Domain-wise biochemical characterization of *Myxococcus xanthus* FrzE

A thesis submitted towards partial fulfillment of the requirements of the BS-MS Dual Degree Program

Submitted by

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CERTIFICATE

This is to certify that this dissertation entitled " Domain-wise biochemical characterization of *Myxococcus xanthus* FrzE " towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Pawara Punam Sundarsing at Indian Institute of Science Education and Research (IISER), Pune under the supervision of Dr. Gayathri Pananghat, Associate Professor, Department of Biology, IISER Pune during the academic year 2021-2022.

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DECLARATION

I hereby declare that the matter embodied in the report entitled " Domain-wise biochemical characterization of *Myxococcus xanthus* FrzE " are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER), Pune under the supervision of Dr. Gayathri Pananghat and the same has not been submitted elsewhere for any other degree.

Pawara Punam Sundarsing

(20171088)

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ABSTRACT

A Gram-negative soil bacterium, *Myxococcus xanthus,* has a complex life cycle that is dependent on its motility. It is known to have two types of directed motility, social motility (S-motility) and gliding or adventurous motility (A-motility). This motility is controlled by the cell polarity reversal frequencies. The frequency of the reversal is modulated by the Frz pathway, a two-component signal transduction system present in *M. xanthus*, . This system consists of proteins homologous to those in the Che system - a chemoreceptor FrzCD, adapter proteins FrzA and FrzB, a fusion protein of a histidine kinase and a response regulator FrzE and a fusion protein of two response regulators FrzZ. FrzE plays a major role in regulating the reversal frequencies by autophosphorylation at the histidine kinase domain upon stimulation by FrzCD via FrzA and transfers the phosphoryl group to the downstream regulators. A histidine residue (His49) in the HPT domain of FrzE gets phosphorylated by the phosphoryl group released from the ATP after its hydrolysis by HATPase domain. Phosphoryl group is transferred from the FrzE_HPT domain to an aspartate residue (Asp709) of the FrzE_Rec domain and inhibits further phosphorylation of FrzE. Biochemical characterization of FrzE will give insights into the mechanism of the Frz pathway and directed motility in *M. xanthus*. In this thesis, domain wise biochemical characterization of FrzE is being carried out. FrzE_Dim_HATPase, FrzE_CheW and FrzE_Rec domains were cloned and purified. ATPase activity for the FrzE_Dim_HATPase was quantified using malachite green assay. The stability of FrzE_Rec was checked using circular dichroism spectroscopy and thermal shift assay. Co-expression for FrzE_Dim_HATPase and FrzE_HPT and FrzE_CheW_Rec and FrzA was carried out for better expression of FrzE_Dim_HATPase and FrzA.

CHAPTER 1
INTRODUCTION

1.1 Motility in *Myxococcus xanthus*:

A Gram-negative, rod shaped, soil bacterium *Myxococcus xanthus*, present in biofilms has a complex life cycle that includes two phases, vegetative growth and development. On nutrient rich surfaces, *M. xanthus* cells are in a vegetative growth phase and show social behavior such as swarms and predation. A swarm is an organized group of cells that take up nutrients or prey on microorganisms by moving in a coordinated manner. A coordinated rhythmic movement of cells known as rippling is observed during the predation, which helps the swarm to remain in the area around the prey, efficiently lyse it and absorb the nutrients (Berleman et al., 2006). During nutrient depletion or unavailability of prey, *M. xanthus* cells enter into the development phase and start to aggregate, forming multicellular mounds that further result in the formation of fruiting bodies. The fruiting bodies contain cells that have differentiated into spherical spores, whereas cells near and outside of the fruiting bodies remain rod shaped, called as peripheral rods, and continue to search for nutrients. On availability of the nutrients, spores germinate to form rod-shaped cells and enter a vegetative growth phase. Motility plays an essential role in the life phases of *M. xanthus*.

M. xanthus is non-motile in liquid since it lacks flagella but is motile on solid surfaces. *M. xanthus* cells move along in the direction of the long axis mediated by two motility systems, Twitching or Social motility (S-Motility) used when the cells are in a group and Adventurous motility (A-motility) used by individual cells (Hodgkin and Kaiser, 1979a,b; Zusman et al., 2007). Motility systems are polarized and have a leading and a lagging pole. Type IV pili is used for social motility (Sun et al., 2000) localized at the leading cell pole which binds to the exopolysaccharides (EPS) present on the neighboring cells or

on the substrates (SS Wu and D Kaiser, 1995). The cycle of expansion and retraction upon binding of type IV pili to the EPS moves the cell in the forward direction. Adventurous motility depends on Agl/Glt focal adhesion complexes (Mignot et al., 2007). These complexes assemble at the leading cell pole and attach to the substrate. It gets translocated toward the lagging pole leading to the forward movement of a cell body.

M. xanthus cells show a reversal, a switch in the direction of the leading pole and the lagging pole. The reversal is in response to external environmental signals, known as chemotaxis, which leads to the biased, directed movement of the cell (Zusman et al., 2007). The frequency of the reversal is controlled by the Frz pathway (Blackhart and Zusman, 1985).

1.2 The Frz pathway:

Frz pathway is a two-component signal transduction system homologous to the chemosensory systems such as the Che system present in enteric bacteria like *Escherichia coli* or *Thermotoga maritima* (McBride et al., 1989). A general Che chemosensory system consists of methyl-accepting chemoreceptors (MCPs), a histidine kinase (CheA), an adaptor protein (CheW), a response regulator (CheY), methylation (CheR) and demethylation (CheB) proteins in adaptation modules. In the Che system, autophosphorylation of CheA is induced due to the stimulation of MCPs by binding to specific ligands. CheA transfers the phosphoryl group to CheY and induces a flagellar rotation switch from counterclockwise to clockwise.

The Frz pathway in *M.xanthus* has FrzCD as the MCP, two adaptor proteins, FrzA and FrzB, FrzE, a fusion protein of histidine kinase and response regulator and two more response regulators, FrzZ and FrzX. In the Frz pathway, FrzE gets autophosphorylated when FrzCD binds to specific ligands. At low stimulation levels, the phosphoryl group is transferred to the Rec domain of FrzE, homologous to CheY. Whereas, at high

stimulation levels, phosphate is transferred to FrzZ, which positively regulates the activity of FrzE and keeps it in the kinase-on state (Inclan et al., 2007, Kaimer et al., 2016). The phosphoryl group is then transferred to FrzX (Guzzo et al., 2018) and eventually the downstream response regulators present in the polarity module that establishes the reversal.

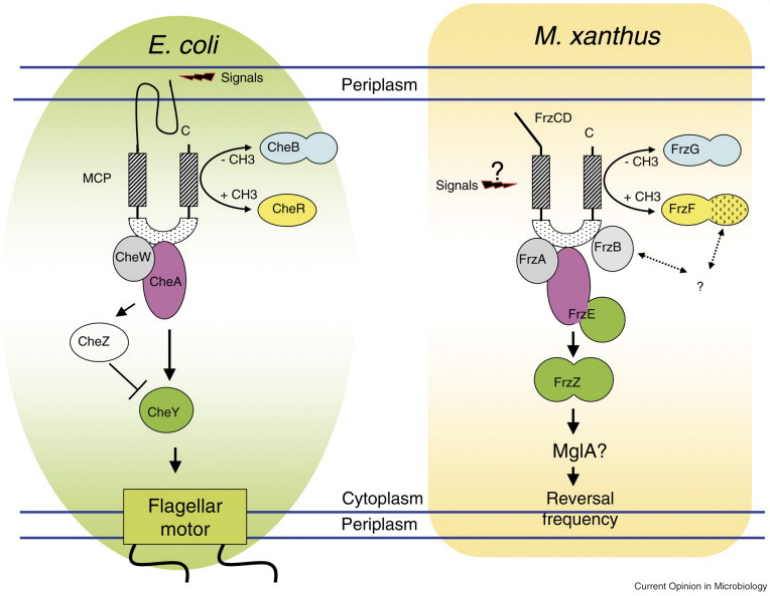


Fig 1.1: Chemosensory systems in *E. coli* and *M. xanthus* (Mauriello et. al., 2007).

FrzE, the histidine kinase, plays an essential role in the signal transduction in the Frz pathway. Hence, understanding the mechanism of FrzE kinase activity and the phosphorylation pathway will provide insights into the regulatory mechanism of the motility reversal in *M. xanthus.*

1.3 FrzE : A fusion protein of histidine kinase and response regulator

FrzE is a fusion of histidine kinase and a response regulator. FrzE protein has five major domains, Histidine Phosphotransfer (HPT), Histidine kinase dimerization, Histidine kinase ATPase (HATPase), CheW-like domain and a response regulatory domain (REC) along with a low-complexity region between HPT and Histidine kinase dimerization domain.

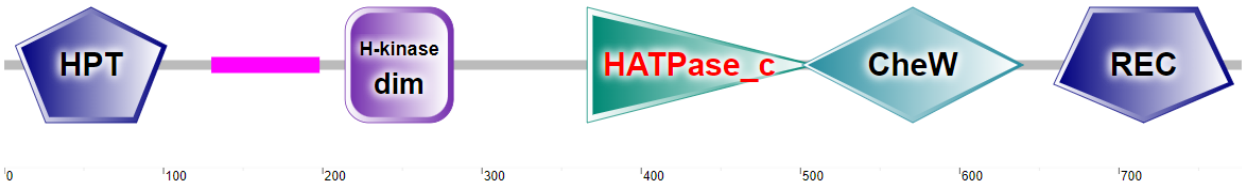


Fig 1.2: Domains of FrzE histidine kinase (from SMART).

FrzE receives a signal from FrzCD via FrzA at a CheW-like domain of FrzE. HATPase domain acts as an ATPase and hydrolyzes ATP releasing the phosphoryl group. FrzE gets phosphorylated at the histidine residue (His49) present in the HPT domain. At low stimulation levels, phosphoryl group from HPT domain is transferred to the aspartate residue (Asp709) of the Rec domain and inhibits further phosphorylation of FrzE (Inclan et al., 2008).

1.3.1 Histidine Phosphotransfer (HPT) domain:

The histidine Phosphotransfer (HPT) domains are known to have a conserved histidine residue that gets phosphorylated. Highly conserved residues, two glycines and glutamic acid, are present in the vicinity of the histidine that has a role in stabilizing the phosphohistidine and transfer of phosphoryl group to the regulatory domain. HPT domain of FrzE has a conserved His49 residue surrounded by Gly53, Gly59 and Glu71 (Xu et al., 1999).

1.3.2 Dimerization and Histidine kinase ATPase (HATPase) domain:

The dimerization domain consists of two helices that interact with the other dimerization domain to form a four-helix bundle, resulting in a homodimer of histidine kinase.

