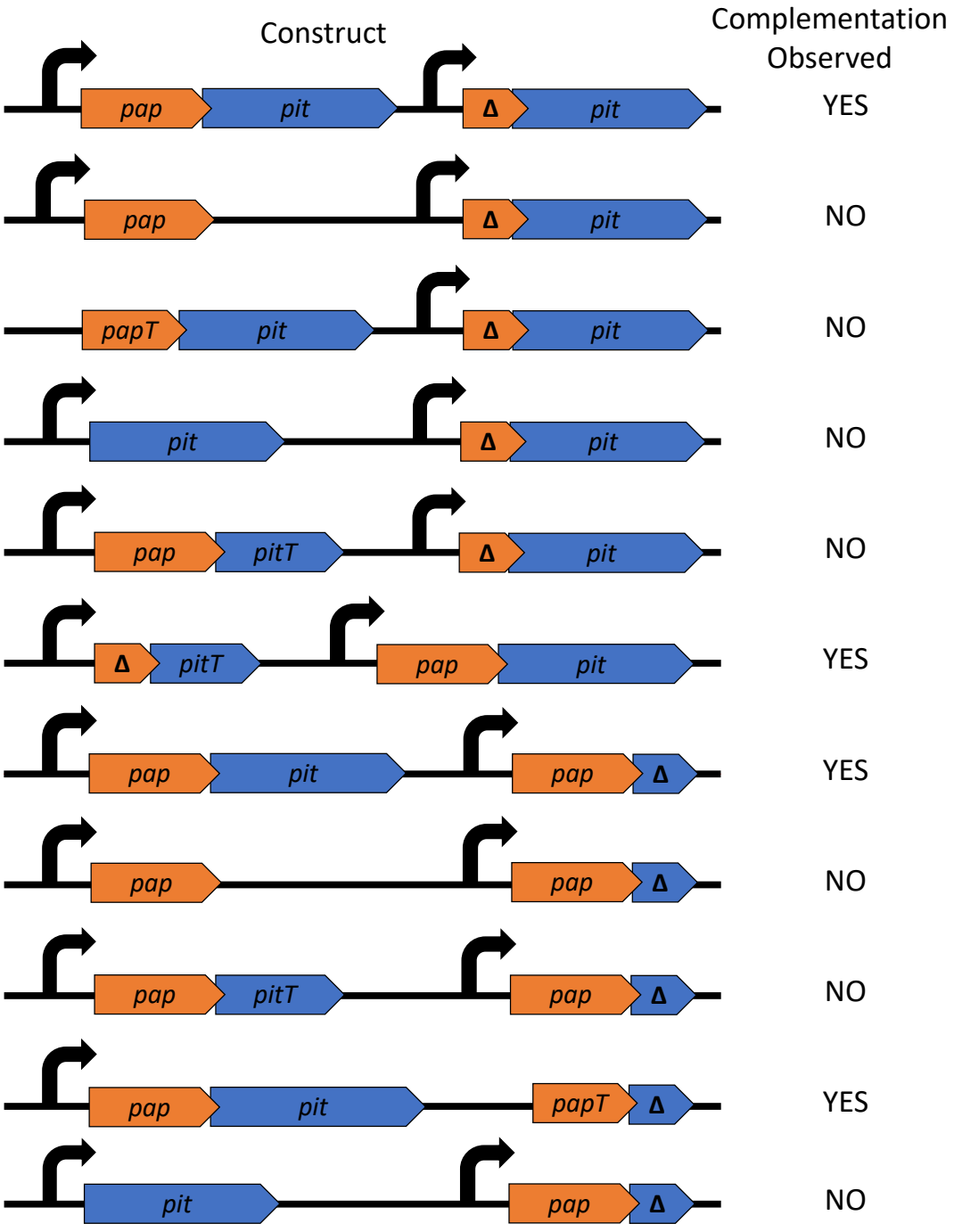


Figure 1.3. Recombinants generated from single homologous recombination of various constructs into *S. meliloti* RmP3924 (*Δpap*) and RmP3925 (*Δpit*). The ability of these constructs to complement was determined by growth in minimal medium with Pi as the only source of phosphate, where only a functional Pap-Pit should allow for growth. Only those constructs that restore a wildtype *pap-pit* operon are able to complement.
*These constructs were developed by Daniel Hsieh.

Table 2.1. Strains and plasmids used in this work

***Sinorhizobium meliloti* Strains**

Strain	Description	Source or reference
Rm1021	*S. meliloti* SU47-derived streptomycin resistant isolate with *pstC* frameshift mutation	
RmG340	Rm1021 *phe232*::Tn5 (Gm^R)	Oresnik *et al.,* 1994
RmP110	Rm1021 with corrected *pstC* mutation	Yuan, Zaheer, and Finan, 2006
RmP611	*phoX*::Tn5 (Nm^R)	Zaheer *et al.,* 2009
RmP635	RmP110 (*pit310*::Tn5), Nm^R	Yuan, Zaheer, and Finan, 2006
RmP1628	RmP110 (Δ*pap*) via pTH2340 double crossover	Zaheer, Unpublished
RmP1629	RmP110 (Δ*pit*) via pTH2341 double crossover	Zaheer, Unpublished
RmP3421	P_{lac}::*pstSCAB-phoUB*, P_{lac}::*phoR*, *lacIq*, *phoC::*ΩSp^R	diCenzo *et al.,* 2017
RmP3923	RmP3421 (φRmG340 *phe232::*Gm^R)	Hsieh, Unpublished
RmP3924	RmP3923 (φRmP1628 Δ*pap*)	Hsieh, Unpublished
RmP3925	RmP3923 (φRmP1629 Δ*pit*)	Hsieh, Unpublished
RmP3926	RmP3923 (φRmP3151 Δ*pap-pit*)	Hsieh, Unpublished
RmP4192	RmP3926 (φRmP611 *phoX*::Tn5), Nm^R	Hsieh, Unpublished

***Escherichia coli* Strains**

Strain	Description	Source or reference
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)U169 φ80d*lacZ*ΔM15	
MT616	Plasmid pRK600 for triparental mating	Finan *et al.,* 1986

Plasmids

E. coli Strain	Plasmid	Description	Source or reference
J556	pRK7813	RK2 derivative broad host range cloning vector, Tc^R	Jones and Gutterson, 1987
J287	pJQ200	p15A origin vector derived from pACYCY184-Gm^R with *Bacillus subtilis sacB*, pUC18 polylinker and *lacZα* fragment, Gm^R	Quandt and Hynes, 1993
N/A	pPLE01	pBluescript II SK(+) with recombinant alkaline phosphatase/β-galactosidase (*phoA/lacZ*) fusion, Amp^R	Islam *et al.*, 2010
N/A	pJET1.2/ blunt	Blunt end cloning vector with MCS in a lethal restriction enzyme gene, pMB1 oriV	Thermo Fisher Scientific
J252	pUCP30T	pRO1600 origin (*Pseudomonas aeruginosa*) broad host-range cloning vector, (suicide vector in *S. meliloti*), Gm^R	Schweizer, 2001
H80	pTH276	pRK7813 (4.8 kb HindIII and SacI fragment of *pap-pit* carrying *sfx1*), Tc^R	Bardin *et al.,* 1998
M411	pTH1522	Reporter gene fusion vector, pBR322 oriV	Cowie *et al.,* 2006
M1257	pTH2340	pJQ200 Δ*pap* deletion vector, Gm^R	Zaheer, Unpublished
M1258	pTH2341	pJQ200 Δ*pit* deletion vector, Gm^R	Zaheer, Unpublished
M2041	pTH2824	pUCP30T (*pap-pit* with 500 bp upstream and downstream) via XbaI/HindIII, Gm^R	Zaheer and Zhao, Unpublished
M2140	pTH2906	pUCP30T (pap^{P75A}-*pit* with 500 bp upstream and downstream) via XbaI/HindIII, Gm^R	Zaheer and Zhao, Unpublished

M2328	pTH3044	pUCP30T (*pap*^{P75fs}*-pit* by insertion of a 'C' at nucleotide 223 of *pap* with 500 bp upstream and downstream) via XbaI/HindIII, Gm^R	Husseininaveh, Unpublished
M2488	pTH3173	pUCP30T ((Δ*pap)-pit*; subcloned from pTH2340 via XbaI/AgeI), Gm^R	Hsieh, Unpublished
M2503	pTH3187	pUCP30T (*Bacteroides pap-pit*) cloned via EcoRI/BamHI, Gm^R	This work
M2516	pTH3198	pUCP30T (*pap-pit~phoA/lacZ*) via XbaI/HindIII, Gm^R	Hsieh, Unpublished
M2592	pTH3246	pUCP30T (*pap*^{P75A}*-pit~phoA*/*lacZ*; *phoA*/*lacZ* from pTH3198 cloned into pTH2906 via StuI/HindIII) Gm^R	This work
M2601	pTH3248	pUCP30T (*sfx1 pap-pit*; *sfx1* promoter from pTH276 cloned into pTH2824 via BamHI/XhoI), Gm^R	This work
M2602	pTH3249	pUCP30T (*sfx1 pap*^{P75A}*-pit*; *sfx1* promoter from pTH276 cloned into pTH2906 via BamHI/XhoI) Gm^R	This work
M2603	pTH3250	pUCP30T (*sfx1 pap-pit~phoA/lacZ*; *sfx1* promoter from pTH276 cloned into pTH3198 via BamHI/XhoI), Gm^R	This work
M2604	pTH3251	pUCP30T (*sfx1 pap*^{P75A}*-pit~phoA/lacZ*; *sfx1* promoter from pTH276 cloned into pTH3246 via BamHI/XhoI), Gm^R	This work
M2605	pTH3252	pUCP30T (*pap-pit~gusA*; *gusA* amplified from pTH1522 and cloned into pTH3198 to replace *phoA/lacZ* via PstI/HindIII), Gm^R	This work
M2606	pTH3253	pUCP30T (*pap*^{P75A}*-pit~gusA*; *gusA* amplified from pTH1522 and cloned into pTH3246 to replace *phoA/lacZ* via PstI/HindIII), Gm^R	This work
M2609	pTH3256	pUCP30T (*sfx1* (Δ*pap*)*-pit*; Δ*pap* amplified from pTH2340 with overlap extension PCR to introduce *sfx1* mutation, cloned into pTH2824 via XbaI/AgeI) Gm^R	This work
M2615	pTH3262	pUCP30T (*sfx1* (Δ*pap*)*-pit~gusA*; Δ*pap* amplified from pTH2340 with overlap extension PCR to introduce *sfx1* mutation, cloned into pTH3252 via XbaI/AgeI) Gm^R	This work

M2618	pTH3266	pUCP30T (*Bacteroides pap-pit* under the *S. meliloti sfx1 pap* promoter amplified from pTH3187 using overlap extension PCR to introduce the *sfx1* mutation), via EcoRI/BamHI, Gm^R	This work
M2656	pTH3295	pUCP30T ((Δ*pap*)-*pit~gusA*; (Δ*pap*)-*pit* from pTH2340 cloned into pTH3252 via XbaI/AgeI) Gm^R	This work
M2657	pTH3296	pUCP30T (*sfx1 pap-pit~gusA*; *sfx1 pap-pit* amplified from pTH3250 and cloned into pTH3252 via XbaI/AgeI), Gm^R	This work
M2658	pTH3297	pUCP30T (*sfx1* pap^{P75A}*-pit~gusA*; *sfx1* pap^{P75A}*-pit* amplified from pTH3251 and cloned into pTH3252 via XbaI/AgeI), Gm^R	This work
M2661	pTH3300	pJET1.2/blunt (*pap-pit* modified sequence geneblock), Amp^R	This work
M2681	pTH3325	pTH2824 (modified *pap-pit* nucleotide sequence) subcloned from pTH3300 via XhoI/AgeI, Gm^R	This work
M2694	pTH3334	pTH3325 (*pit* RBS changed back to native sequence (GAAAA → GAGAA)), via XbaI/HindIII, Gm^R	This work
M2695	pTH3335	pUCP30T (*pap*-RBS-*pit*), via XbaI/HindIII, Gm^R	This work
M2696	pTH3336	pUCP30T (*pap* (no stop)-*pit*), via XbaI/HindIII, Gm^R	This work
M2697	pTH3337	pUCP30T (*pap*-no overlap-*pit*), via XbaI/HindIII, Gm^R	This work
M2698	pTH3338	pUCP30T (*pap* (no stop)-GGSG-*pit*), via XbaI/HindIII, Gm^R	This work
M2699	pTH3339	pUCP30T (*pap* (no stop)-*pit* (no start)), via XbaI/HindIII, Gm^R	This work
M2700	pTH3340	pUCP30T (*pap* (no stop)-GGSG-*pit* (no start)), via XbaI/HindIII, Gm^R	This work
M2702	pTH3342	pUCP30T (pap^{M1L}*-pit*), via XbaI/HindIII, Gm^R	This work

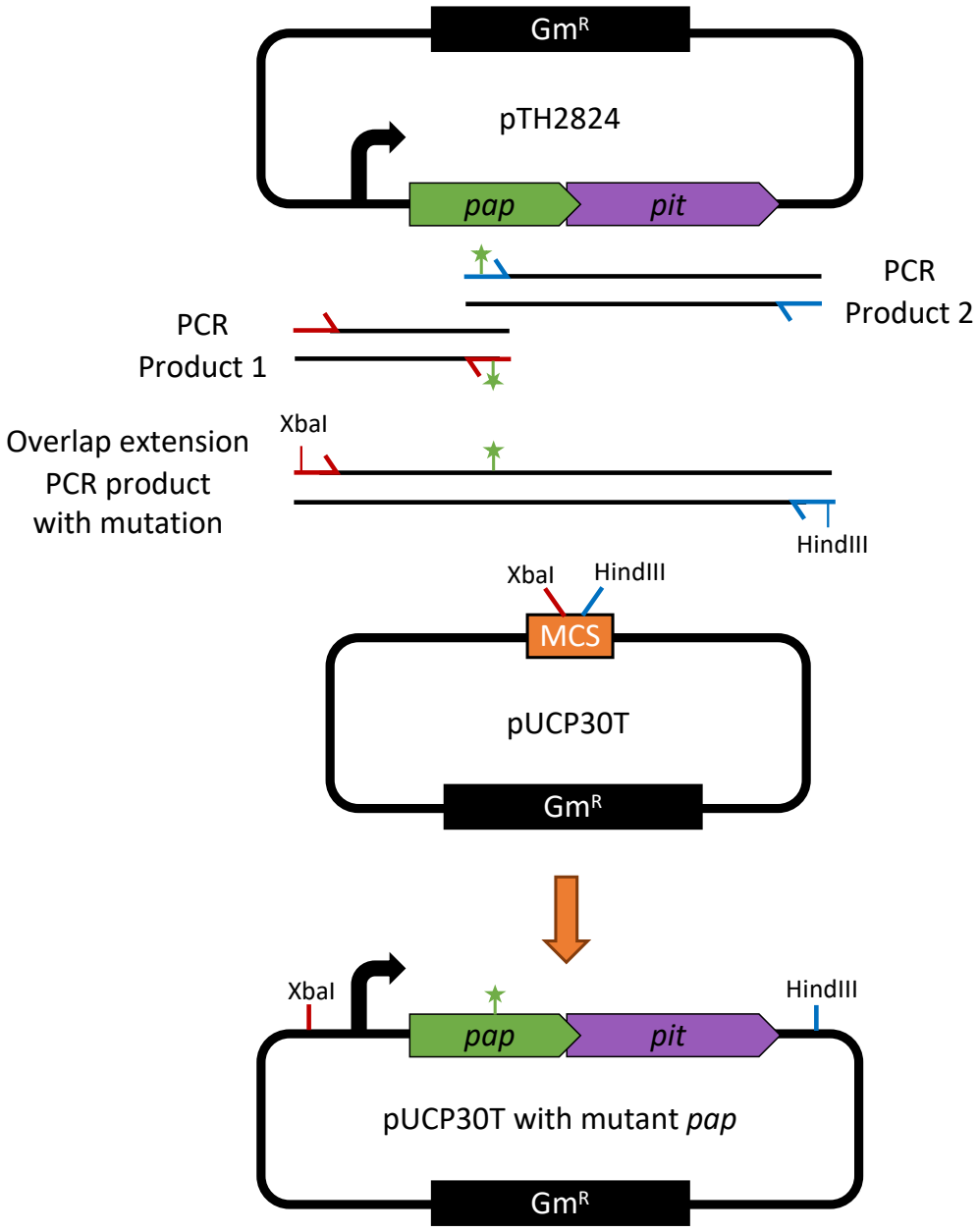
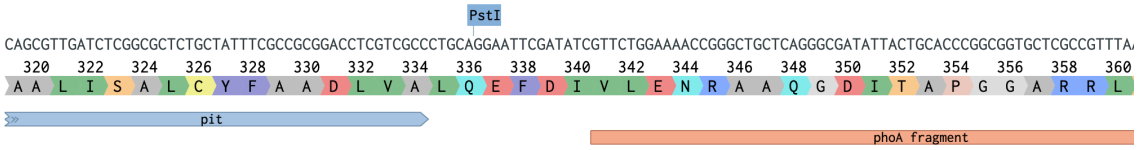