Histidine kinase ATPase is the main catalytic domain which hydrolyses the ATP. It has an ATP binding pocket where ATP and magnesium bind and hydrolysis takes place. Histidine kinases have four highly conserved motifs, N box, G1 box, F-box and G2 box (Bilwes et al., 1999). Conserved hydrophilic residues present in these boxes are involved in the magnesium and ATP binding, its stabilization and borders the ATP binding pocket, while the conserved hydrophobic residues play a role in lining the ATP binding site. The N box has a conserved Asparagine residue which helps stabilize the magnesium ion during the ATPase activity. Conserved glycine residues present in the G1 box are involved in protecting the adenosine of ATP from water molecules by clustering around the adenosine. The phosphate group in ATP is secured by the conserved glycine residues in the G2 box and the phenylalanine residues in the F box (Bilwes et al., 1999).

In FrzE, the histidine kinase domain, HATPase, contains the conserved motifs with Asn377 in the N box, Gly419 and Gly421 in the G1 box, Gly472 and Gly474 in the G2 box and Phe455 in the F box (Meenakshi PV, MS thesis, May 2022).

1.3.3 CheW-like domain:

The CheW-like domain of a histidine kinase is homologous to the adaptor protein, CheW and interacts with CheW. This interaction is necessary for receiving the signal from MCP and stimulating the kinase activity (Zhao et al., 2006). The CheW-like domain of histidine kinase and CheW interacts via two interfaces where at one interface, the subdomain-1 of the CheW-like domain interacts with the subdomain-2 of CheW while at the second interface, subdomain-2 of the CheW-like domain interacts with the

subdomain-1 of CheW. This interface contains the conserved hydrophobic residues on both CheW-like and CheW subdomains.

The Frz pathway contains two adaptor proteins, FrzA and FrzB. FrzE is shown to interact with FrzA but not with FrzB because of the lack of amino acid sequence that interacts with the CheW domain of FrzE (Guiseppi et al., 2019).

1.3.4 Response regulatory (Rec) domain:

Response regulatory or receiver (Rec) domains function as an output of the chemosensory systems and have conserved Aspartate residue. Phosphoryl group from the histidine residue of the HPT domain transfers to the aspartate residue of the regulatory domain. There are four known regulatory domains present in the Frz pathway, one of them is the Rec domain in FrzE. This domain acts as a phosphate sink at low stimulation levels and prevents the noisy activation of the Frz pathway by negatively regulating the autophosphorylation activity of FrzE (Inclan et al., 2008).

Table 1.1: Domains of FrzE and their functions.

Domain	Function
Histidine Phosphotransfer (HPT)	mediates phosphotransfer reactions
Histidine kinase dimerization	homodimer interface
Histidine kinase ATPase (HATPase)	ATPase
CheW-like	Interacts with adapter protein FrzA
Response regulatory (REC)	negatively regulates the autophosphorylation of the kinase domain

In order to characterize the kinase activity and the phosphotransfer event of FrzE, it is essential to understand the role of individual domains. Therefore, we wanted to biochemically characterize the individual domains. This thesis reports the cloning, expression and purification of FrzE_Dim_HATPase, FrzE_CheW and FrzE_Rec domain. Co-exprssion was carried out for FrzE_Dim_HATPase and FrzA. Further, assays were performed to obtain kinetic parameters of ATP hydrolysis activity of the ATPase domain.

CHAPTER 2
MATERIAL AND METHODS

To characterize the individual domains, FrzE_Dim_HATPase, FrzE_CheW and FrzE_Rec constructs of individual domains and a combination of domains with affinity tags were obtained. Protein expression of the constructs was standardized and then followed by the purification trials. Assays were performed with the purified protein to check their activity. Detailed steps for cloning, expression check, purification and the assays are listed below.

2.1 Domain boundary determination:

Individual domain boundaries were determined to clone the FrzE_CheW and FrzE_Rec domain. FrzE_Dim_HATPase domain boundary was identified by Meenakshi PV (MS thesis, May 2022). Standard protein BLAST (a sequence similarity search program) (McGinnis et al., 2004) alignment of the protein sequence of individual domains was performed against PDB data to identify the homologous proteins with available structures. Structures of the homologous proteins were obtained from PDB (rcsb.org). The domain boundaries of the homologous proteins were checked in UCSF Chimera (Pettersen et al., 2014) and the corresponding residues were identified for domains FrzE_CheW and FrzE_Rec, based on the alignment. Secondly, the secondary structure information of the domains was obtained from PSIPRED (McGuffinet al., 2000) and used to confirm the residues at the start and end of secondary structures flanking the domains. Based on this information, domain boundaries were determined and primers were designed as listed in Table 2.1.

2.2 Cloning of the domains:

FrzE_Dim_HATPase, FrzE_CheW, FrzE_Rec and FrzE_CheW_Rec domains and kan constructs, pHis17_FrzE_HPT_NHis and pHis17_FrzA_CHis were cloned using the restriction-free cloning method (van den Ent and Lowe, 2006). Table 2.1 lists the primers and templates used for the specific cloning strategies.

Table 2.1: List of the primers and template used for the cloning (G.S: Gene specific primer; OH primer: Overhang; RF PCR: Restriction free PCR)

Clone	Forward primer	Reverse primer	Template
pHis17_FrzE_Dim_ HATPase_ CStrep	G.S.: 5'-CCGAGCGCCGCG AAGTCCGCGGTGGC CGA-3' OH: 5'-CTTTAAGAAGGAG ATATACATATGCCGAG CGCCGCGAAGTCCG CGGTGGCCGA-3'	G.S.: 5'-CGGCAGGCGCAG GGTGATGGTGGAGC CA-3' OH: 5'-ATTTTTCGAACTG AGGATGAGACCAGG ATCCCGGCAGGCG CAGGGTGATGGTGG AGCCA-3'	PCR1: pETPhos_FrzE_ΔRec RF PCR: pHis17_RomX_CStrep
pHis17_FrzE_CheW_ CStrep	G.S.: 5'-TCGCTGGCGTTGA TGAAGGTGCTGCT-3' OH: 5'-CTTTAAGAAGGAG ATATACACATGCGTG GCCATATGTCGCTGG CGTTGATGAAGGTG CTG-3'	G.S.: 5'-CTGGGTGACGGG GCGGGCCATCCGTC -3' OH: 5'-TTATTTTTCGAAC TGAGGATGAGACCA GGATCCCTGGGTGA CGGGGCGGGCCAT-3'	PCR1: pETPhos_FrzE_ΔRec RF PCR: pHis17_RomX_CStrep
pHis17_FrzE_Rec_ CStrep	G.S.: 5'-CGCCTCCGGGTG CTGCTGGTGGACGA-3' OH:	G.S.: 5'-GGTCAGCCGGTC GATGGCCTGCGCGA GAAC-3' OH:	PCR1: Genomic DNA RF PCR: pHis17_RomX_CStrep

	5’-CTTTAAGAAGGAG ATATACACATGCGTG GCCATATGCGCCTCC GGGTGCTGCTGGTG GAC-3;	5’-TTATTTTTCGAAC TGAGGATGAGACCA GGATCCGGTCAGCC GGTCGATGGCCTGC GCGA-3’	
pHis17_FrzE_ CheW_Rec_ CStrep	5’-CTTTAAGAAGGAG ATATACACATGCGTG GCCATATGTCGCTGG CGTTGATGAAGGTG CTG-3’	5’-CACCAGCAGCAC CCGGAGGCGCTTG GCGGCGGGGGCCT GGGTGACGGGGCG GGCCAT-3’	PCR1: pHis17_FrzE_ CheW_CStrep RF PCR: pHis17_RomX_CStrep
pHis17_FrzE_Dim_ HATPase_N377A_ CStrep	5’-TGCACCTGCTGCG CGCATCGGTGGACC ACGGC-3’	5’-CGGCAGGCGCAG GGTGATGGTGGAGC CA-3’	PCR1 AND RF PCR: pHis17_FrzE_Dim_ HATPase_ CStrep
pHis17_FrzE_HPT_ NHis (Kan)	5’-GCTAAAGTTGTTAA AGACTTTAAATTGCC GCGCGGCAGCCACA TG-3’	5’-GTATATATGAGTAA ACTTGGTCTGACAG TTAGAAAAACTCATC GAGCATC-3’	RF PCR: pHis17_FrzE_HPT_ NHis (Amp)
pHis17_FrzA_CHis (Kan)	5’-GCTAAAGTTGTTAA AGACTTTAAATTGCC GCGCGGCAGCCACA TG-3’	5’-GTATATATGAGTAA ACTTGGTCTGACAG TTAGAAAAACTCATC GAGCATC-3’	RF PCR: pHis17_FrzA_CHis (Amp)

2.2.1 Cloning of FrzE_Dim_HATPase domain:

The FrzE_Dim_HATPase gene was amplified from the FrzE_ΔRec construct present in the pETPhos vector (obtained from Dr Emilia Mauriello’s lab). A gradient PCR (polymerase chain reaction) was carried out at annealing temperatures 55 ^{o}C, 58 ^{o}C and 61 ^{o}C using the gene specific primers, to optimize the temperature for the reaction. The PCR product was run on 1 % agarose gel to check for bands corresponding to the size of the *FrzE_Dim_HATPase* domain. Successfully amplified PCR reactions were pooled and PCR purified using a Qiagen kit. Vector specific overhangs were introduced into the

purified PCR product by PCR using overhang specific primers. The PCR product, along with the desired amplicon, had multiple non-specific bands, hence the band corresponding to the *FrzE_Dim_HATPase* domain with the vector overhangs was purified by gel extraction. pHis17 plasmid containing *romX* gene and a C-terminal strep tag was used as a template for the linear amplification of pHis17 from the 5' end of the megaprimer. PCR for a test containing a megaprimer, i.e. the *FrzE_Dim_HATPase* gene with overhangs, and control without megaprimer was performed at an annealing temperature of 60 ^{o}C. The PCR product was run on 1% agarose gel and *DpnI* digestion of the PCR product was carried out to remove the methylated parent plasmid. A reaction for both test and control was set up, containing 9 µL of PCR product and 1 µL of *DpnI* and was incubated at 37 ^{o}C for 3 hours. The digested product was transformed into NEB electrocompetent cells through electroporation. Six colonies, along with positive control with pETPhos and negative control with a colony from the control plate, were screened by colony PCR at an annealing temperature of 60 ^{o}C. In colony PCR, 1 µL of culture grown from individual colonies was used as a template. Plasmid purification of the positive clones from colony PCR was performed using a Qiagen kit and sent for sequencing for further confirmation.

2.2.2 Cloning of FrzE_CheW domain:

FrzE_CheW domain was amplified from the pETPhos_FrzE_ΔRec construct by gradient PCR at annealing temperatures, 57^{o}C and 60^{o}C using gene specific primers. The PCR product was run on 1 % agarose gel and PCR products were pooled and PCR purified using a Qiagen kit. Vector specific overhangs were introduced into the purified PCR product by PCR using overhang specific primers and the PCR product was run on 1 % agarose gel. PCR products were pooled and PCR purified. PCR for the linear amplification of the pHis17 vector was performed with pHis17_romX_CStrep as a template and *frzE_cheW* amplicon with vector specific overhang as a megaprimer at an annealing temperature of 58 ^{o}C. The PCR product was run on 1 % agarose gel and *DpnI* digestion was carried out. The digested product was transformed into NEB

electrocompetent cells through electroporation. Five colonies were screened by colony PCR at annealing temperature of 58 ºC. Plasmid purification was done for the positive clones from colony PCR and further screened by restriction enzyme digestion check. A 10 µL reaction containing 1 µL of plasmid (concentration around 150 ng/µL), 1 µL of 10X Cutsmart buffer and 0.5 µL of both *NdeI* and *BamHI* restriction enzymes was incubated at 37ºC for 12 hours. The digested products were run on the 1% agarose gel to check for the digested band size corresponding to the *frzE_cheW* gene. Positive clones were further confirmed by sequencing.

2.2.3 Cloning of FrzE_Rec domain:

Since the pETPhos_FrzE_ΔRec construct lacks the Rec domain, genomic DNA from *Myxococcus xanthus* was used as a template for cloning.

Genomic DNA purification of *Myxococcus xanthus:*

M. xanthus (strain: DK1622) cells stored in the glycerol stock were plated on the CYE-AMP agar plate and incubated at 32 ºC for four days. The cells were inoculated in 10 ml CYE media with 0.1 mg/ml ampicillin and incubated at 32 ºC for four days. The culture was pelleted down and CTAB (Cetyl trimethylammonium bromide) (Coleman Lab, 2021) method was used to purify the genomic DNA.

The genomic DNA of *M. xanthus* was used for the amplification of the FrzE_Rec domain. Different PCR trials were carried out with gradient annealing temperatures (52 ºC, 54 ºC, 57 ºC and 62 ºC) using gene specific primers, where 1:10, 1:30 and 1:50 dilutions of genomic DNA were used as a template with varying dNTPs, primers and DMSO concentrations. The PCR products were run on 1 % agarose gel, the PCR product with band corresponding to the *FrzE_Rec* gene size was PCR purified and used

as a template for the re-amplification of the *frzE_rec* domain by PCR at an annealing temperature of 62 ºC to get more amplicon. This was run on 1% agarose gel and the specific band was gel extracted, followed by PCR to introduce vector specific overhang. Linear amplification of the vector, pHis17, was carried out in a PCR with a template pHis17_romX_CStrep and megaprimer at an annealing temperature of 58º. *DpnI* digestion of the RF PCR products was carried out followed by the transformation in NEB electrocompetent cells through electroporation. Seven colonies were screened by colony PCR at an annealing temperature of 62 ºC. Plasmid purification of positive clones from the PCR screen was performed and sent for sequencing.

2.2.4 Cloning of FrzE_CheW domain by restriction enzyme digestion and ligation method:

Restriction enzyme digestion and ligation method was used to clone the FrzE_CheW domain into the pHis17 vector with N-terminal and C-terminal 6x-histidine tag, respectively. Two pHis17 vectors with *FrzB* gene between restriction sites *NdeI* at the N-terminal and *BamHI* at the C-terminal, one of them containing N-terminal His-tag and other C-terminal His-tag, were used as vectors for ligation. pHis17_frzE_cheW_CStrep construct was used to obtain the insert *FrzE_CheW* gene. 100 µL digestion reactions for vectors pHis17_frzE_cheW_CStrep, pHis17_frzB_NHis, pHis17_frzB_CHis were set up containing 10 µL of 10x Cutsmart buffer, 5 µL of *NdeI,* 5 µL of *BamHI* and 7000 ng of each vector and was incubated at 37ºC for 6 hours. After the digestion, the reaction was run on 1% agarose gel. Digested vector backbone of pHis17_FrzB_NHis, pHis17_FrzB_CHis and insert FrzE_CheW were gel extracted. Thermostable alkaline phosphatase (TSAP) digestion of both vector backbones was carried out to dephosphorylate the 5’ end. The reaction containing 1 µL of 10X multicore buffer, 1 µL of TSAP enzyme and 340 ng of each vector was incubated at 37 ºC for 40 minutes. After 40 minutes, the reaction was incubated at 72 ºC for 10 minutes to irreversibly inactivate the TSAP. The total vector and insert amount needed for the ligation reaction at a 1:5 ratio was determined using the NEBioCalculator tool for ligation. In the ligation

reaction, 1 µL 10X ligase buffer, 1 µL of ligase enzyme, 170 ng of vector and 160 ng of insert in the test was added. A control reaction without the insert was also kept. The ligation reaction for test and control for each vector was incubated at 16 °C for 12 hours. The ligation reaction was transformed into NEB electrocompetent cells through electroporation and the colonies were screened for positive clones. Plasmid purification was done for positive clones and clones were sent for sequencing.

2.2.5 Cloning of FrzE_CheW_Rec domain:

A reverse linker primer containing the C-terminal DNA sequence of the *frzE_cheW* domain and the N-terminal DNA sequence of the *frzE_rec* domain was used to fuse both these domains. Firstly, the *frzE_cheW* gene was amplified from the pHis17_cheW_CStrep construct by gradient PCR at an annealing temperature of 57°C and 60°C using forward overhang primer and reverse linker primer. PCR product was run on 1% agarose gel, reactions were pooled and PCR purified. The *frzE_CheW* domain was introduced upstream of the *frzE_rec* domain into the vector pHis17_rec_CStrep by PCR. In this PCR, pHis17_rec_CStrep was used as a template and *frzE_cheW* with forward vector overhang and a linker was used as a megaprimer to amplify the pHis17 vector along with the *frzE_rec* domain. PCR for the test with megaprimer and control without megaprimer was performed at an annealing temperature of 60 °C and the product was run on 1 % agarose gel. *DpnI* digestion of the PCR product was carried out and transformed into NEB electrocompetent cells through electroporation. Seven colonies were screened by colony PCR at an annealing temperature of 60 °C and plasmid purification of positive clones was done. Positive clones were further screened by restriction enzyme digestion check. The digested product was run on the 1% agarose gel to check positive clones and positive clones were sent for sequencing.

2.2.6 Cloning of the FrzE_Dim_HATPase_N377A mutant:

Asparagine (Asn377) present in the FrzE_HATPase domain was found to be conserved residue which stabilizes the magnesium ion during the ATPase activity (Meenakshi PV, MS thesis, May 2022). Hence asparagine was mutated to alanine by introducing a point mutation. Forward primer containing a DNA sequence from the point of mutation and alanine instead of asparagine was designed and used along with the gene specific reverse primer to amplify HATPase_N377A from the site of mutation to the C-terminal end of the domain. HATPase_N377A was amplified from pHis17_Dim_HATPase_CStrep by gradient PCR at annealing temperatures of 58 ^{o}C, 60 ^{o}C and 62 ^{o}C and the PCR product was run on 1 % agarose gel. PCR products were pooled, PCR purified and used as a megaprimer in the PCR for linear amplification of the pHis17 vector along with the frzE_dim_HATPase domain upto the point of mutation. Vector pHis17_dim_HATPase_CStrep was used as a template and PCR for test and control was performed at an annealing temperature of 58^{o}C. This PCR product was run on 1% agarose gel, *DpnI* digested and transformed into NEB electrocomp cells through electroporation. Colony PCR was performed for three colonies and positive clones were plasmid purified. A restriction enzyme digestion check was carried out for further screening and positive clones were sent for sequencing.

2.2.7 Cloning of FrzE_HPT and FrzA with Kanamycin resistance:

Both constructs should have two distinct antibiotic resistance to co-express. FrzE_HPT was co-expressed with FrzE_Dim_HATPase to increase the expression of FrzE_Dim_HATPase and FrzE_CheW_Rec was coexpressed with FrzA for the expression of FrzA. It was decided to change the ampicillin resistance of pHis17_FrzE_HPT_NHis and pHis17_FrzA_CHis to kanamycin while pHis17_FrzE_Dim_HATPase_CStrep and pHis17_FrzE_CheW_Rec_CStrep will remain ampicillin resistant. Kanamycin resistance was introduced into the vectors using the RF cloning method. First, the kanamycin resistance gene was amplified from the kanamycin

resistance vector, pHis17_RomR_CHis using the primers containing the pHis17 vector backbone and DNA sequence of the kanamycin gene. The PCR reaction was run on 1% agarose gel, PCR containing successful amplicons were PCR purified (Qiagen kit) and was used as a megaprimer in the next linear amplification reaction of pHis17_FrzE_HPT_NHis and pHis17_FrzA_CHis. The PCR reaction for the test with pHis17_FrzE_HPT_NHis/pHis17_FrzA_CHis as a template and megaprimer and control without megaprimer was set up at 60 ^{o}C. The PCR reactions were run on 1% agarose gel and the 9 µL of PCR product was directly transformed into the NEB electrocompetent cells through electroporation. Here, the plating was done on the LB-Kan agar plates, as only the colonies with successfully inserted kanamycin resistance will grow. Colony PCR was performed to check the presence of the FrzE_HPT domain and FrzA and positive clones were sent for sequencing.

2.3 Protein expression check and standardization:

Expression of protein was checked for the positive clones of FrzE_Dim_HATPase_CStrep, FrzE_CheW_CStrep, FrzE_Rec_CStrep, FrzE_CheW_NHis, FrzE_CheW_CHis, FrzE_CheW_Rec_CStrep and the ATPase mutant FrzE_Dim_HATPase_N377A_CStrep. The plasmid of the positive clone was transformed into competent expression cell lines. The culture was grown by inoculating a patch of colonies into the 10 ml LB media containing 0.1 mg/ml ampicillin and incubating at 37 ^{o}C with shaking at 180 RPM until the OD_{600} reached 0.6. 5 ml of this culture was taken out in a separate tube and induced with 0.2 % arabinose for BL21-AI strains and 0.5 mM IPTG for BL21-DE3, Rosetta, and C43 strains, whereas the rest of the culture was kept uninduced. Cultures were incubated at various temperatures post induction and were pelleted down at 13,000 RPM for 1 min after the incubation. 500 µL of lysis buffer containing 50 mM Tris, pH 8, 200 mM NaCl and 10% glycerol was used to resuspend the pellet. Sonication was performed at 60% amplitude with a pulse of 1 second on, 3 seconds off for 1 minute. 20 µL of the sonicated sample was taken out to make the samples for the total fraction. After sonication, samples were centrifuged at

14,000 RPM at 4 ºC for 20 min to get a soluble fraction. 20 µL of the soluble fraction was taken out to make the samples used to load on the SDS-PAGE gel. 20 µL of 2x SDS dye was added to both the 20 µL of total and soluble fractions and samples were incubated at 99 ºC for 10 min. Samples were loaded on SDS-PAGE gel and run at 230 V. The expression of protein was checked by comparing the uninduced and induced fractions and solubility of protein was checked by comparing the total and soluble fractions of the induced fractions. Expression strains and conditions with the best protein expression and solubility were selected for the purification of the respective proteins.