Figure 2.2. Overlap extension PCR to generate mutant *pap-pit* plasmids. Two primer sets were used to generate two independent PCR products (1 and 2). The two internal primers (one red and one blue) share partial homology, typically around 20 bp, and carry the mutation that is to be introduced into the construct (indicated by the green star). The mutation could be a point mutation or a small insertion or deletion. The two external primers are then used to amplify the final PCR product using overlap extension. The final product was cloned into the multiple cloning site (MCS) of pUCP30T using XbaI and HindIII restriction sites. Mutations were made in the promoter region, in addition to the *pap-pit* coding regions using this method.

A.



B.

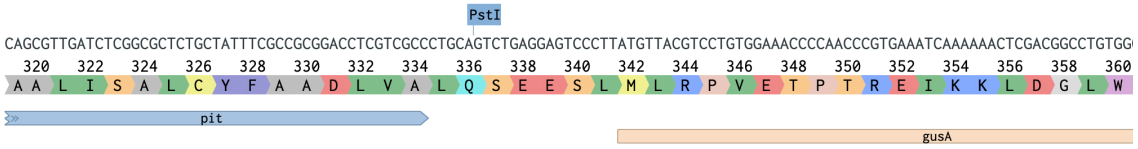


Figure 2.3. Nucleotide and protein sequence of the *phoA/lacZα* and *gusA* translational fusions to *pit*. A. The *S. meliloti pap-pit* genes were cloned into pPLE01 via PstI to create a C-terminal translational fusion of *pit* to the *phoA/lacZα* fragment. 500 bp of downstream *pit* sequence was also cloned via HindIII downstream of *phoA/lacZα*. The *pap-pit~phoA/lacZ* fusion was subcloned into pTH2824 via StuI and BspEI to generate pTH3198. A six amino acid linker sequence is present following the last amino acid of the *pit* coding sequence. The *phoA/lacZα* fragment does not include the N-terminal signal sequence**. B.** The *gusA* fragment was PCR amplified from pTH1522 and subcloned into pTH3198 via PstI and HindIII, replacing the *phoA/lacZ* sequence to give a *pit~gusA* translational fusion (pTH3252). A seven amino acid linker sequence was used to connect *pit* and *gusA*. The 500 bp of downstream *pit* sequence present in pTH3198 is not present in pTH3252.

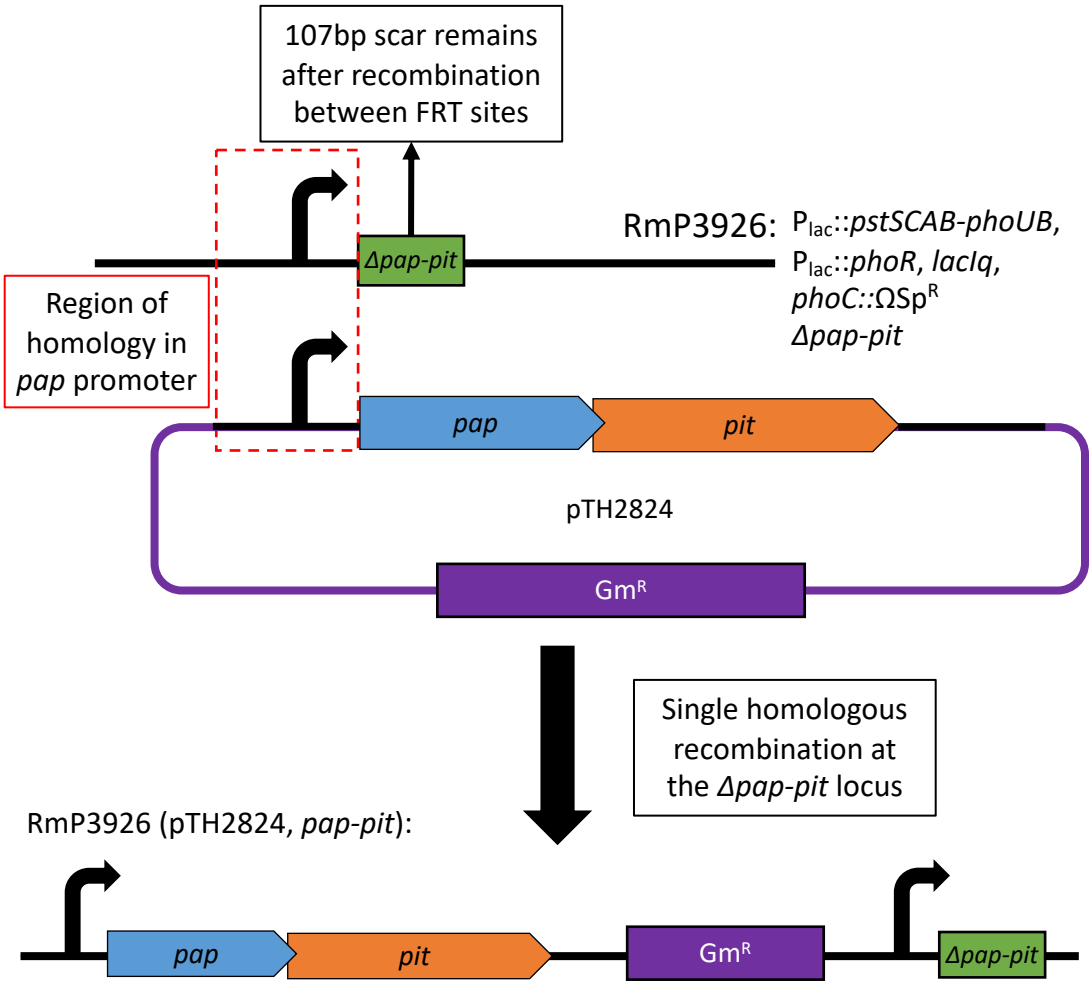


Figure 2.4. Recombination of a *pap-pit* plasmid construct into RmP3926 (*Δpap-pit*). A plasmid carrying an FRT-kanamycin-FRT cassette with 500 bp homology to the upstream and downstream regions of *pap-pit* was integrated into RmP110. Double crossover recombinants (Kan^RGm^S) were selected for; these would contain a deletion of the *pap-pit* genes. Recombination between the FRT sites resulted in deletion of the gene conferring kanamycin resistance, leaving a 107 bp scar (*Δpap-pit*). This *Δpap-pit* deletion was transduced into RmP3421 resulting in RmP3926. The sequence immediately preceding the scar region was the *pap* promoter sequence which had approximately 500 bp of homology to the plasmid constructs and thus, the plasmids are able to recombine into the chromosome at the *Δpap-pit* locus. This results in the expression of the integrated *pap-pit* constructs from the native *pap* promoter.

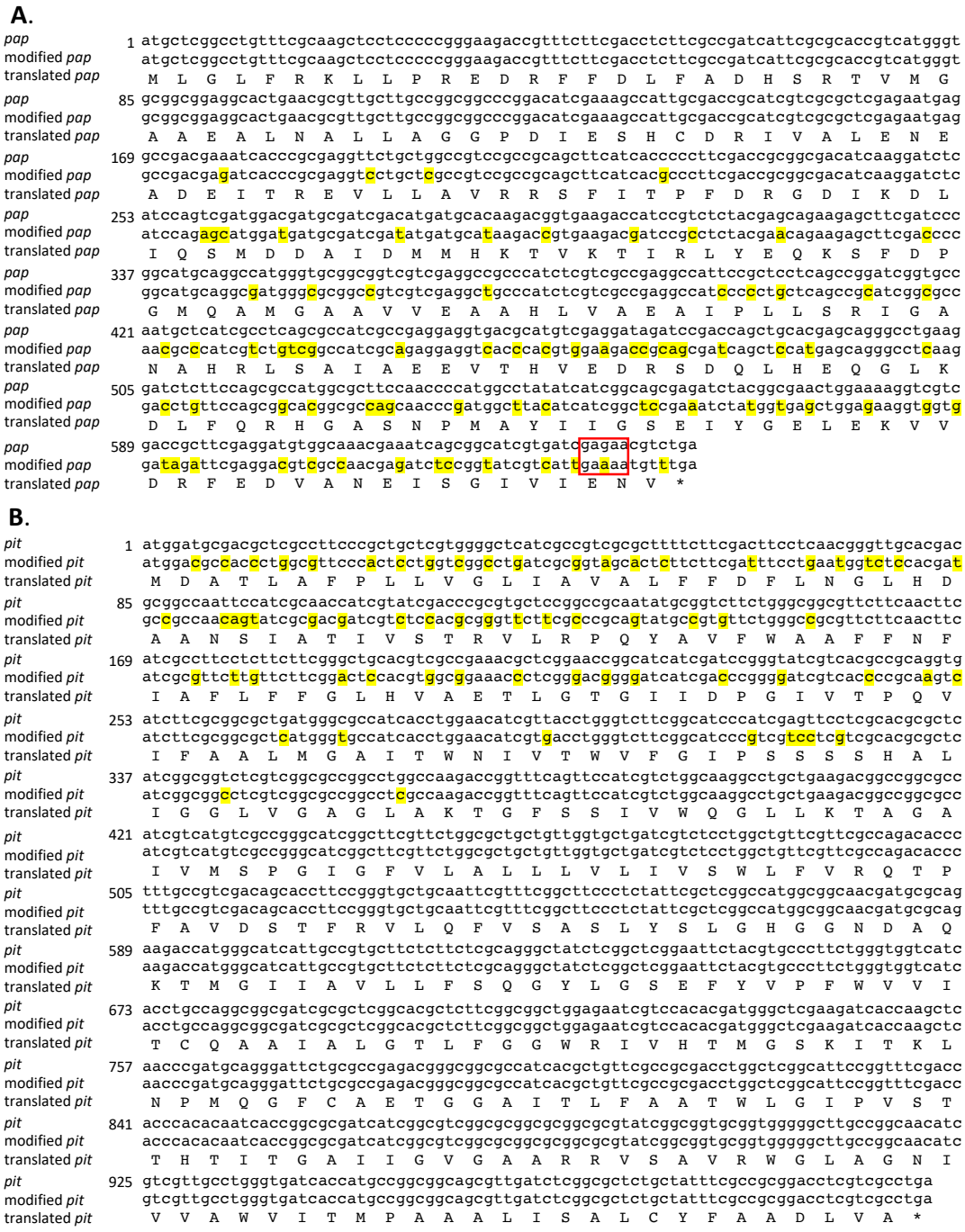


Figure 2.5. Modified *S. meliloti pap-pit* nucleotide sequence. The codon usage and amino acid sequence (given for each gene) was maintained. Changes between the wildtype *pap-pit* sequence and the modified sequence are highlighted in yellow. **A.** The modified *pap* nucleotide sequence in comparison to the wildtype *pap* sequence. The red box outlines the proposed RBS for *pit*. In the modified sequence (pTH3325), GAAAA was used. It was changed back to the native GAGAA in pTH3334. **B.** The modified *pit* nucleotide sequence in comparison to wildtype *pit* sequence.

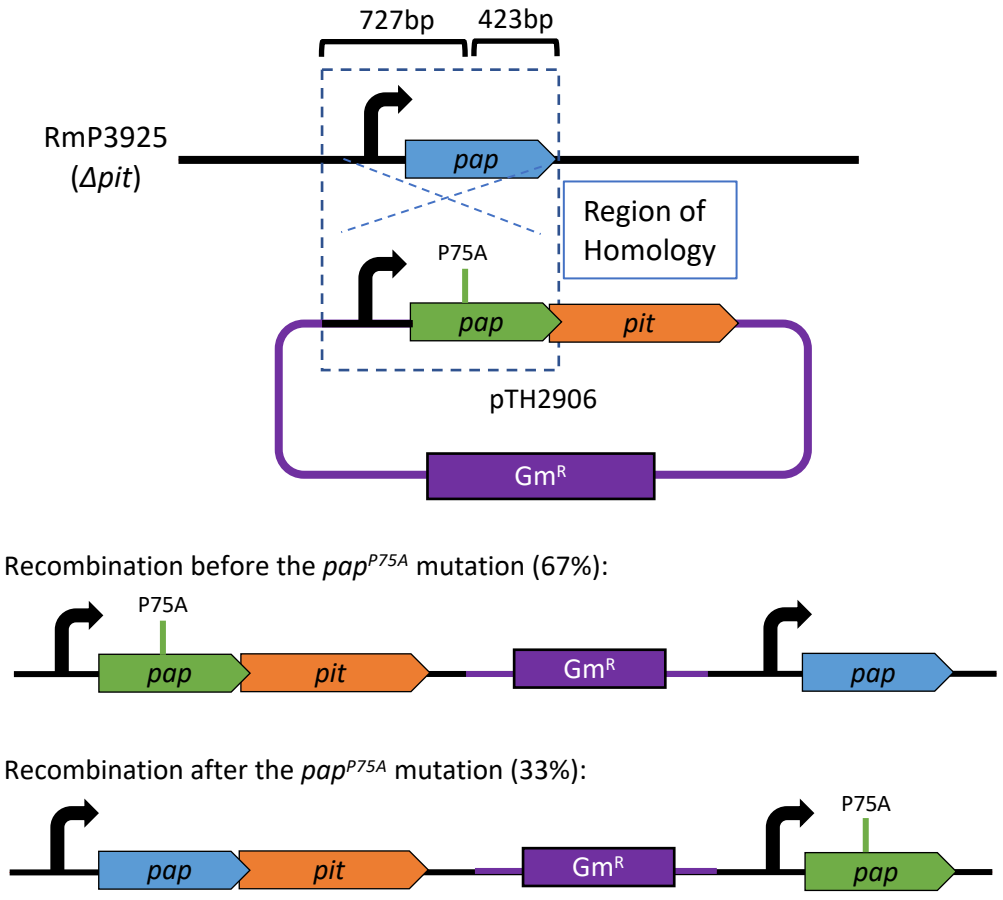


Figure 2.6. Recombination into of *pap*^{P75A}*-pit* (pTH2906) into RmP3925 (*Δpit*). Two different recombinants can result from the recombination of pTH2906 into RmP3925. One in which a *pap*^{P75A}*-pit* operon is maintained, and a wildtype *pap* is present under a separate promoter downstream (67% of exconjugants) and another construct in which a wildtype *pap-pit* operon is generated and *pap*^{P75A} is under a separate promoter downstream (33% of exconjugants). Similar recombinants were obtained using *pap*^{P75fs}*-pit* (pTH3044) and *pap*^{M1L}*-pit* (pTH3342), however the percentage of each recombination event varied.