2.4 Co-expression:

To co-express the two proteins, constructs with distinct antibiotic resistance, i.e. ampicillin and kanamycin, were transformed into the BL21-AI strain with equal amounts of plasmid. Transformed cells were plated on LB-Amp-Kan agar plates, so that only the cells carrying both transformed plasmids will grow. Plates were incubated at 37 ºC for 12 hours. 10 ml culture was grown by inoculating the colonies in LB media with 0.1 mg/ml ampicillin and kanamycin and incubated at 37 ºC till OD_{600} 0.6. Separate 5 ml culture was induced with 0.2 % arabinose and both induced and uninduced cultures were incubated at 18 ºC for 12 hours, then pelleted down at 13,000 RPM. To check the co-expression of proteins, a similar procedure was performed as that of protein expression check.

2.5 Protein purification:

Purification of the protein was carried out using the Strep-tag affinity chromatography and the protein was further purified using size-exclusion chromatography. 1 liter culture for FrzE_CheW, 4 liter culture for FrzE_Rec and FrzE_CheW_Rec and 6 liter culture for FrzE_HATPase and FrzE_HATPase_N377A was grown at standardized expression

conditions. BL21-AI strain was used for all the proteins, culture was induced with 0.2% arabinose at OD_{600} 0.6 and grown at 18^{o}C with 180 RPM shaking for 12 hours. The culture was pelleted down at 5000 RPM, 4^{o}C for 20 mins (Rotor ID: JA-10). Lysis buffer containing 50 mM Tris, pH 8, 150 mM NaCl and 10% glycerol was used to resuspend the pellet. Sonication was performed at 60% amplitude with a pulse of 1 second on, 3 seconds off for 4 minutes. The second round of sonication with the same parameters was performed after a gap of 5 minutes. The lysate was pelleted down at 15,000 RPM, 4^{o}C for 50 min (Rotor ID: JA-25.50) to remove cell debris and get the soluble protein in the supernatant. The supernatant was loaded onto the HiTrap Strep column (5 ml) equilibrated with the equilibration buffer containing 50 mM Tris, pH 8 and 150 mM NaCl. While loading the lysate, a protein with the C-terminal Strep-tag will bind to the Strep-Ttactin present in the column. After loading the lysate, the column was washed with the equilibration buffer to remove the residual nonspecific bound proteins. Flow-through and wash were collected. 5 ml of 2.5 mM desthiobiotin in equilibration buffer was injected to elute the protein. Desthiobiotin competes with the Strep-tag to bind to the biotin binding pocket of Strep-Tactin present in the Strep column and protein with the Strep-tag gets eluted. Two rounds of elution were done. 2 ml fractions of eluted protein were collected and samples of fractions, flow-through and wash were prepared to load on the SDS-PAGE gel by incubating 10 µL of samples with the 2X SDS dye at 99^{o}C for 10 min. The SDS-PAGE gel was run to check the protein present in the fractions. Fractions with the protein were pooled and concentrated upto 1 ml using the 5 kDa centricon for FrzE_Rec and 10 kDa centricon for FrzE_HATPase, FrzE_HATPase_N377A and FrzE_CheW_Rec .

To further purify the protein, size-exclusion chromatography was carried out. In size exclusion chromatography, proteins are separated according to the size, where proteins with higher molecular weight will elute first and vice versa. Superdex75 and SEC650 columns were used for the purification of FrzE_Rec, whereas Superdex200 column was used for the purification of FrzE_HATPase, FrzE_HATPase_N377A and FrzE_CheW_Rec. Equilibration buffer containing 50 mM Tris, pH 8 and 50 mM NaCl was used to equilibrate the column. 1 ml protein concentrated from the previous purification step was injected into the column. An elution profile was captured by

monitoring the UV absorbance at 280 nm as the protein got eluted and fractions were collected. 10 µL sample of the fractions corresponding to the UV absorbance peaks in the profile were incubated along with the 2X SDS dye at 99ºC for 10 min and run on the SDS-PAGE gel. Fractions with the protein were pooled and concentrated upto 200-500 µL using the centricon. The concentrated protein was stored at -80ºC after flash freezing 10-20 µL aliquots in liquid nitrogen.

2.6 Bradford assay:

Bradford assay was used to estimate the concentration of proteins before using it for the assays. 1X and 5X dilutions for FrzE_Rec, 5X and 10X dilutions for FrzE_Dim_HATPase and 10X, 20X and 30X dilutions for FrzE_∆Rec protein were used. 5 µL of each dilution were added to the wells of a 96 well plate. Three trials for each dilution were taken. Standards of BSA (stock concentration 1 mg/ml) were made with varying concentration (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mg/ml) and 5 µL of each standard BSA concentration was added in the wells. 250 µL of Bradford reagent was added in all dilutions and standards and absorbance at 595 nm was measured using the microplate reader (CLARIOstar plus). A standard curve of concentration versus absorbance was plotted to obtain the slope value. The concentrations of each dilution were calculated by dividing the respective absorbance value by the slope and multiplying it with the dilution factor. The average value was taken to estimate the protein concentration.

2.7 Circular Dichroism Spectroscopy (CD spectroscopy):

Far-UV CD spectroscopy was carried out for FrzE_Rec to check secondary protein structure information. Jasco J-720 spectropolarimeter was used for the measurements of far-UV CD spectra of 10 µM of protein at 1 nm step resolution, 100 nm/min scan speed and 1 nm bandwidth. CD data was plotted and analyzed using CAPITO.

2.8 Thermal shift assay:

Thermal shift assay (Ericsson et al., 2006) was carried out to check the presence of tertiary folded structure and stability of the protein. A 25 µL reaction containing 20 µL of 5 µM protein and 5 µl of 50× SYPRO Orange (S5692; Sigma-Aldrich) reaction was set up in 96-well PCR plates (Bio-Rad) sealed with a microseal (Bio-Rad). The plate was centrifuged for 30 s at 4°C and incubated at 4°C for 10 min in the Bio-Rad CFX96 Real-Time machine. The fluorescence of SYPRO Orange was measured at a temperature range of 4–95°C with an increase of 0.4°C every 20 s. The fluorescence resonance energy transfer channel (excitation at 470 nm and emission at 569 nm) was used to collect data for the change in the fluorescence emission of SYPRO. The first derivative of the fluorescence (obtained from the raw data) with respect to the temperature ($-(\mathrm{d}F)/\mathrm{d}T$) was calculated and plotted against the temperature.

2.9 Malachite assay:

Malachite assay was used to quantify the amount of free phosphate in a reaction. A reaction was set up containing the 0.5 µM of the protein FrzE_HATPase, 50 mM Tris, pH 8, 50 mM NaCl, 1 mM $MgCl_2$ and 1 mM ATP. Controls without protein and ATP were also set up and the reaction was incubated at 25^{o}C. 20 µL of the reaction was taken out at four 20 min intervals and stopped using 5 µL of 0.5 M EDTA. The standards were made with increasing concentrations of NaH_2PO_4, i.e. 0 µM, 25 µM, 50 µM, 75 µM, 100 µM, 150 µM, and 200 µM. After 60 minutes, 50 µL of malachite green solution containing malachite green, 7.5% ammonium molybdate and 11% tween20, was added to the standards and the reactions. Malachite green with ammonium molybdate forms a complex with free phosphate resulting in a change in color to green. Absorbance at 630 nm was measured using the microplate reader (CLARIOstar plus) for the standards and the reactions. A standard curve of concentration versus absorbance was plotted to get the value of slope. The concentration of free phosphate released in each reaction was

calculated by dividing the normalized absorbance value of each reaction by the slope from the standard curve. A graph of the concentration of free phosphate for the reactions with respect to the time was plotted. k_{cat} value for the reaction was calculated by dividing the phosphate concentration by protein concentration and time. Three trails were taken.

A similar assay was performed for 0.5 μM of FrzE_ΔRec protein purified from the construct pETPhos_FrzE_ΔRec (Meenakshi PV, MS thesis, May 2022).

CHAPTER 3
RESULTS AND DISCUSSION

3.1 Domain boundary determination:

Domain boundaries were determined for the domains FrzE_CheW and FrzE_Rec.

3.1.1 Domain boundary determination of FrzE_CheW:

A standard protein BLAST against PDB data for the protein sequence of FrzE_CheW showed that the chemotaxis protein, CheA, of *Thermotoga maritima* was the best homologous protein to the FrzE_CheW domain with the identity score 37 % (Fig 3.1A, B). The P5 (CheW) domain in CheA (Fig 3.1C) was found to be homologous to the FrzE_CheW domain. By analyzing the structure of CheA (PDB ID: 1B3Q), it was found that the P5 domain starts from alanine (A) at residue 542 and ends at residue 669 with glycine (G) (Fig 3.1A). Ala510 in FrzE_CheW was aligned with Ala542 in CheA and whereas, Asp638 in FrzE_CheW was aligned with Gly669 in CheA.

Next, secondary structure prediction of the FrzE_CheW from PSIPRED showed that the secondary structure, a β-sheet, starts from Ala510. Asp638 is flanked by an α-helix ending with Met646 (Fig 3.1D).

From the alignment with CheA and structure of the P5 domain in CheA and the predicted secondary structure of the FrzE_CheW domain, the boundary of the domain was determined from Ala510 to Met646.