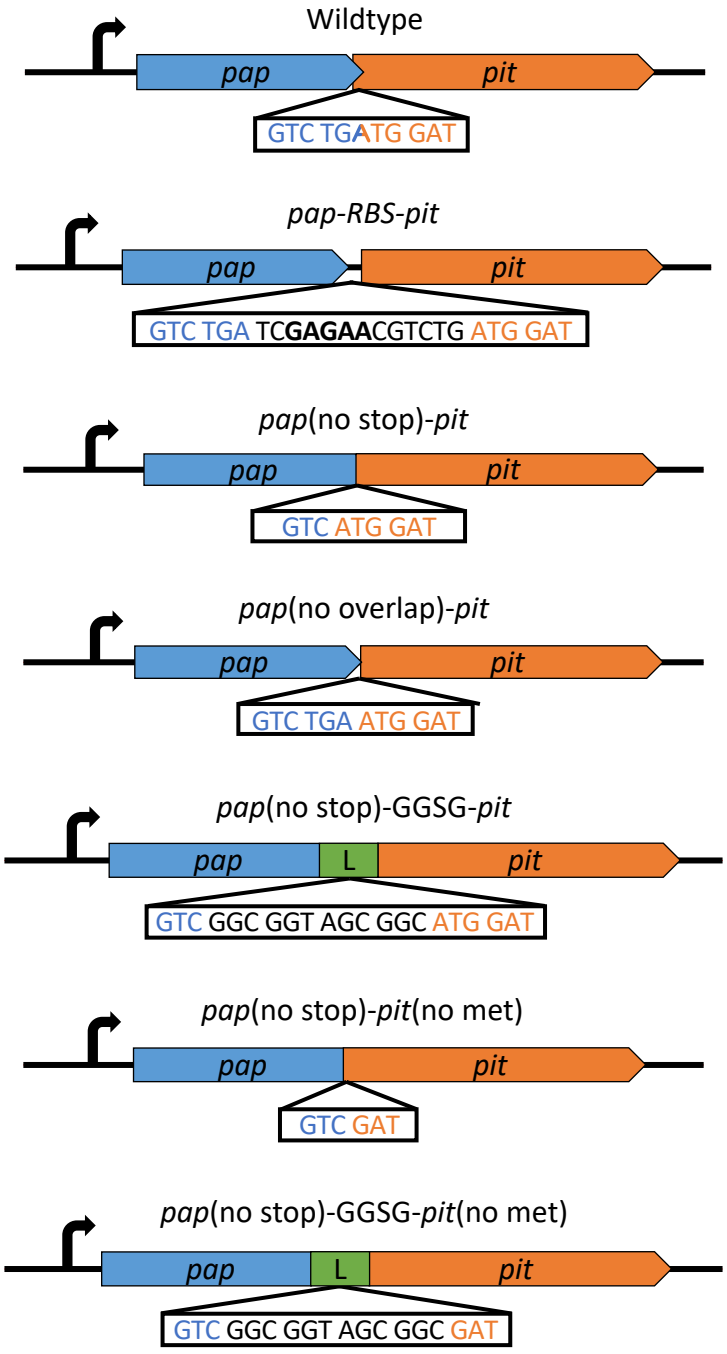


Figure 2.7. *S. meliloti pap-pit* gene fusion constructs. Constructs were developed using overlap extension PCR and cloned into pUCP30T using XbaI and HindIII. Plasmids were conjugated into RmP3926 and recombined into the chromosome at the Δ*pap-pit* site.

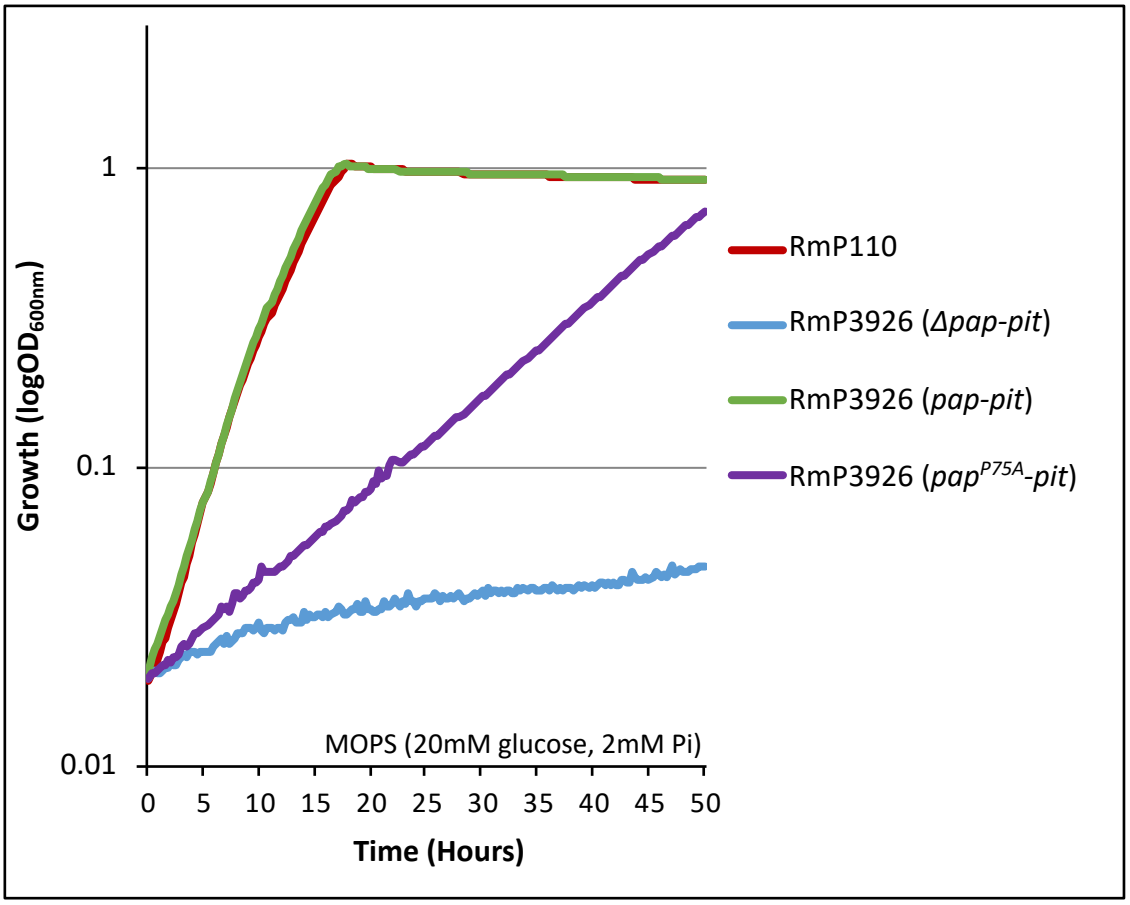


Figure 3.1. Growth in glucose minimal medium with 2 mM Pi of *S. meliloti Δpap-pit* with a *pap^{P75A}-pit* point mutation construct in minimal medium. RmP3926 with the *pap^{P75A}-pit* construct did not grow as well as RmP3926 with wildtype *pap-pit* in MOPS-P2, indicating this point mutation had an effect on Pi transport and growth.

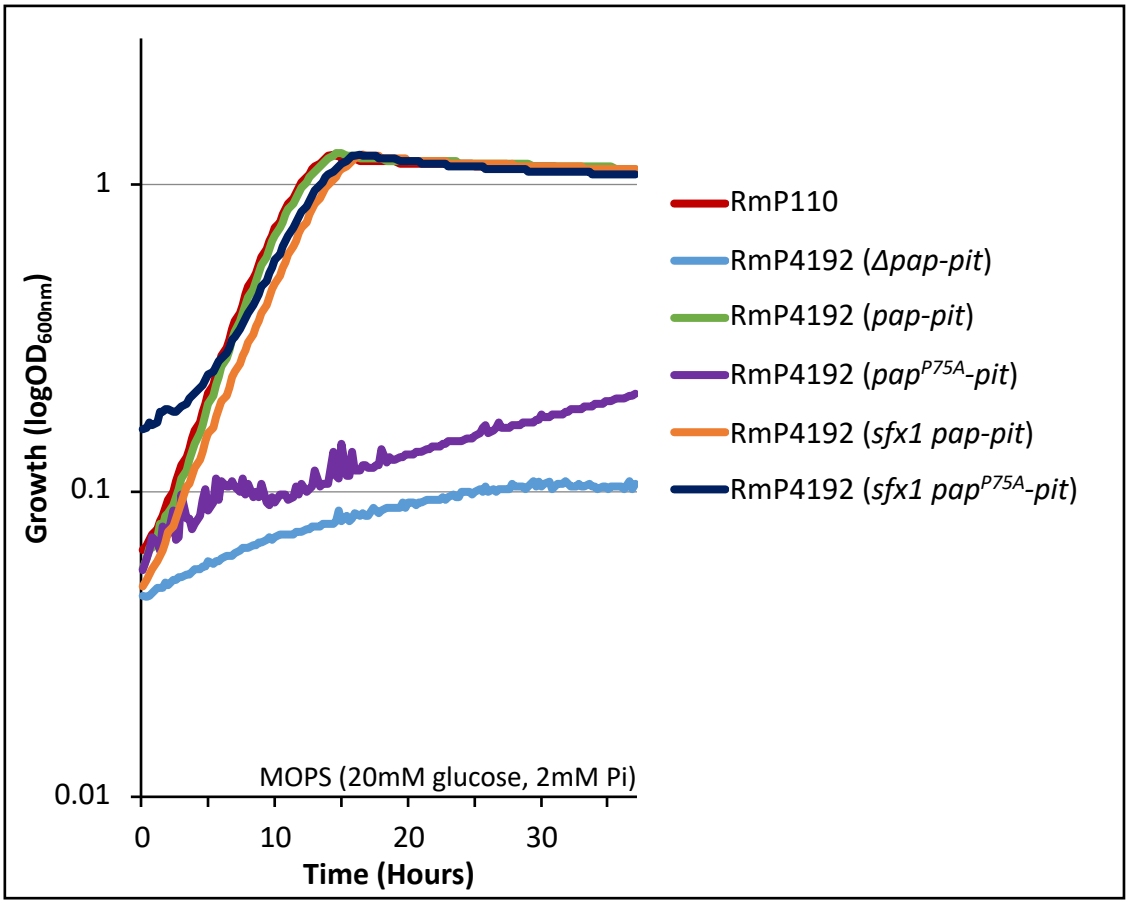


Figure 3.2. Expression of *pap^{P75A}-pit* under the *sfx1* promoter allows for complementation of *S. meliloti Δpap-pit.* Growth of RmP4192 (RmP3926 *phoX*::Tn5) with *pap^{P75A}-pit* under the *sfx1* promoter allowed for growth similar to RmP4192 with wildtype *pap-pit*. RmP4192 with *pap^{P75A}-pit* under the wildtype pap promoter grew moderately well, indicating overexpression using the *sfx1* promoter restored a wildtype level of Pi transport and growth.

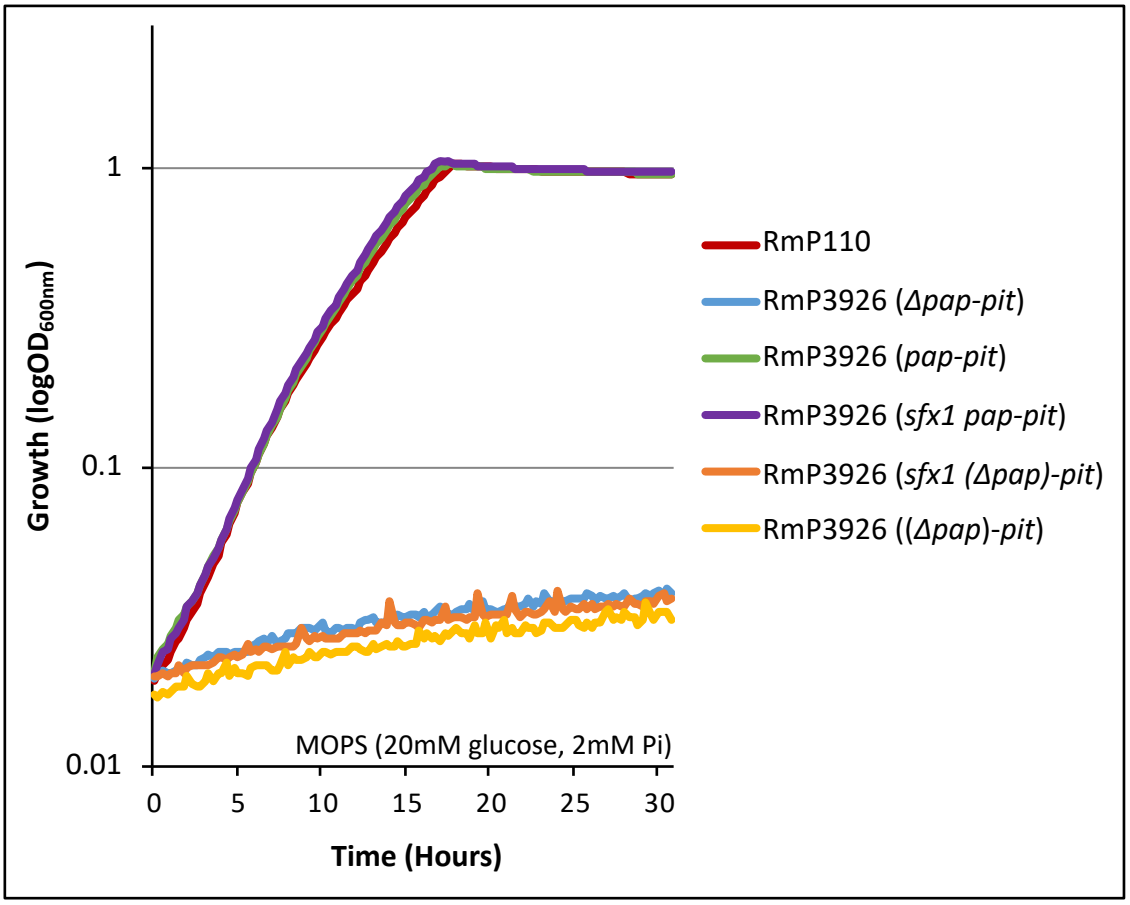


Figure 3.3. Growth of *S. meliloti Δpap-pit* with *Δpap* constructs in minimal medium. Complementation of *S. meliloti* RmP3926 was observed for *pap-pit* (pTH2824) and *sfx1 pap-pit* (pTH3248) but not for *sfx1* (Δ*pap)-pit* (pTH3256) and (Δ*pap)-pit* (pTH3173). Increasing the expression of a (Δ*pap)-pit* mutant did not allow for increased Pi transport and growth.