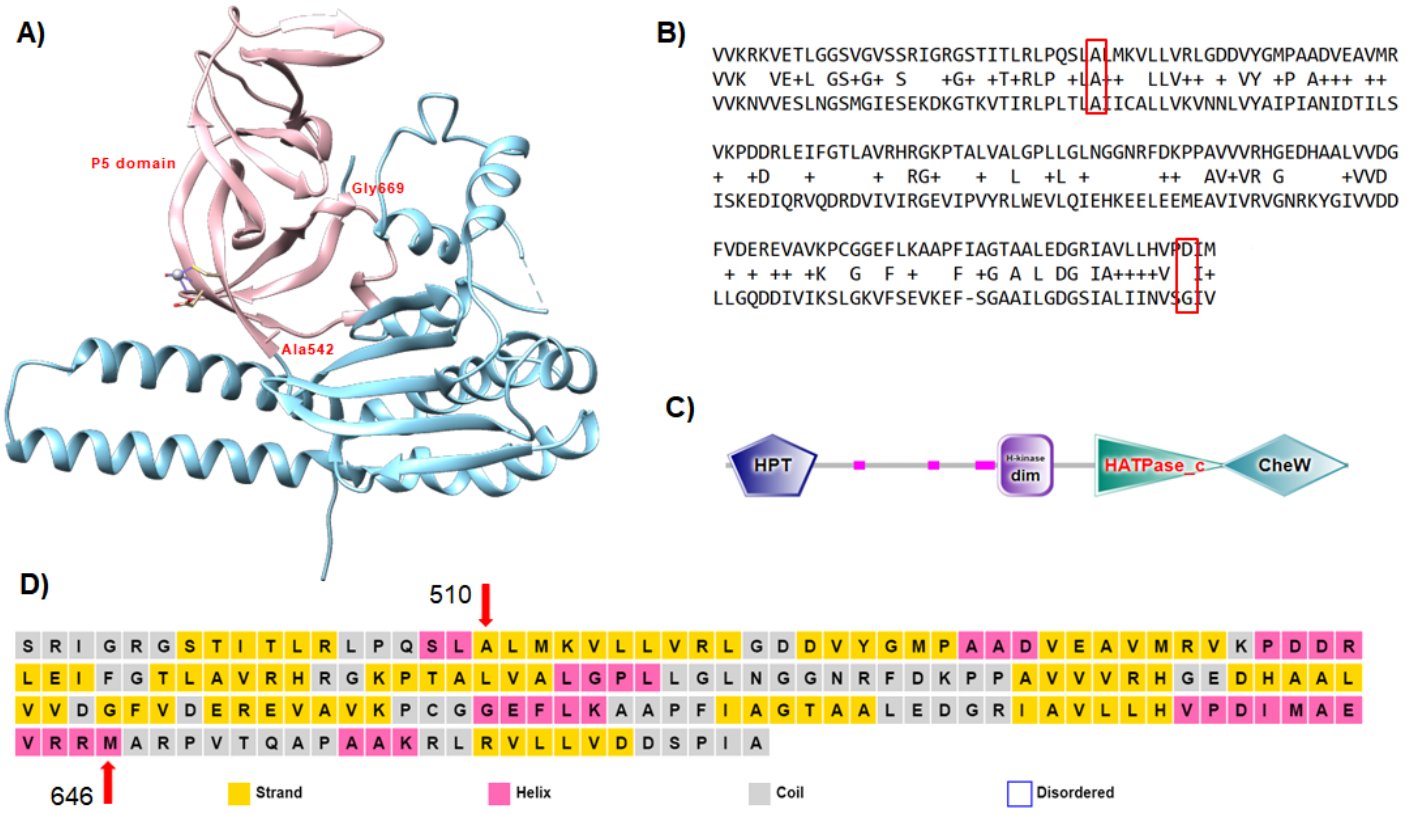


Fig 3.1: Determination of domain boundary for FrzE_CheW.

A) Structure of CheA of *Thermotoga maritima* (PDB ID: 1B3Q) generated using UCSF Chimera. P5 domain is highlighted in pink. Alanine present at the start of the P5 domain and Glycine at the end of the P5 domain are labeled. B) Alignment of CheA and FrzE_CheW protein sequence from BLAST. C) Domains of CheA (from SMART). D) Secondary structure of FrzE_CheW predicted from PSIPRED.

3.1.2 Domain boundary determination of FrzE_Rec:

Sensor protein from *Syntrophus aciditrophicus* was homologous to the FrzE_Rec with the highest identity score, 39 %. The sequence and structure of the sensor protein (PDB ID: 3GT7) showed that it starts at the glutamic acid (E) which is aligned to Arg660 in FrzE_Rec (Fig3.2.A, B). The secondary structure of FrzE_Rec predicted from PSIPRED showed the start of β-sheet from Arg660 (Fig.3.2.C). As FrzE_Rec was the last domain of FrzE, the end of the FrzE protein sequence at Thr777 was determined to be the end of the Frz_Rec domain. Hence, the domain boundary of FrzE_Rec was determined from Arg660 to Thr777.

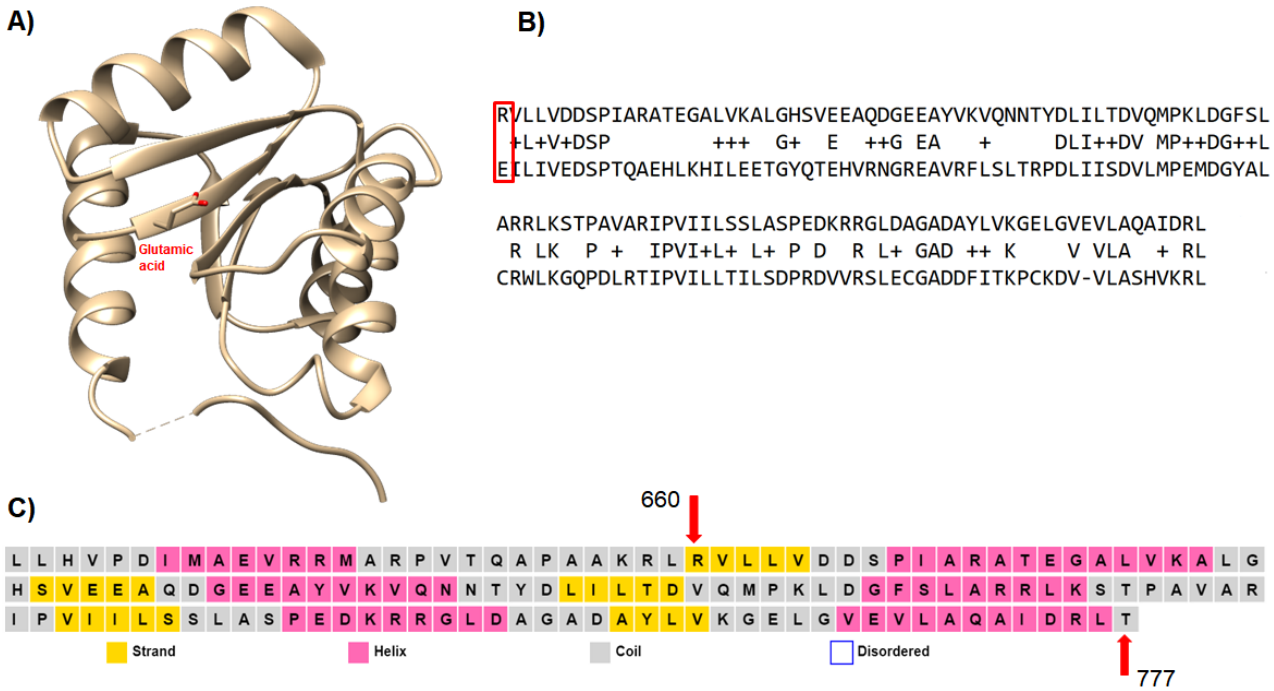


Fig 3.2: Determination of domain boundary for FrzE_Rec. A) Structure of Sensor protein of *Syntrophus aciditrophicus* (PDB ID: 3GT7) generated using UCSF Chimera. Glutamic acid (E) is present at the start of the protein structure. B) Alignment of sensor protein and FrzE_Rec protein sequence from BLAST. C) Secondary structure of FrzE_Rec predicted from PSIPRED.

3.2 Cloning for FrzE_Dim_HATPase, FrzE_CheW, FrzE_Rec, FrzE_CheW_Rec and FrzE_Dim_HATPase_N377A and kanamycin resistant constructs, pHis17_FrzE_HPT_NHis and pHis17_FrzA_CHis:

frzE_dim_HATPase and *frzE_cheW* were amplified from pETPhos_FrzE_ΔRec construct, bands at 906 bp for *frzE_Dim_HATPase* (Fig 3.3A) and 435 bp for *frzE_cheW* (Fig 3.3B) were observed on the agarose gel image for PCR-1. Similarly, a 471 bp band for *frzE_cheW_linker* amplification from pHis17_frzE_cheW_CStrep was observed on the agarose gel image (Fig 3.3D). No bands were observed at 360 bp for *frzE_rec* amplification from 1:10, 1:30 and 1:50 dilutions of genomic DNA where 2% and 3% DMSO was used in the PCR containing 1:30 dilution of genomic DNA and 3% DMSO was used in the PCR containing 1:50 dilution of genomic DNA. In the next trial, dNTPs concentration was increased to 100 µM, 3% DMSO was added and 1:30 dilution of genomic DNA was used as a template. In this trial, bands at 360 bp corresponding to the *frzE_rec* domain were observed at annealing temperature 62 ^{o}C (Fig 3.3C). The amplification from the site of point mutation (N377) to the C-terminal end of the FrzE_HATPase domain was carried out in the PCR-1 to get FrzE_Dim_HATPase_N377A mutant and the 390 bp bands corresponding to this amplification were observed on the agarose gel (Fig 3.3E). 830 bp band corresponding to the kanamycin gene with pHis17 vector overhangs was observed in the agarose gel after the PCR-1 (Fig 3.3F).

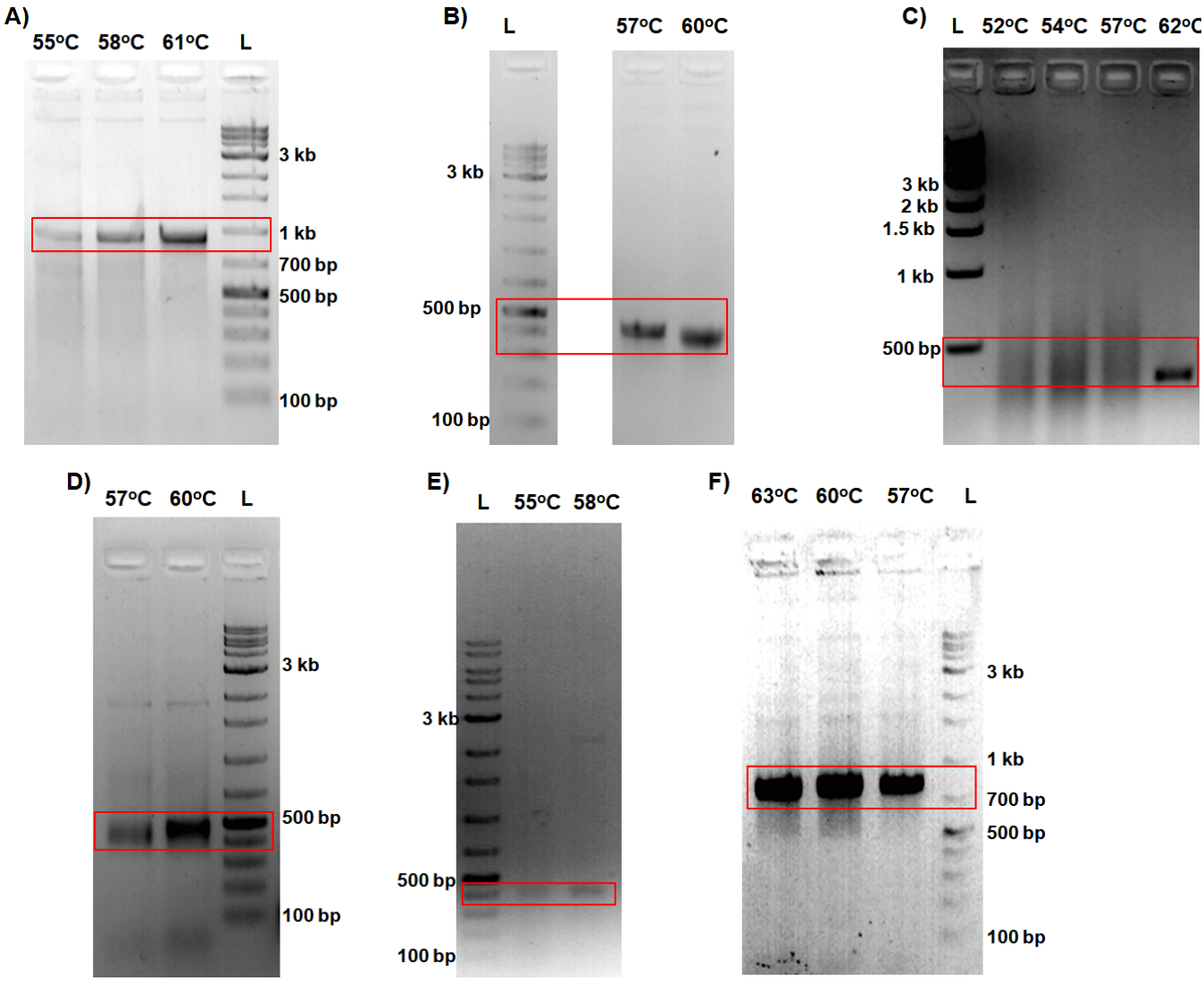


Fig 3.3: **Agarose gel images of PCR-1.** A) 906 bp band of *FrzE_Dim_HATPase* has been amplified in lanes 1, 2 and 3. Lane 4 is 1 kb plus DNA ladder. B) Lane 1 is 1 kb plus DNA ladder. 435-bp band of *frzE_cheW* has been amplified in lanes 2 and 3. C) First lane is a 1 kb DNA ladder. 360-bp band of *frzE_rec* has been amplified in lane 4 at 62ºC. D) 471 bp band for *frzE_cheW_rec* has been amplified in lanes 1 and 3. Lane 3 is 1 kb plus DNA ladder. E) 390 bp band corresponding to the amplicon from N377 to the C-terminal end of pHis17_Dim _HATPase_CStrep has been amplified in the first lane. Second lane is 1 kb plus DNA ladder. F) 830 bp *kanamycin* gene along with pHis17 vector backbone has been amplified in the first three lanes, 4th lane is 1 kb plus DNA ladder.

Bands for megaprimer at 950 bp with other non-specific bands for *frzE_dim_HATPase*, 504 bp for *frzE_cheW* and 429 bp for *frzE_rec* were observed on the image of agarose gel for PCR-2.

A smear above the megaprimer was observed in the test reaction of linear amplification PCR for *frzE_dim_HATPase*, *frzE_cheW*, *frzE_rec*, *frzE_cheW_rec, frzE_dim_HATPase_N377A and kanamycin*. The absence of this smear in the control PCR suggested that PCR has worked and the *frzE_dim_HATPase*, *frzE_cheW* and *frzE_rec* genes were introduced in the pHis17_romX_CStrep vector. *frzE_cheW* gene into the vector pHis17_frzE_rec_CStrep and point mutation N377A into the vector pHis17_frzE_dim_HATPase_CStrep were also successfully introduced. *Kanamycin* gene was introduced into the vectors pHis17_FrzE_HPT_NHis and pHis17_FrzA_CHis. Three positive clones for the FrzE_CheW (Fig 3.4B), FrzE_CheW_Rec (Fig 3.4D) and FrzA (Fig 3.4F), one for FrzE_Rec (Fig 3.4C) and FrzE_HPT (Fig 3.4G), two positive clones for FrzE_Dim_HATPase_N377A (Fig 3.4E) and FrzE_Dim_HATPase (Fig 3.4A) were obtained, based on the observations from the colony PCR, as bands corresponding to the gene size were observed on the agarose gel. Two clones out of three for FrzE_CheW_Rec, one clone out of three for FrzE_CheW and both clones for FrzE_Dim_HATPase_N377A from the colony PCR were observed to release the band size corresponding to the gene and were found to be positive from the restriction enzyme digestion check. Sequencing results showed clones for FrzE_CheW, FrzE_Rec and FrzE_HPT to be positive. Only one out of two clones was positive for FrzE_Dim_HATPase, FrzE_Dim_HATPase_N377A and FrzE_CheW_Rec. All three clone of FrzA were positive.

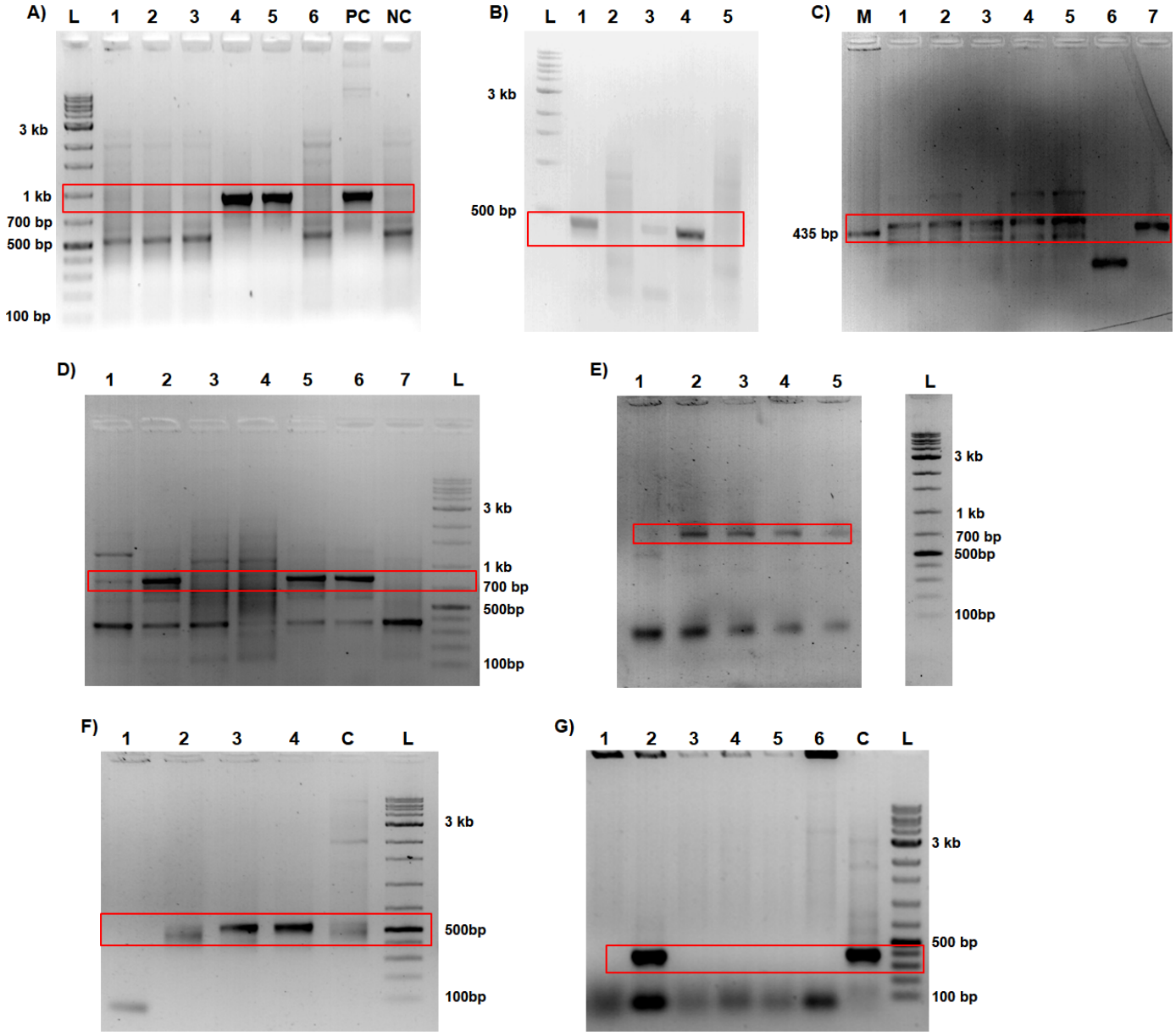


Fig 3.4: Agarose gel images of the colony PCR. A) 906 band for FrzE_Dim_HATPase in lane 5 and lane 6. Lanes 1, 8 and 9 are 1 kb-Plus DNA ladder, positive control and negative control, respectively. B) 435 band for FrzE_CheW in lanes 2, 4 and 5. Lane 1 is a 1 kb DNA ladder. C) Lane 1 is a 360 bp marker. Lane 8 has a 360 bp band for FrzE_Rec. D) 810 bp band for FrzE_CheW_Rec in lanes 2, 5 and 6. Lane 8 is a 1 kb-Plus DNA ladder. E) Lane 2, 3 and 5 contains amplified 906 band for FrzE_Dim_HATPase_N377A, lane 7 is 1 kb plus DNA ladder. F) Lane 2,3 and 4 have amplified the 500 bp FrzA band, lane 6 is 1 kb plus DNA ladder. G) Lane 2 has 384 bp band corresponding to the FrzE_HPT domain. Lane 8 is 1 kb plus DNA ladder.

3.3 Cloning of FrzE_CheW_NHis and FrzE_CheW_CHis:

pHis17_frzE_cheW_CStrep, pHis17_frzB_NHis and pHis17_frzB_CHis were set for *NdeI* and *BamHI* restriction enzyme digestion and the reaction was run on the agarose gel for gel extraction. The agarose gel image showed the *frzE_cheW* band at 435 bp, 336 bp band for digested FrzB gene and 2.6 kb band for the digested linear pHis17 vector backbone (Fig 3.5A). Insert *frzE_CheW* and the vectors, pHis17_NHis and pHis17_CHis were gel extracted with the concentration 14 ng/µL, 43 ng/µL and 77 ng/µL, respectively. A ligation reaction was performed for the test with the insert and control without the insert for both vectors and transformed. The test plate for both pHis17_NHis and pHis17_CHis vectors had colonies while the control was clean. This shows that the ligation reaction of the insert FrzE_CheW and vectors has worked. Agarose gel image for colony PCR of FrzE_CheW_NHis and FrzE_CheW_CHis showed three clones had amplified 435 bp for FrzE_CheW (Fig 3.5B). Sequencing results showed that two out of three clones were positive for both FrzE_CheW_NHis and FrzE_CheW_CHis.

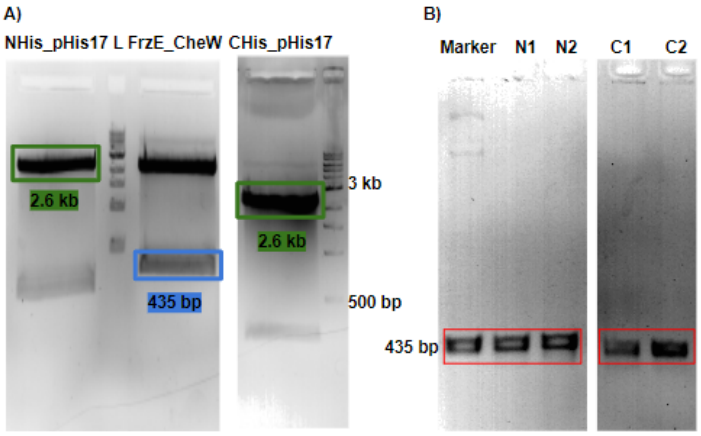


Fig. 3.5: A) Agarose gel images after the digestion of vectors and FrzE_CheW. Lane 1, 2, 3, in the first gel, is digested pHis17_NHis at 2.6 kb, 1kb DNA ladder, digested FrzE_CheW at 435 bp, respectively. In gel 2, digested pHis17_CHis is present at 2.6 kb and lane 2 is 1 kb DNA ladder. B) Agarose gel image of colony PCR for pHis17_FrzE_CheW_NHis and pHis17_FrzE_CheW_CHis. Lane 1 is a 435 bp marker. 435 bp band for FrzE_CheW is present in lanes 2 and 3 in the first gel and lanes 1 and 2 in the second gel.