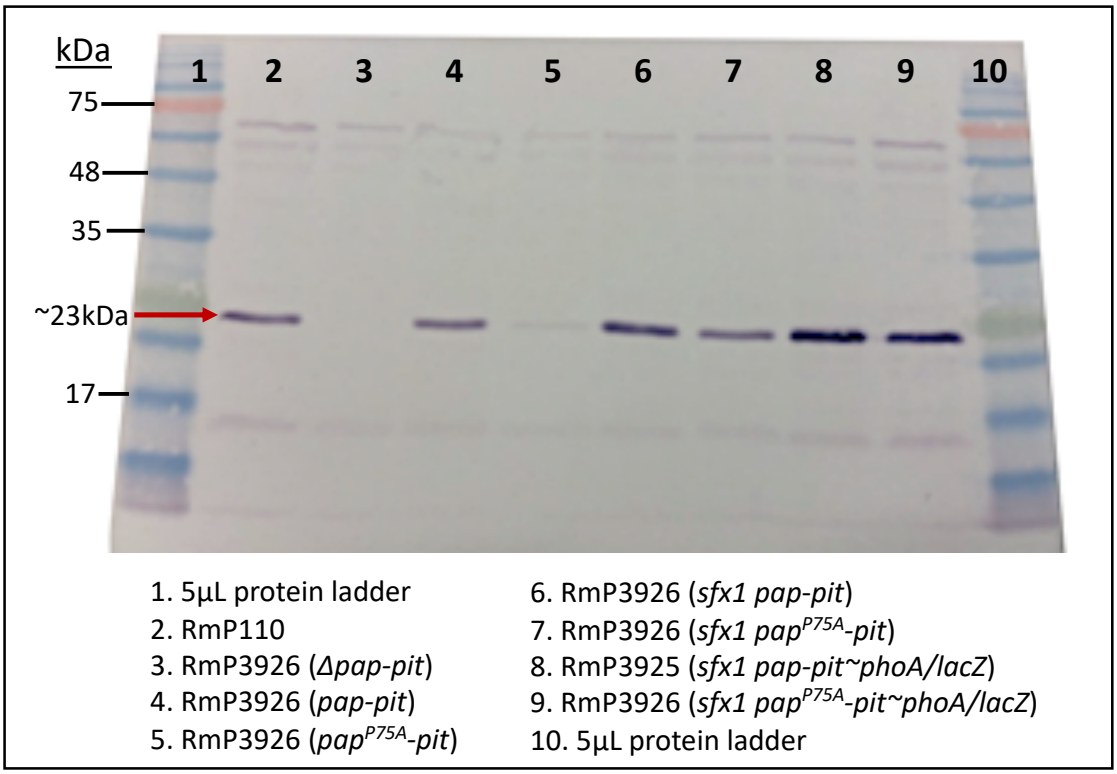


Figure 3.4. Western blot of *S. meliloti* RmP3926 with *pap^{P75A}-pit* under wildtype and *sfx1 pap* promoters. RmP110 was used as a positive control for wildtype Pap with the red arrow indicating the band of the appropriate size for Pap at approximately 23 kDa. RmP3926 (Δ*pap-pit*) was used as a negative control for Pap. RmP3926 with wildtype *pap-pit* integrated into the chromosome gave a band similar to RmP110. RmP3926 with *pap^{P75A}-pit* under the wildtype promoter gave a faint band at the correct size for Pap. RmP3926 with the *sfx1* promoter upstream of wildtype *pap-pit* (lanes 6 and 8) showed a band darker in colour than RmP110, indicating increased transcription and translational of *pap*. RmP3926 with *pap^{P75A}-pit* under the *sfx1* promoter (lanes 7 and 9) gave a darker band than when under the wildtype promoter, indicating increased transcription and translational of Pap^{P75A} to a level similar to RmP110.

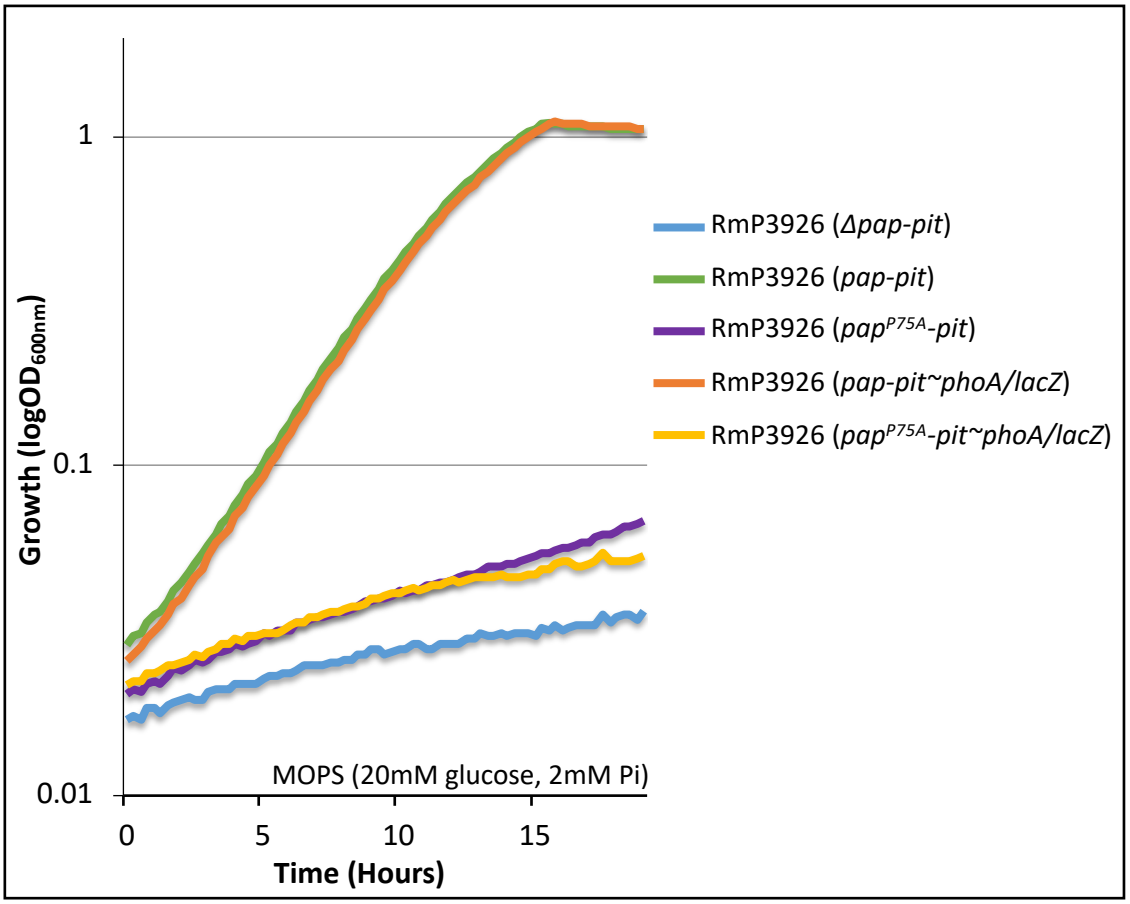


Figure 3.5. Growth of *S. meliloti Δpap-pit* with constructs containing a *phoA*/*lacZ* fusion to *pit*. The *phoA*/*lacZ* fusion did not have an effect on growth in MOPS-P2 as strains with and without the translational reporter grew similarly. RmP3926 with *pap-pit* and *pap-pit~phoA*/*lacZ* both grow well. RmP3926 with *pap*^{P75A}*-pit* and *pap*^{P75A}*-pit~phoA*/*lacZ* also grew similarly.

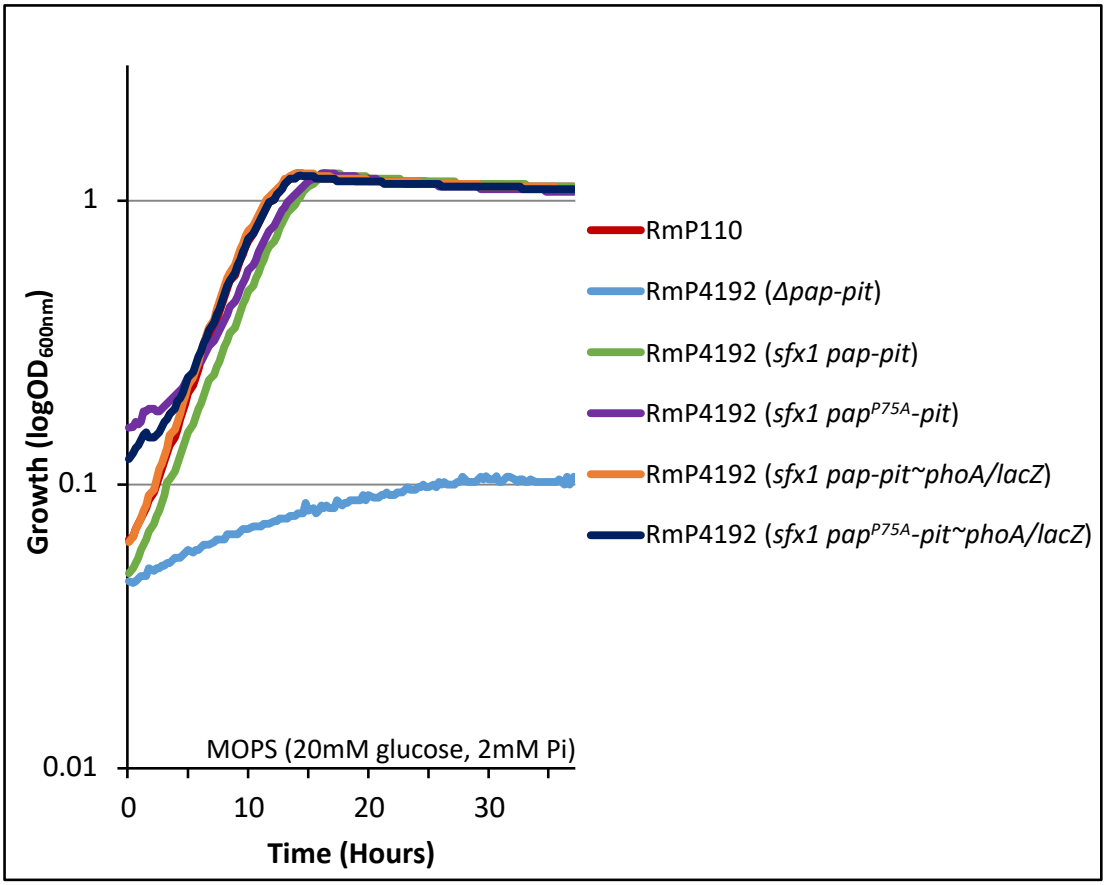


Figure 3.6. Complementation of *S. meliloti Δpap-pit* by *pap-pit* constructs under the *sfx1* promoter. Growth was observed in MOPS-P2 for RmP4192 with *pap-pit* constructs under the *sfx1* promoter regardless of the presence or absence of the *phoA*/*lacZ* fusion to *pit*. Strains with the *pap*^{P75A}*-pit* mutation under the *sfx1* promoter also grew the same as wildtype *pap-pit*.

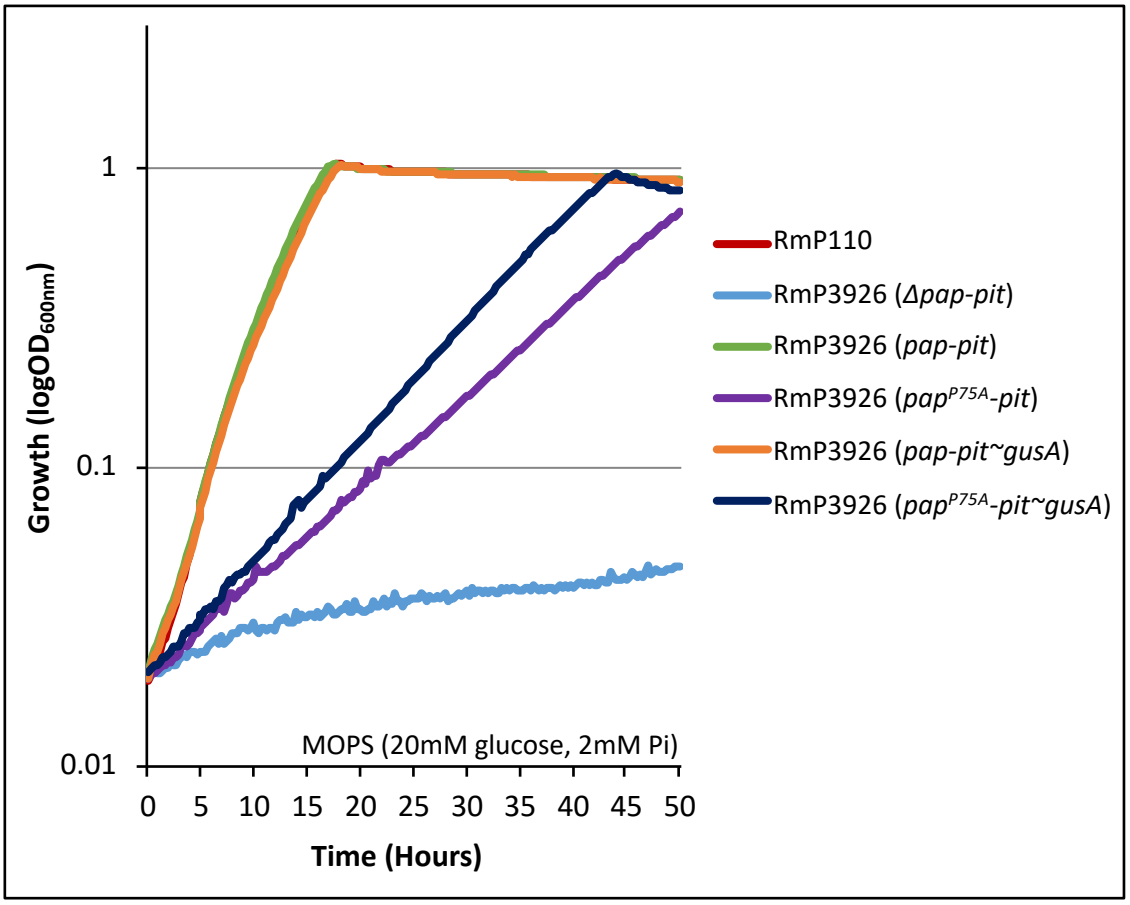


Figure 3.7. Growth of *S. meliloti Δpap-pit* strains carrying a *gusA* translational fusion to *pit*. The *gusA* fusion did not affect the growth of RmP3926 with *pap-pit* as both *pap-pit* and *pap-pit~gusA* grew similarly in MOPS-P2. However, for pap^{P75A}*-pit* constructs, the *pit~gusA* translational fusion slightly improved the growth of pap^{P75A}*-pit* in MOPS-P2, but both strains did not grow as well as wildtype.