3.4 Protein expression check and standardization for positive clones:

A 35 kDa band for FrzE_Dim_HATPase was observed on 12% SDS-PAGE (Fig 3.6A). Protein expression was less as the band present at 35 kDa was thin. Expression in BL21-AI cells at 18 ºC was slightly better than expression in other cell lines and conditions. Hence, this condition was selected for growing the larger culture and protein purification.

FrzE_CheW protein was not expressed as a 16 kDa band was not observed on 15% SDS-PAGE gel (Fig 3.6B). This was the case for BL21-AI and BL21-DE3 strains at 18 ºC and 25 ºC. Rosetta and C43 strains were tried for the protein expression and the expression was not observed. Hence, it was decided to change the C-terminal strep tag of the protein with the N-terminal and C-terminal Histidine tag. Protein expression was not observed for FrzE_CheW_NHis and FrzE_CheW_CHis as well. The expression could be affected and protein can degrade inside the cell if the domain is incomplete, resulting in no expression of the protein. Protein FrzE_CheW_Rec contains FrzE_CheW and that can get expressed along with the FrzE_Rec domain, hence cloning of the FrzE_CheW and FrzE_Rec domain together was done.

A 30 kDa band corresponding to the molecular weight of FrzE_CheW_Rec was observed on 12% SDS-PAGE gel (Fig 3.6E). Protein was expressed in BL21-AI and BL21-DE3 cell lines at 18 ºC for FrzE_CheW_Rec. Expression in the BL21-AI strain was better than BL21-DE3, hence this condition was selected for the purification of the protein.

Protein expression of FrzE_Rec at 14 kDa was observed in the induced culture on 15% SDS-PAGE gel (Fig 3.6D) for BL21-AI cells at 18ºC and these conditions for growth were used for the protein purification.

Mutant FrzE_Dim_HATPase_N377A was expressed at 35 kDa in the induced culture on 12% SDS-PAGE gel in BL21-AI cells at 18ºC with less expression (Fig 3.6C).

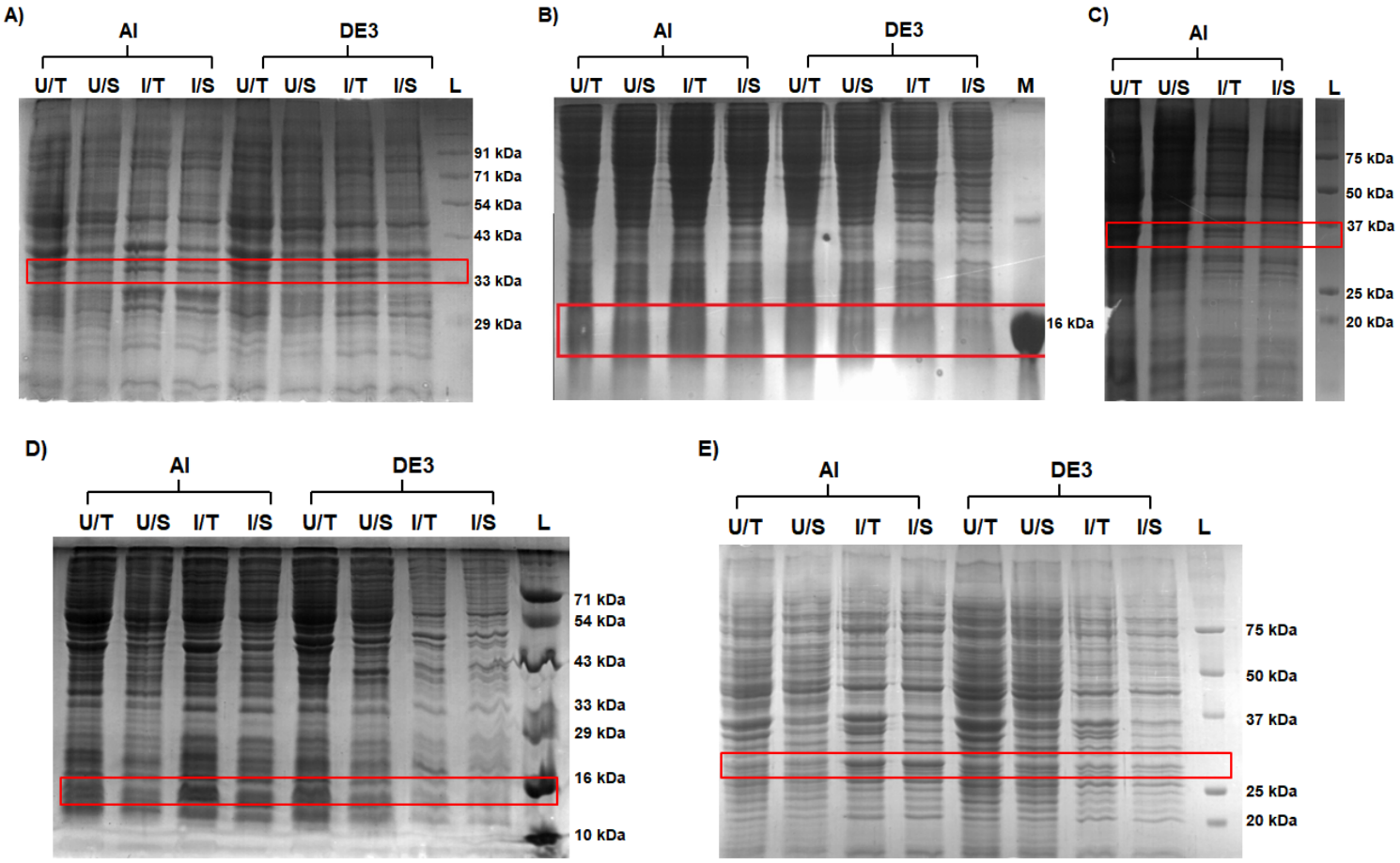


Fig 3.6: SDS-PAGE gel for the expression check of FrzE_Dim_HATPase, FrzE_Dim_HATPase_N377A, FrzE_CheW, FrzE_Rec and FrzE_CheW_Rec. A) 35 kDa for the FrzE_Dim_HATPase band in lane 4 (soluble fraction of induce) is darker than in lane 2 (soluble fraction of uninduced). Lane 9 is a protein ladder. B) 16 kDa band for FrzE_CheW is not observed in lane 4 (soluble fraction of induce). Lane 9 is a 16.8 kDa marker. C) Faint 35 kDa for HATPase_N377A is present in lane 4 (soluble fraction of induced). It is absent in lane 2 (insoluble fraction of uninduced). D) 14 kDa for FrzE_Rec band in lane 4 (soluble fraction of induce) is darker than in lane 2 (soluble fraction of uninduced). Lane 9 is a protein ladder. E) 30 kDa band for FrzE_CheW_Rec in lane 4 (soluble fraction of induce) is darker than in lane 2 (soluble fraction of uninduced). Lane 9 is a protein ladder.

3.5 Protein purification of FrzE_Dim_HATPase:

Strep tag affinity chromatography was performed for the purification of FrzE_Dim_HATPase with C-terminal strep tag. The protein eluted in the fractions from the strep column was checked on 12% SDS-PAGE gel. Bands at 35 kDa, which is the molecular weight of the FrzE_Dim_HATPase protein, were observed in 4^{th}, 5^{th}, 6^{th}, 7^{th} and 8^{th} fractions and a small amount of protein was also observed in 9^{th}, 10^{th} and 11^{th} fractions (Fig 3.7A). No significant band at 35 kDa was present in the flow-through or wash fractions, indicating that almost all the protein was bound to the strep column. Impurities with higher molecular weight were present in all the fractions. This could be due to various proteins present in the lysate that can interact with the FrzE_Dim_HATPase protein and eluted along with it. Fractions containing the protein were pooled and concentrated upto 1 ml in the 10 kDa centricon.

Further purification of the FrzE_Dim_HATPase protein was done to remove the impurities from the previous purification step. Size exclusion chromatography with the Superdex200 column was used in the next round of purification of the protein as it separates the molecules according to size. The elution profile showed a UV absorbance peak between 7 to 9 ml volume which corresponds to the void (Fig 3.7C). Presence of protein in the void indicates that protein might have formed the higher order aggregates, or interacts with the other proteins forming molecules with higher molecular weight. Small peaks at 12 and 14 corresponding to ~ 300 kDa and 70 kDa, respectively, were also present (Fig 3.7C). Protein was collected as 1 ml fractions and was checked on 12% SDS-PAGE gel. 35 kDa band was present in 7^{th}, 8^{th}, 9^{th}, 10^{th}, 11^{th} and 12^{th} fractions (Fig 3.7B). These protein fractions were relatively purer than the protein after strep-tag affinity purification. Fractions containing the protein were pooled and concentrated upto 250 µL. 20 µL aliquots were stored at -80^{o}C after flash freezing in liquid nitrogen.

The concentration of the purified protein was estimated by Bradford assay and found to be 11 µM. So, 250 µL of 11 µM purified FrzE_Dim_HATPase protein was obtained from the 6 liter culture.