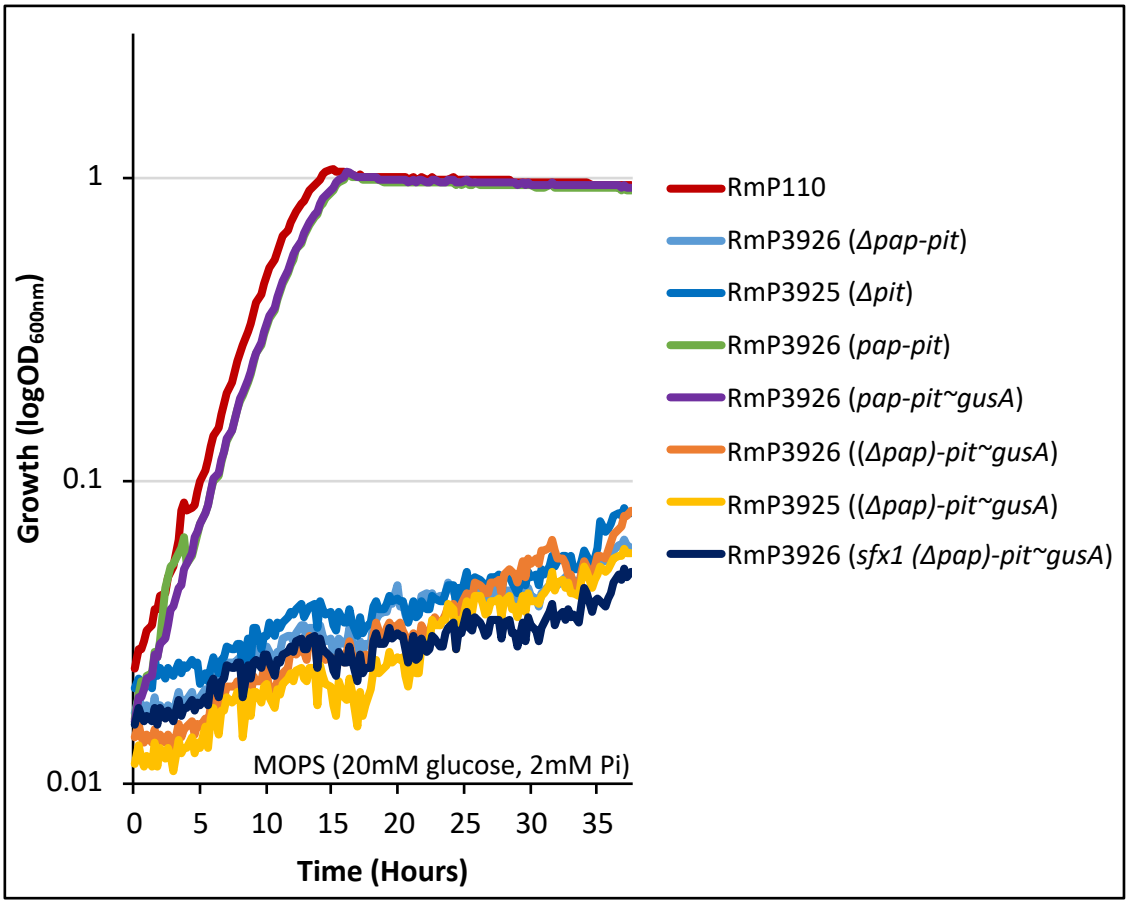


Figure 3.8. Growth of *S. meliloti* RmP3926 (*Δpap-pit*) and RmP3925 (*Δpit*) with (*Δpap*)-*pit~gusA* constucts under wildtype and *sfx1* promoters in minimal medium. RmP3926 with (Δ*pap*)-*pit~gusA* did not grow regardless of if it was under the wildtype or *sfx1* promoter, indicating increasing its expression had no effect on Pi Transport or growth. RmP3925 with (Δ*pap*)-*pit~gusA* also did not grow even though both *pap* and *pit* are present on the chromosome (under separate promoters). This may indicate that *pit* was not being transcribed or translated in a (Δ*pap*)-*pit~gusA* construct.

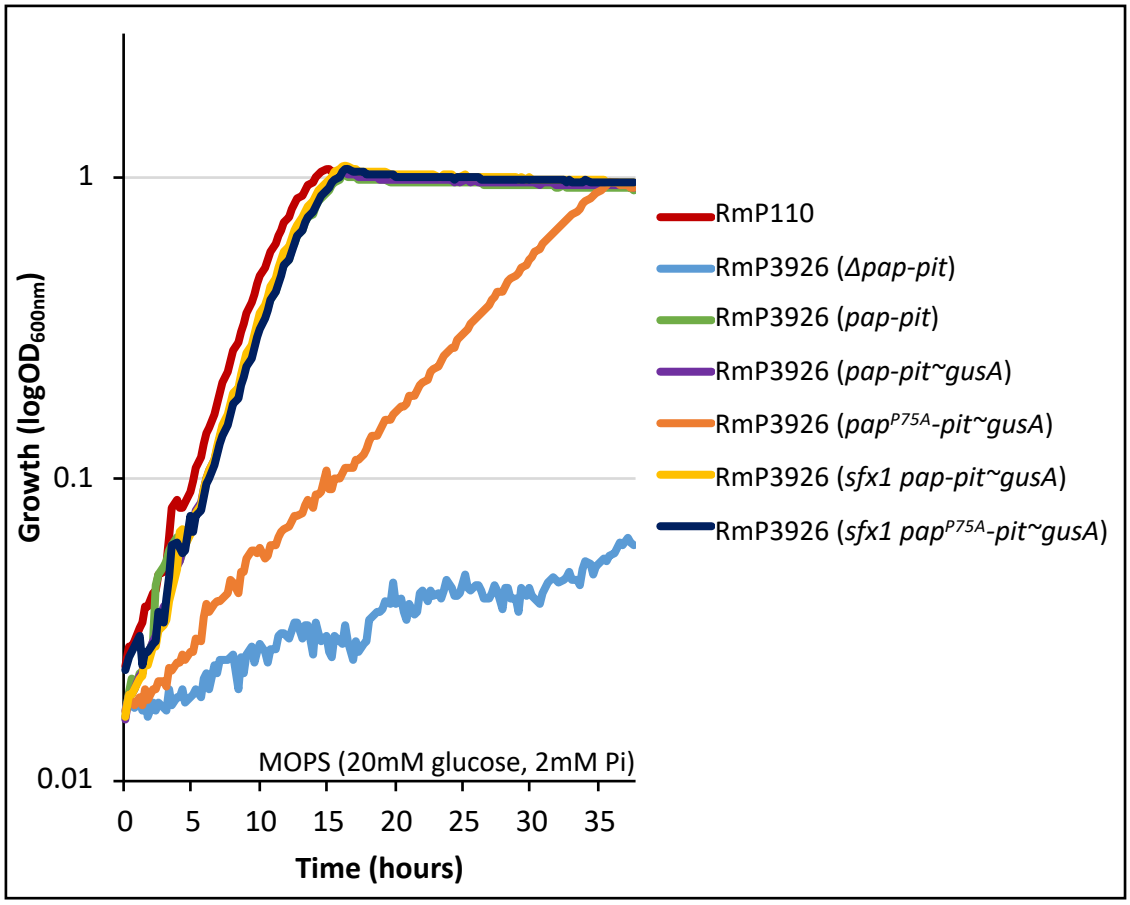


Figure 3.9. Growth of *S. meliloti* RmP3926 (*Δpap-pit*) with *pap^{P75A}-pit~gusA* under wildtype and *sfx1* promoters. The *sfx1* promoter allowed for wildtype-like growth of *pap^{P75A}-pit~gusA*, while the wildtype promoter only allowed for moderate growth. The *pit~gusA* translational fusion did not affect the growth of any of the strains.

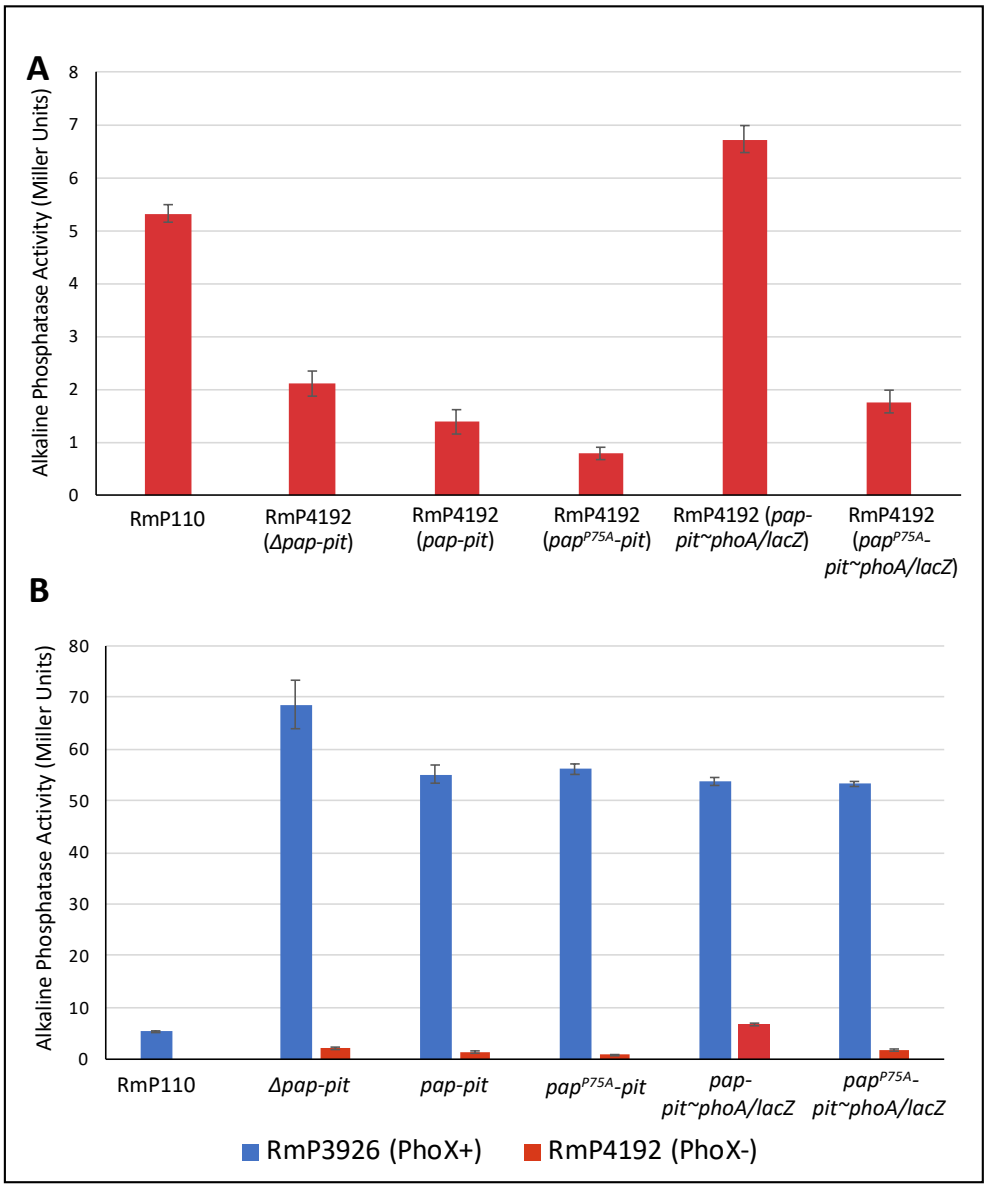


Figure 3.10. Alkaline phosphatase activity of *S. meliloti* strains with and without a *pit~phoA/lacZ* translational fusion. A. Alkaline phosphatase activity of RmP4192 (Δ*pap-pit, phoX*-) with *pit~phoA/lacZ* fusions was measured. The overall level of activity was very low (less than 7 MU) and the strains with the *pit~phoA/lacZ* translational fusion did not have a much higher level of activity compared to those that did not have a fusion. The *pap-pit~phoA/lacZ* strain showed higher levels of activity than the *pap*^{P75A}*-pit~phoA/lacZ*, suggesting that the level of pit translation in the *pap*^{P75A} mutant is lower than in wildtype. **B.** Alkaline phosphatase activity comparison of *pit~phoA/lacZ* constructs in a *phox*+ (RmP3926) and *phoX*- (RmP4192) background. The background level of activity in the RmP3926 background was much higher making differences between the constructs harder to identify. Thus, the RmP4192 background was better for testing the *pit~phoA/lacZ* constructs. Values are the average of three technical replicates +/- standard error.

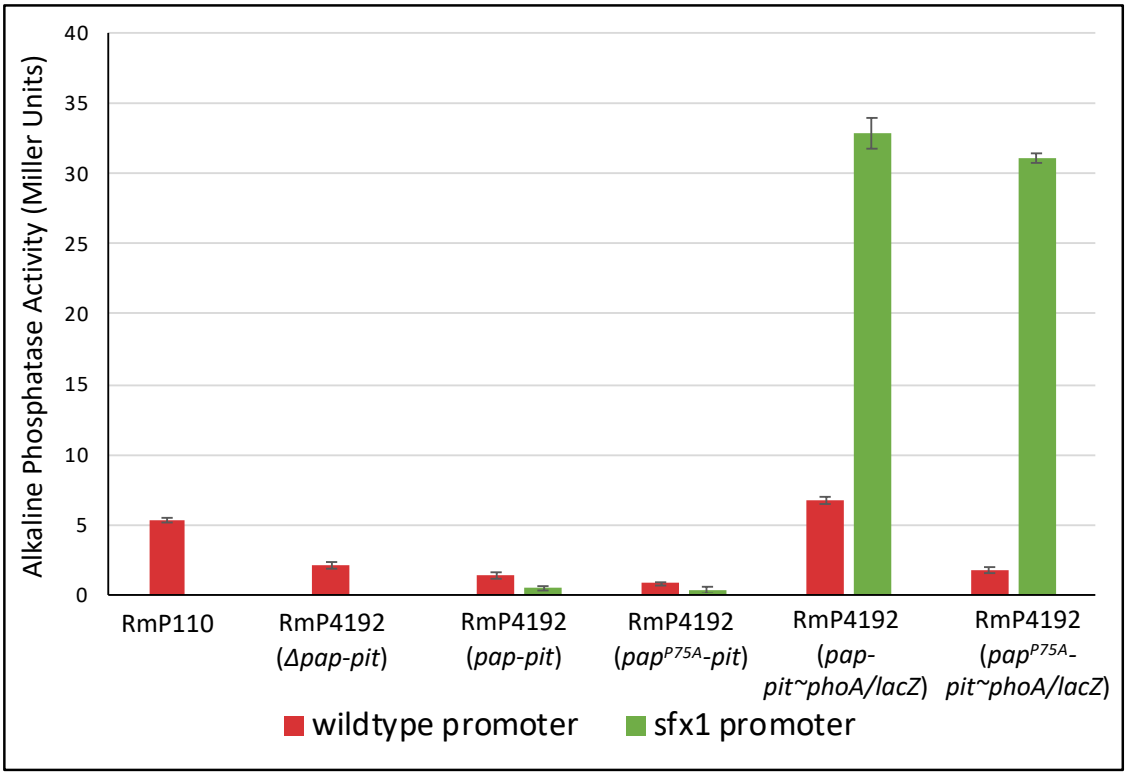


Figure 3.11. Alkaline phosphatase activity of RmP4192 (*phoX*-) strains with *pit~phoA/lacZ* translational fusions under wildtype and *sfx1* promoters. The *sfx1* promoter greatly increased the level of alkaline phosphatase activity of the *pit~phoA/lacZ* translational fusion strains indicating increased levels of Pit translation. The level of activity in the *sfx1 pap-pit~phoA/lacZ* and *sfx1 pap*^{P75A}*-pit~phoA/lacZ* was similar between the two strains suggesting similar levels of Pit in the cell. The level of activity of strains without a *pit~phoA/lacZ* translational fusion is not increased under the *sfx1* promoter, as expected. Values are the average of three technical replicates +/- standard error.

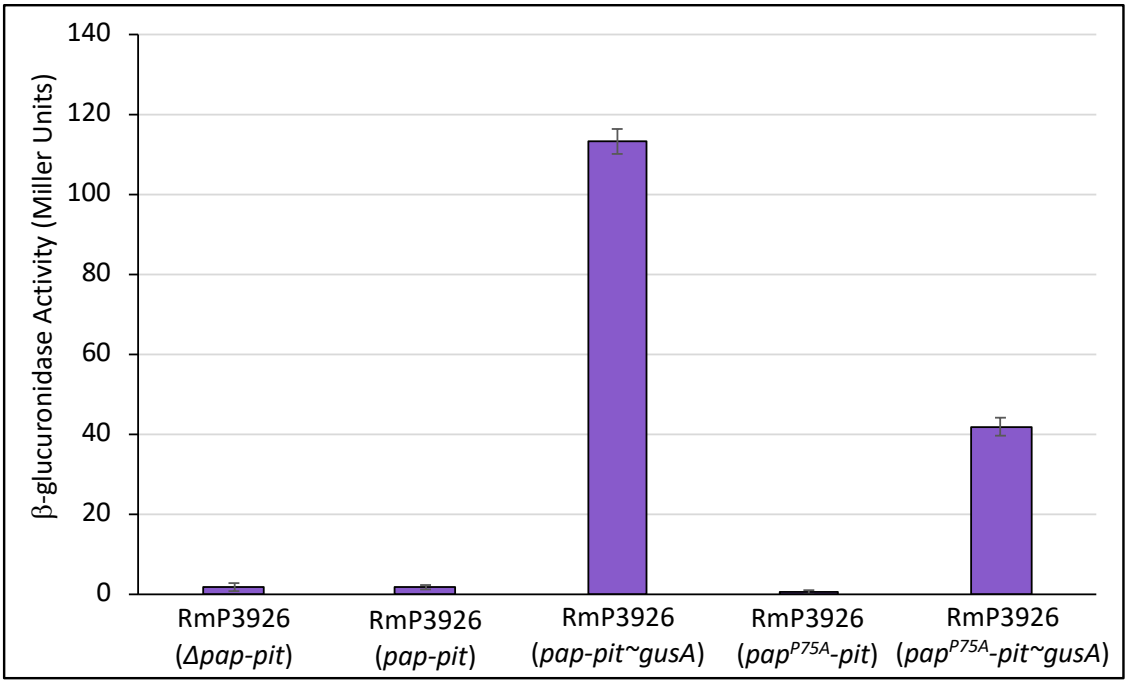


Figure 3.12. β-glucuronidase activity of *S. meliloti* RmP3926 (*Δpap-pit*) strains with and without a *pit~gusA* translational fusion. Strains without a *pit~gusA* translational showed low levels of background activity (2 MU or less). RmP3926 with *pap-pit~gusA* showed a higher level of β-glucuronidase activity than RmP3926 with pap^{P75A}*-pit~gusA*, indicating less Pit translation in the pap^{P75A} mutant. Values are the average of three technical replicates +/- standard error.

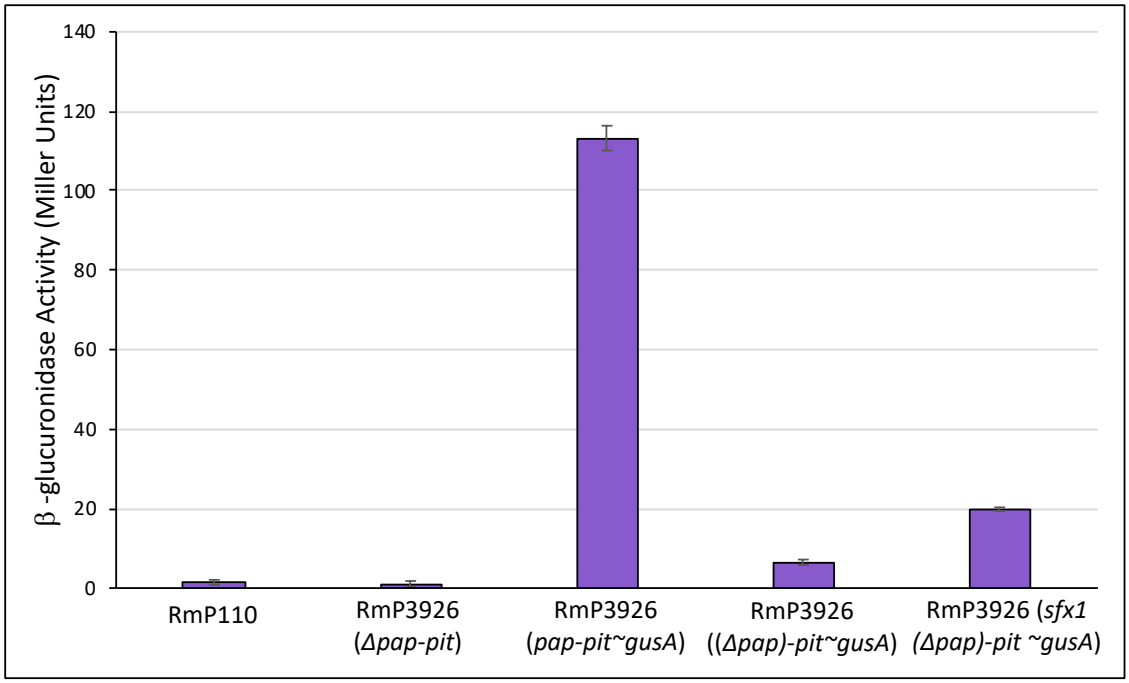


Figure 3.13. β-glucuronidase activity of *S. meliloti* RmP3926 (*Δpap-pit*) strains with a (*Δpap*)-*pit~gusA* translational fusion under wildtype and *sfx1* promoters. Strains without a *pit~gusA* translational showed low levels of background activity (2 MU or less). RmP3926 with *pap-pit~gusA* showed a higher level of β-glucuronidase activity than RmP3926 with (Δ*pap*)-*pit~gusA* under both the wildtype and *sfx1* promoters, indicating less Pit translation in the Δ*pap* mutant. The *sfx1* promoter did increase *pit* translation slightly of the (Δ*pap*)-*pit~gusA* construct but it was still much less than wildtype. Values are the average of three technical replicates +/- standard error.

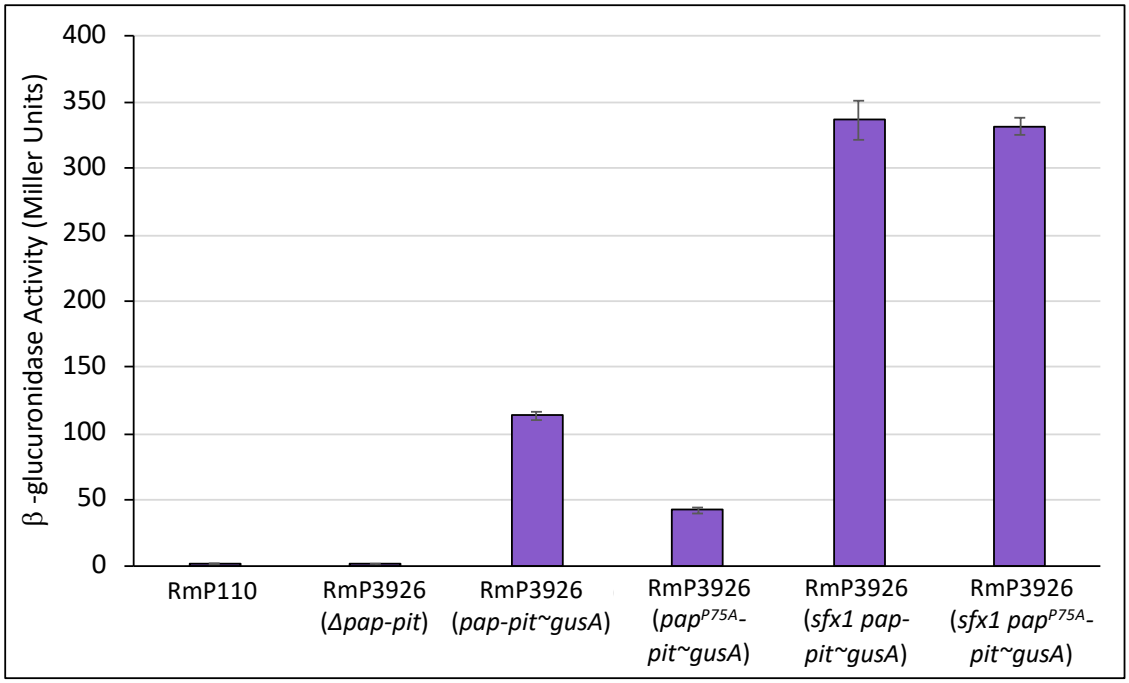


Figure 3.14. β-glucuronidase activity of *S. meliloti* RmP3926 (*Δpap-pit*) strains with a *pap^{P75A}-pit~gusA* translational fusion under wildtype and *sfx1* promoters. Strains without a *pit~gusA* translational showed low levels of background activity (2 MU or less). RmP3926 with *pap-pit~gusA* showed a higher level of β-glucuronidase activity than RmP3926 with *pap^{P75A}-pit~gusA* under the wildtype *pap* promoter, indicating less Pit translation in the *pap^{P75A}* mutant. However, under the *sfx1* promoter, both *pap-pit~gusA* and *pap^{P75A}-pit~gusA* showed similar levels of activity that were approximately three times greater than that of *pap-pit~gusA* under the wildtype promoter. This suggested increased translation of Pit under the *sfx1* promoter. Values are the average of three technical replicates +/- standard error.

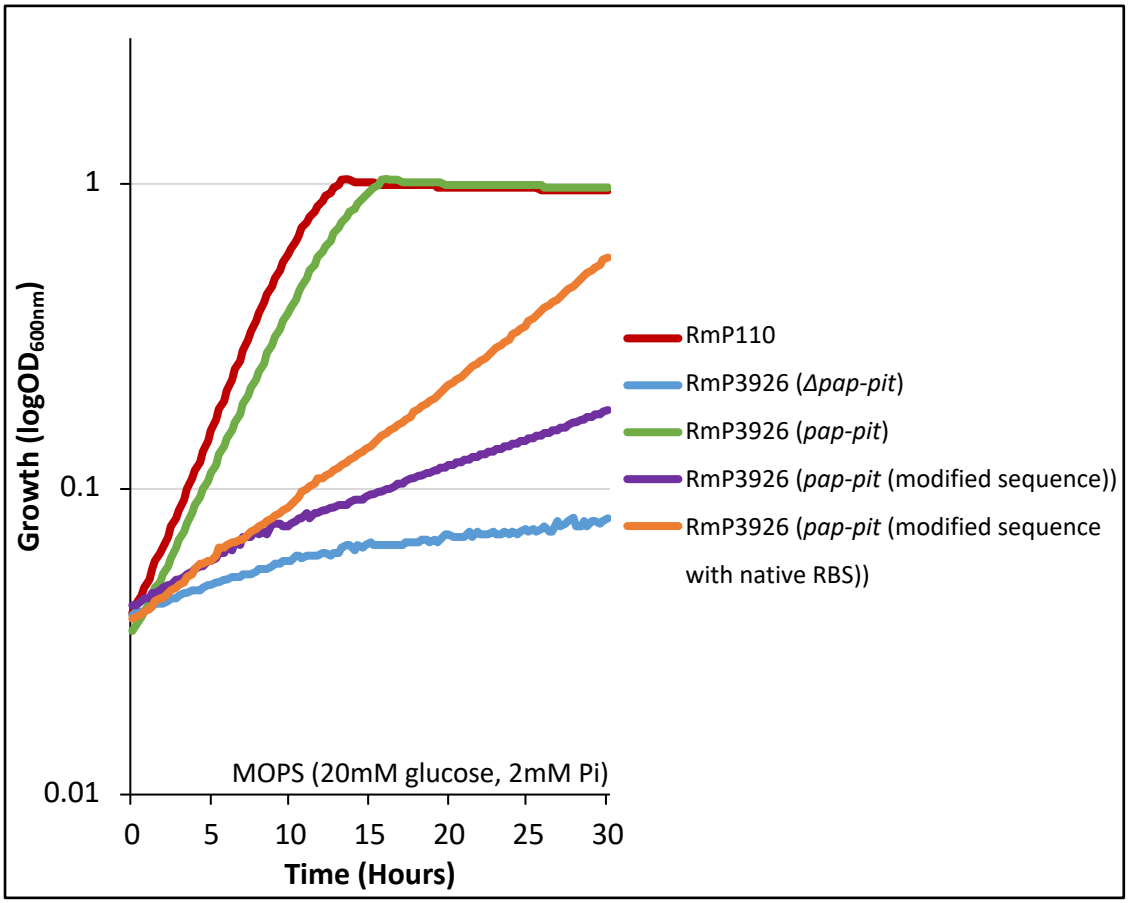


Figure 3.15. Growth of *S. meliloti* RmP3926 (*Δpap-pit*) with a modified *pap-pit* nucleotide sequence in minimal medium. *S. meliloti pap-pit* was reconstructed to create a new nucleotide sequence but maintained the same amino acid sequence and codon usage of each gene (pTH3325). A variant was also made with the modified sequence but with the native RBS (pTH3334). Neither plasmid was able to fully complement RmP3926 (Δ*pap-pit*), but RmP3926 with pTH3334 grew better than RmP3926 with pTH3325.

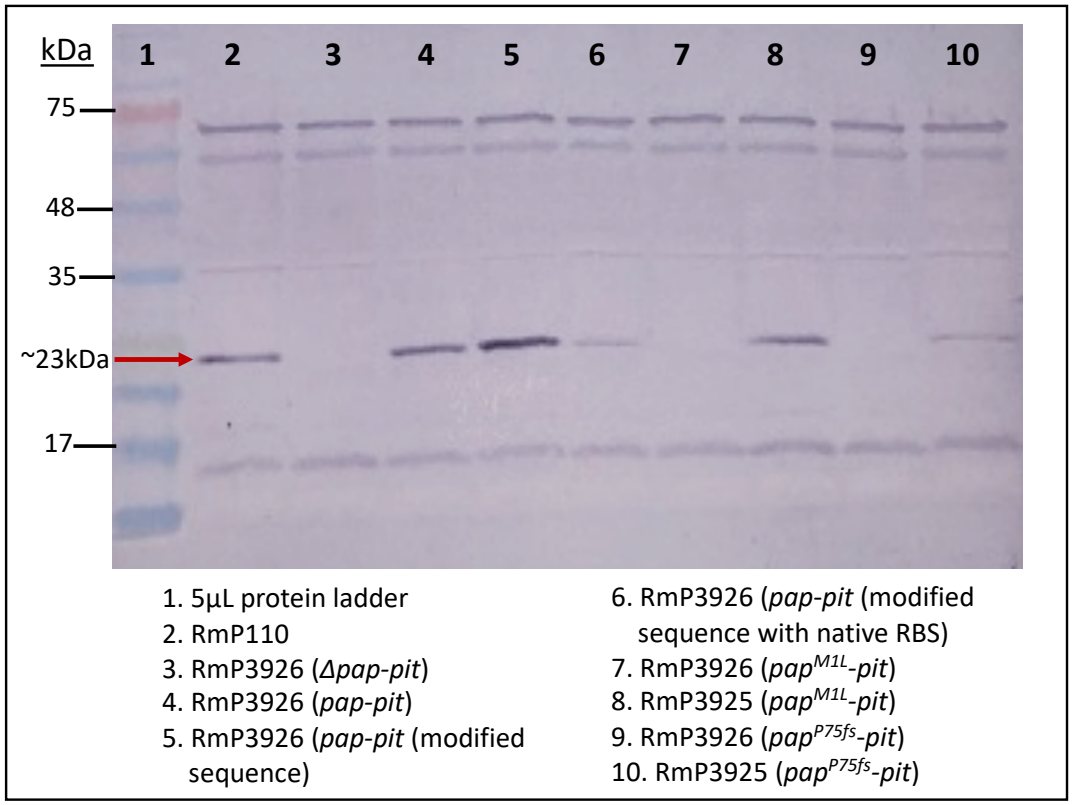


Figure 3.16. Western blot of *S. meliloti* RmP3926 (*Δpap-pit*) with *pap* point mutants and modified *pap-pit* sequence. RmP110 was used as a positive control for wildtype Pap with the red arrow indicating the band of the appropriate size for Pap at approximately 23 kDa. RmP3926 (Δ*pap-pit*) was used as a negative control for Pap. RmP3926 with wildtype *pap-pit* integrated into the chromosome gave a band similar to RmP110. RmP3926 with *pap-pit* with the modified sequence (pTH3325) gave a band at 23 kDa that was darker than the wildtype band. RmP3926 with *pap-pit* with the modified sequence with the native RBS for *pit* (pTH3334) gave a band that was lighter than wildtype at 23 kDa. RmP3926 (Δ*pap-pit*) with *pap^{M1L}-pit* did not give a band for Pap, but RmP3925 (Δ*pit*) with *pap^{M1L}-pit* gave a band similar to wildtype, likely coming from the wildtype *pap* under the separate promoter. RmP3926 with *pap^{P75fs}-pit* did not give a band for Pap, but RmP3925 with *pap^{P75fs}-pit* did give a faint band at 23 kDa for Pap likely coming from the wildtype *pap* under the separate promoter.

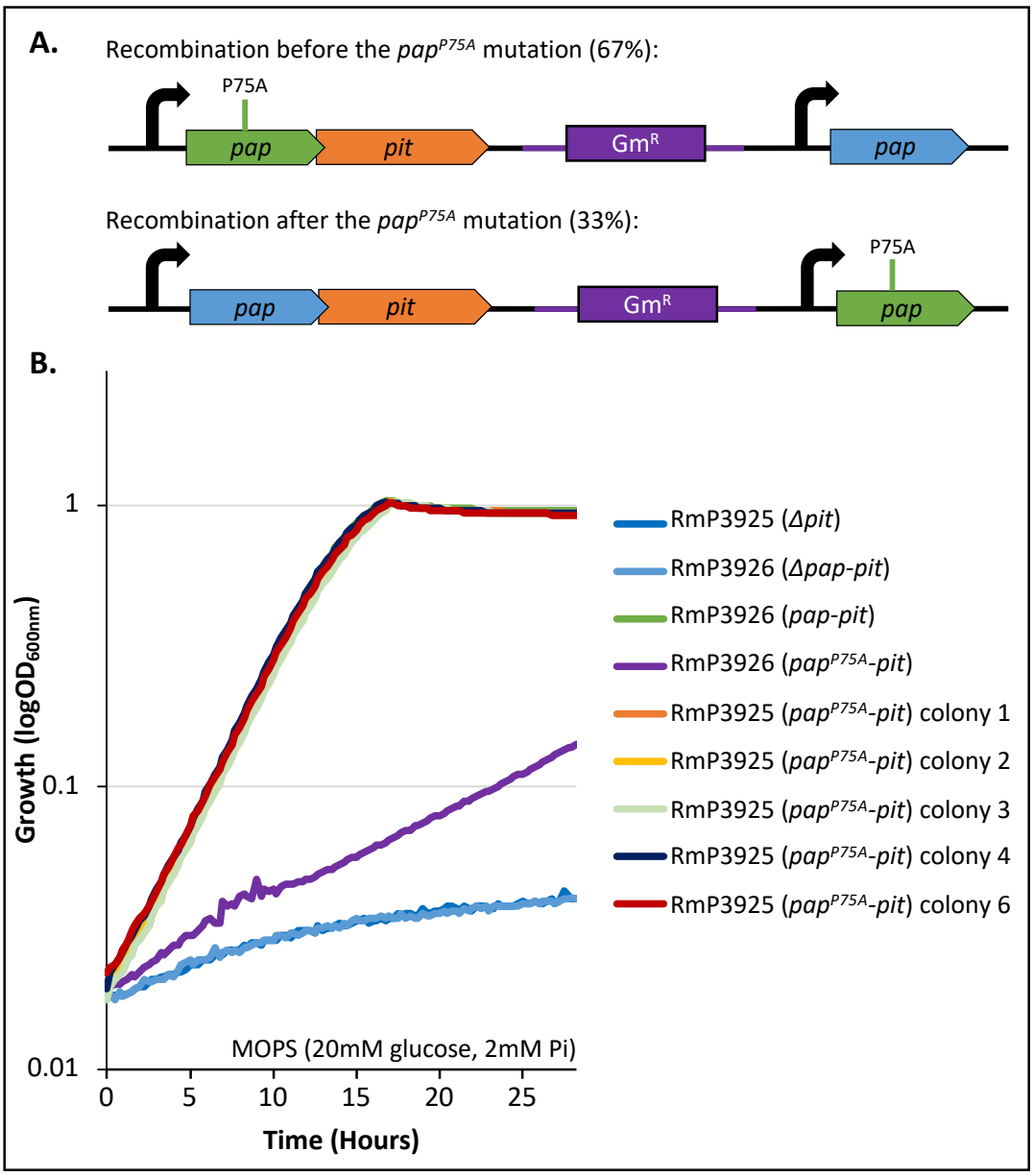


Figure 3.17. Growth of *S. meliloti* RmP3926 (*Δpap-pit*) and RmP3925 (*Δpit*) with *pap^{P75A}-pit* integrated into the chromosome in minimal medium. A. Integration of *pap^{P75A}-pit* into RmP3925 resulted in two recombinants: one in which the wildtype *pap-pit* operon was restored with *pap^{P75A}* under a separate promoter and a second in which wildtype *pap* was under a separate promoter from *pap^{P75A}-pit*. **B.** RmP3926 with *pap^{P75A}-pit* grew moderately well, but not as well as RmP3926 with wildtype *pap-pit*. All five recombinants of *pap^{P75A}-pit* in RmP3925 grew like RmP3926 with wildtype *pap-pit* and after sequencing, 4 of the 5 colonies were found to have the *pap^{P75A}-pit* operon with wildtype *pap* downstream. This indicated that the wildtype *pap* was able to restore the normal functioning of the *pap^{P75A}-pit* operon, even though it was transcribed and translated from a separate promoter.

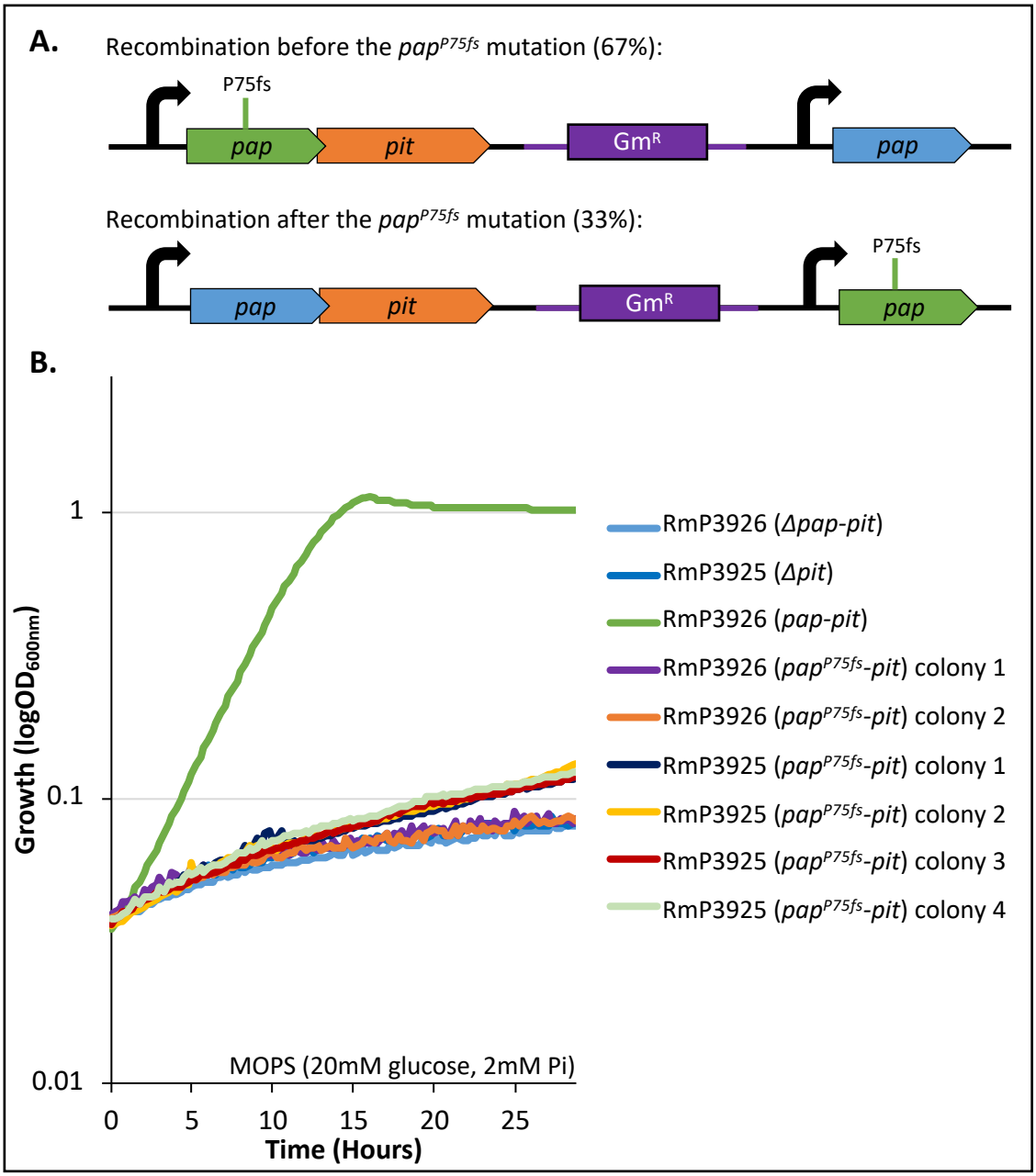


Figure 3.18. Growth of *S. meliloti* RmP3926 (*Δpap-pit*) and RmP3925 (*Δpit*) with *pap^{P75fs}-pit* integrated into the chromosome in minimal medium. A. Integration of *pap^{P75fs}-pit* into RmP3925 resulted in two recombinants: one in which the wildtype *pap-pit* operon was restored with *pap^{P75fs}* under a separate promoter and a second in which wildtype *pap* was under a separate promoter from *pap^{P75fs}-pit*. **B.** RmP3926 with *pap^{P75fs}-pit* grew poorly in MOPS-P2. All four recombinants grew poorly with *pap^{P75fs}-pit* in RmP3925. Colony 1 and 2 were sequenced for RmP3925 (*pap^{P75fs}-pit*) and confirmed to have *pap^{P75fs}-pit* as an operon with wildtype *pap* downstream. This wildtype *pap* did not allow for functional Pi transport and growth in these recombinants.

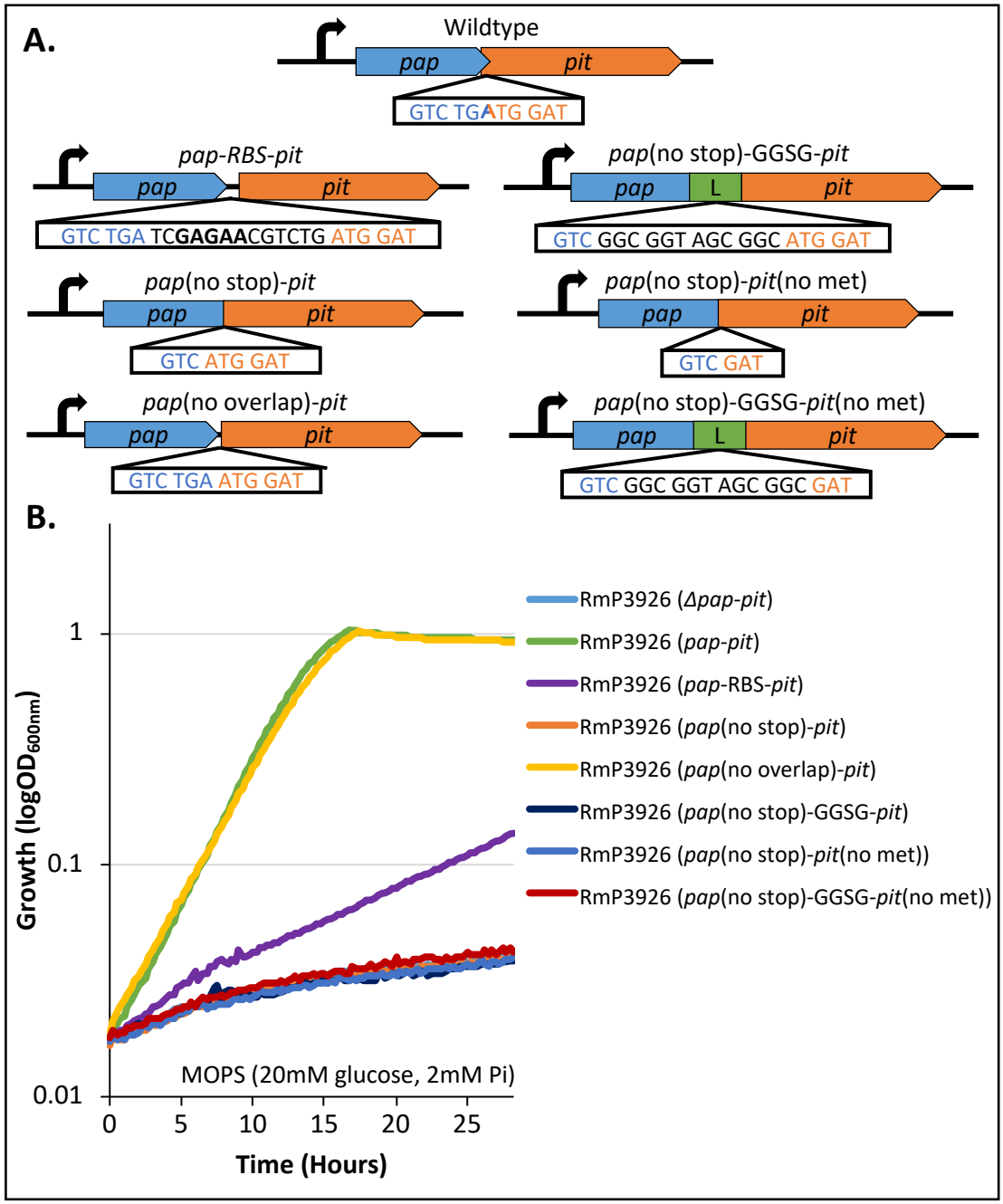


Figure 3.19. Growth of *S. meliloti* RmP3926 (*Δpap-pit*) with *pap-pit* gene fusion constructs in minimal medium. A. *S. meliloti pap-pit* gene fusion constructs that were cloned into pUCP30T and recombined into RmP3926 (Δ*pap-pit*). **B.** RmP3926 with *pap*-RBS-*pit* (pTH3335) grew moderately well and RmP3926 with *pap*(no overlap)-*pit* (pTH3337) grew like RmP3926 with wildtype *pap-pit*. These constructs were the only two that still had the *pap* stop codon. RmP3926 with *pap*(no stop)-*pit* (pTH3336), or *pap*(no stop)-GGSG-*pit* (pTH3338), or *pap*(no stop)-*pit*(no met) (pTH3339), or *pap*(no stop)-GGSG-*pit*(no met) (pTH3340) did not grow.

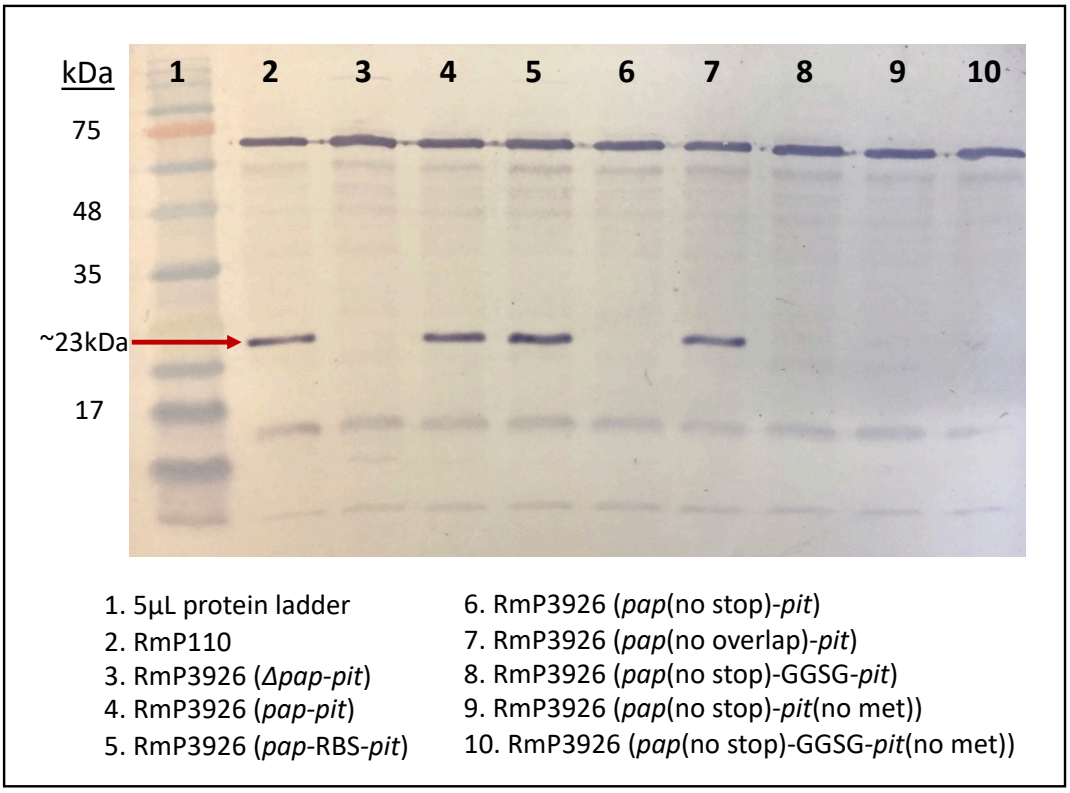


Figure 3.20. Western blot of *S. meliloti* RmP3926 (*Δpap-pit*) with *pap-pit* gene fusions. RmP110 was used as a positive control for wildtype Pap with the red arrow indicating the band of the appropriate size for Pap at approximately 23 kDa. RmP3926 (Δ*pap-pit*) was used as a negative control for Pap. RmP3926 with wildtype *pap-pit* integrated into the chromosome gave a band similar to RmP110. RmP3926 with *pap*-RBS-*pit* (pTH3335) and RmP3926 with *pap*(no overlap)-*pit* (pTH3337) gave bands for the appropriate size of Pap. These constructs were the only two that still had the *pap* stop codon. RmP3926 with *pap*(no stop)-*pit* (pTH3336), or *pap*(no stop)-GGSG-*pit* (pTH3338), or *pap*(no stop)-*pit*(no met) (pTH3339), or *pap*(no stop)-GGSG-*pit*(no met) (pTH3340) did not give any band at 23 kDa for Pap.

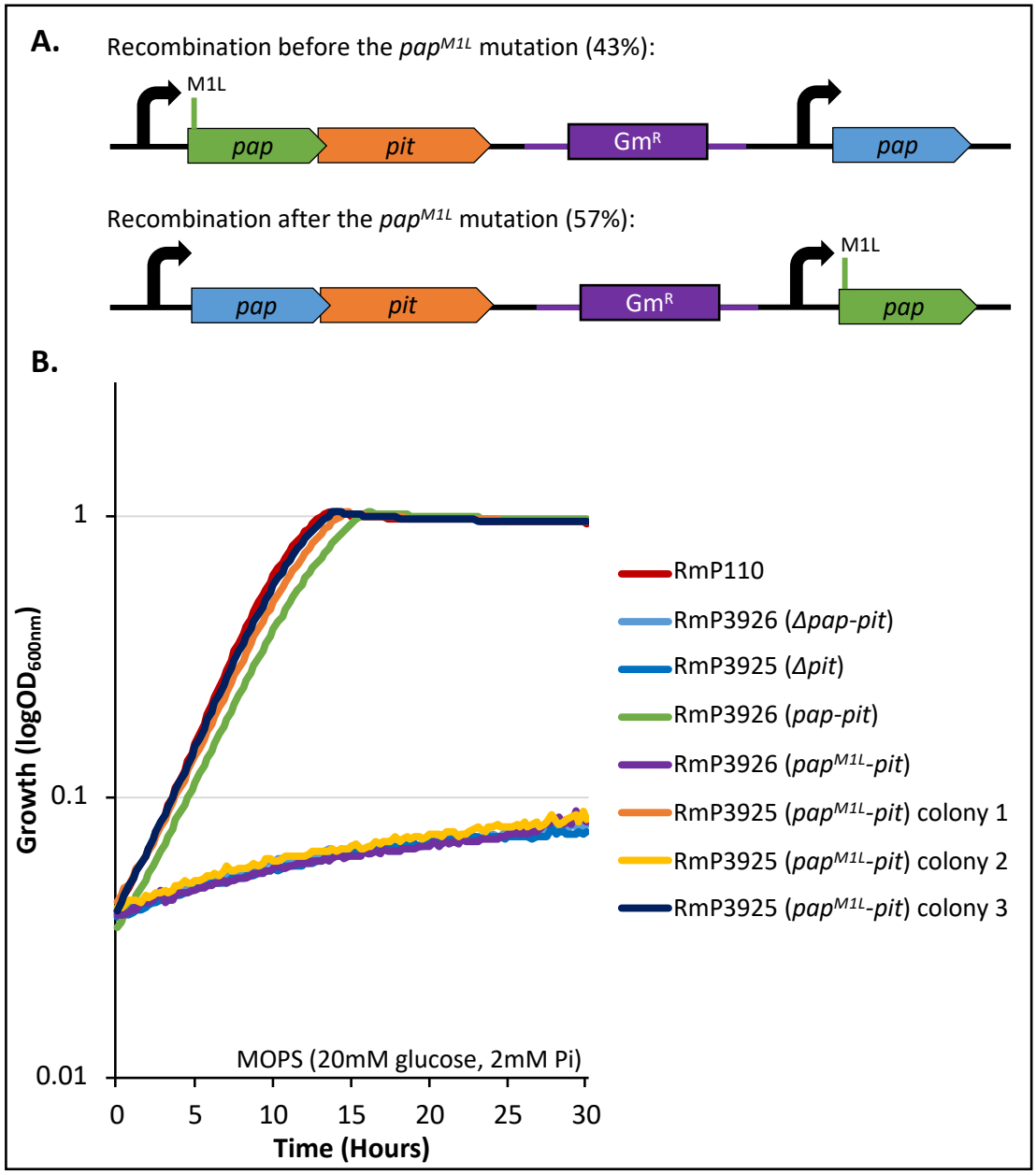


Figure 3.21. Growth of *S. meliloti* RmP3926 (*Δpap-pit*) and RmP3925 (*Δpit*) with *pap^{M1L}-pit* integrated into the chromosome in minimal medium. A. Integration of *pap^{M1L}-pit* into RmP3925 (Δ*pit*) resulted in two recombinants: one in which the wildtype *pap-pit* operon was restored with *pap^{M1L}* under a separate promoter and a second in which wildtype *pap* was under a separate promoter from *pap^{M1L}-pit*. **B.** RmP3926 with *pap^{M1L}-pit* grew poorly in MOPS-P2. Three colonies were tested for recombination of *pap^{M1L}-pit* into RmP3925 and two grew like wildtype and the last grew poorly. The colony that grew poorly likely has *pap^{M1L}-pit* with wildtype *pap* downstream while the two colonies that grew well likely have *pap-pit* with *pap^{M1L}* downstream. This result suggested that the wildtype *pap* downstream of *pap^{M1L}-pit* was not able to allow for Pi transport and growth.

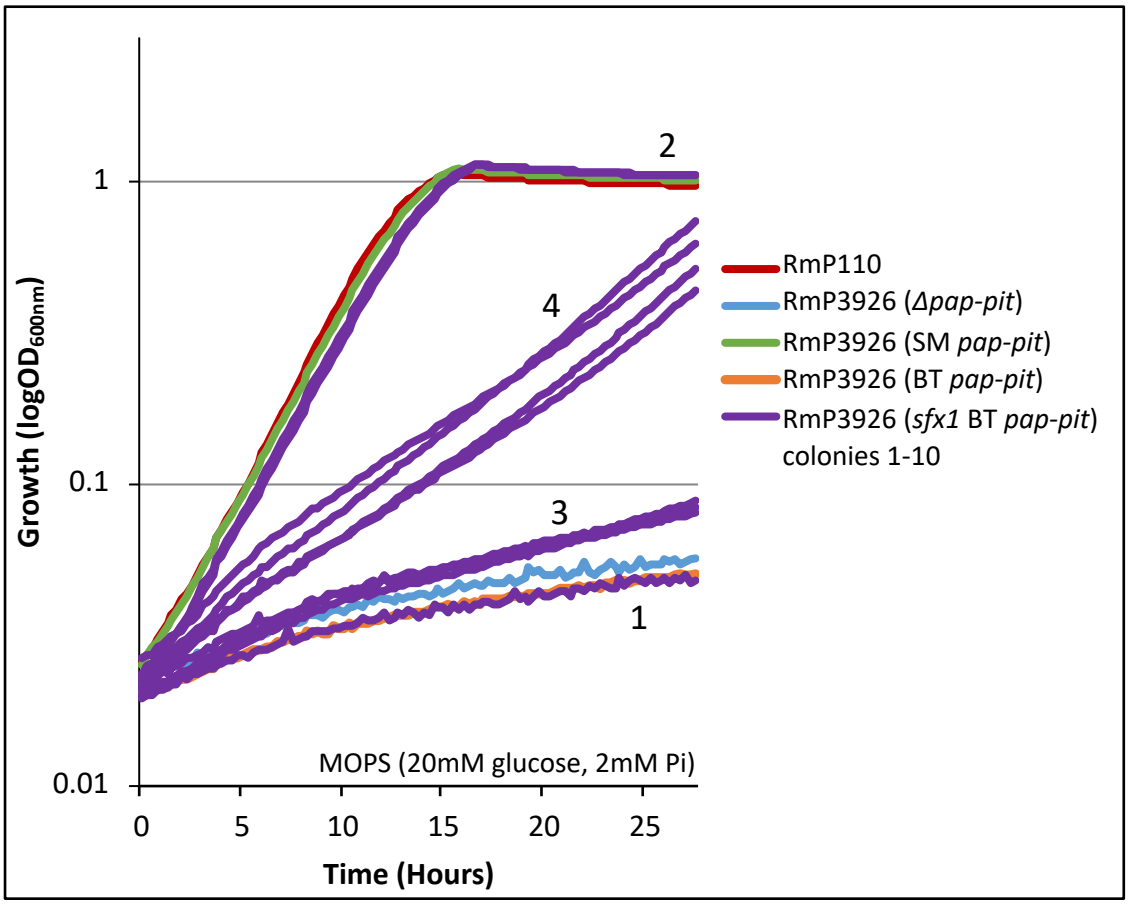


Figure 3.22. Growth of *S. meliloti* RmP3926 (*Δpap-pit*) with *Bacteroides pap-pit* under wildtype and *sfx1 S. meliloti pap* promoters in minimal medium. RmP3926 (Δ*pap-pit*) with *Bacteroides pap-pit* under the wildtype promoter did not grow in MOPS-P2. RmP3926 with *Bacteroides pap-pit* under the *sfx1* promoter showed 3 different growth phenotypes: 2 colonies grew like *S. meliloti* wildtype, 4 grew moderately well, and 4 grew poorly. After investigation, it appeared as though the moderate growth phenotype is the most likely representation of *S. meliloti* RmP3926 with *Bacteroides pap-pit* under the *sfx1* promoter.