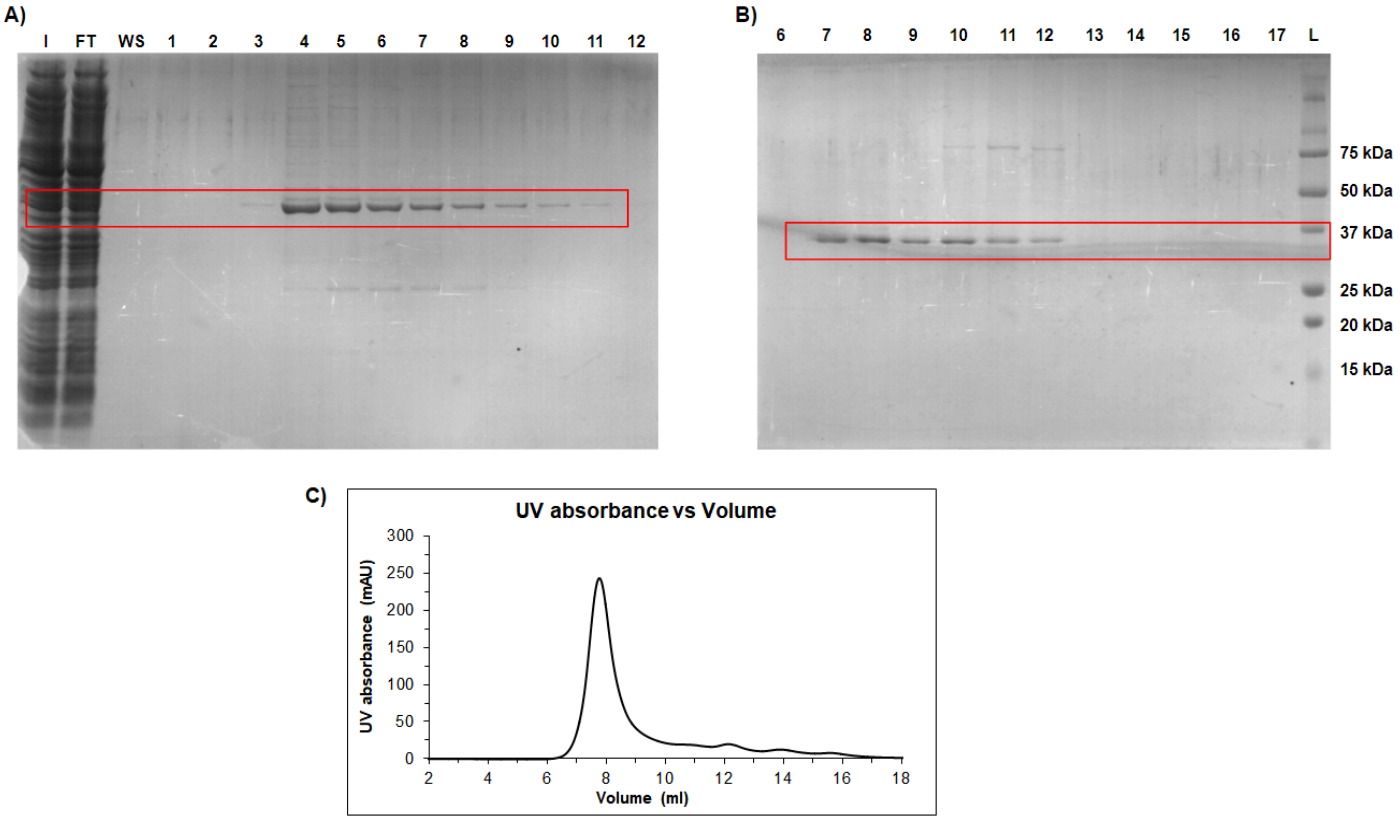


Fig 3.7: Purification of FrzE_Dim_HATPase. A) Image of SDS-PAGE gel after strep-tag affinity purification. Lane 1, 2 and 3 are input, flow-throwgh and wash fractions, respectively. 35 kDa band corresponding to the FrzE_Dim_HATPase is present in lane 7 to 13. B) SDS-PAGE gel image after passing protein through the Superdex 200. 35 kDa band corresponding to the FrzE_Dim_HATPase is present in lane 2 to 7. Lane 13 is a protein ladder. C) Elution profile after passing protein through the Superdex 200 column.

3.6 Protein purification of FrzE_Rec:

FrzE_Rec was purified by strep tag affinity chromatography as it contained a C-terminal strep tag. 15% SDS-PAGE gel was used to check the presence of protein in the fractions eluted from the strep column. 14 kDa bands corresponding to the molecular weight of FrzE_Rec were present in the 3rd, 4th, 5 th ,6 th,7 th and 8 th fractions (Fig 3.8A). Flow-through and wash did not have significant 14 kDa bands indicating the binding of

almost all the protein to the strep column. Impurities with higher molecular weight were present in all the fractions. Protein was concentrated upto 1 ml in the 5 kDa centricon.

Next, the protein was purified using size exclusion chromatography with the Superdex75 column. The elution profile showed UV absorbance peaks at 7, 8 and 9, corresponding to the void (Fig 3.8D). A small peak at 12 was present, which corresponds to ~ 14 kDa protein (Fig 3.8D). Protein was collected as 500 µL fractions and corresponding to the peak was checked on 15% SDS-PAGE gel. Protein was present in the 14^{th}, 15^{th}, 16^{th}, 18^{th} and a small amount in the 20^{th}, 22^{th}, 24^{th},and 26^{th} fractions (Fig 3.8B). The impurities were still present. Also, the protein might have formed the higher order as it is a 14 kDa protein that came in void corresponding to molecular weight higher than ~ 70 kDa. The formation of aggregates or proteins interacting with other impurities is also a possibility. To check if higher orders were present, the next round of size exclusion chromatography using the SCE650 column with a separation range from 5 kDa to 650 kDa was carried out. Fractions with protein were pooled and concentrated upto 1 ml, to inject in the SEC650 column.

Protein was present in the void after using the SEC650 column as well, as UV absorbance peaks were between 8 to 10 in the elution profile (Fig 3.8E). 500 µL fractions were collected and checked on 15% SDS-PAGE gel. 14 kDa bands were present in the 17^{th}, 18^{th},19^{th}, 20^{th} and 21^{st} fractions (Fig 3.8C). Presence of protein in the void after using the SEC650 column indicates that protein might have formed aggregates. Another possibility is interactions with impurities leading to the higher molecular weight and hence eluting it in the void. This could happen if the protein is unstable and misfolded. A CD spectroscopy or a thermal shift assay would show the instability of the protein. Hence, this protein was collected and stored to perform the assays. Fractions with protein were pooled, concentrated upto 500 µL, and stored in -80^{o}C as 20 µL aliquots after flash freezing.

Bradford assay estimated the concentration to be 35 µM. So, 500 µL of 35 µM purified FrzE_Rec protein was obtained from the 4 liter culture.

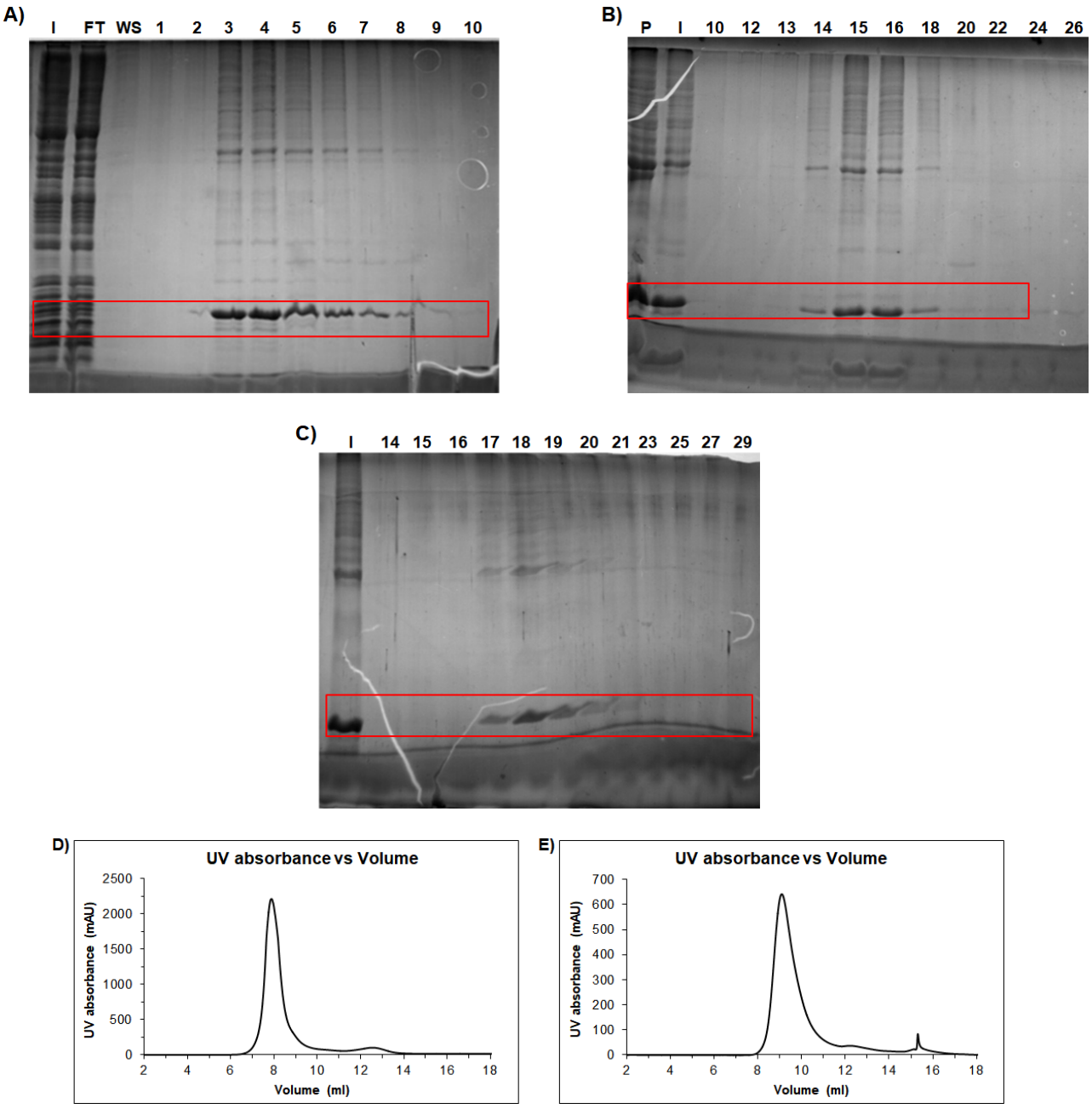


Fig 3.8: Purification of FrzE_Rec. A) Image of SDS-PAGE gel after strep-tag affinity purification. Lane 1, 2 and 3 are input, flow-through and wash fractions, respectively. 14 kDa band for FrzE_Rec is present in lane 7 to 12. B) SDS-PAGE gel image after Superdex 75. Lane 1 and 2 are pellet and input fractions, respectively. The 14 kDa band for FrzE_Rec is present in lane 6 to 9. C) SDS-PAGE gel image after SEC650. Lane 1 is input. The 14 kDa band for FrzE_Rec is present in lane 5 to 8. D) Elution profile after Superdex 75. E) Elution profile after SEC650.

3.7 Protein purification of FrzE_CheW_Rec:

Purification of FrzE_CheW_Rec was performed in a similar way as that of FrzE_HATPase and FrzE_Rec. Strep tag affinity chromatography was used as FrzE_CheW_Rec has a C-terminal Strep tag. 30 kDa band of the protein was observed in the 4th, 5th ,6th and 7th fractions and a small amount in 8th and 9th fractions in 12% SDS-PAGE gel after the elution (Fig 3.9A). Impurities were present in the fractions. Fractions containing the protein were pooled and concentrated upto 1 ml in the 10 kDa centricon.

Further purification was carried out using size exclusion chromatography with the Superdex200 column. UV absorbance peak was present from 7 to 9, corresponding to the void and a small peak at 12 corresponding to ~ 300 kDa (Fig 3.9C). Protein was collected as 1 ml fractions and was checked on 12% SDS-PAGE gel. 30 kDa band was present in 7th, 8th, 9th and 10th fractions (Fig 3.9B). Impurities were still present in the factions. Fractions containing the protein were pooled and concentrated upto 250 µL.

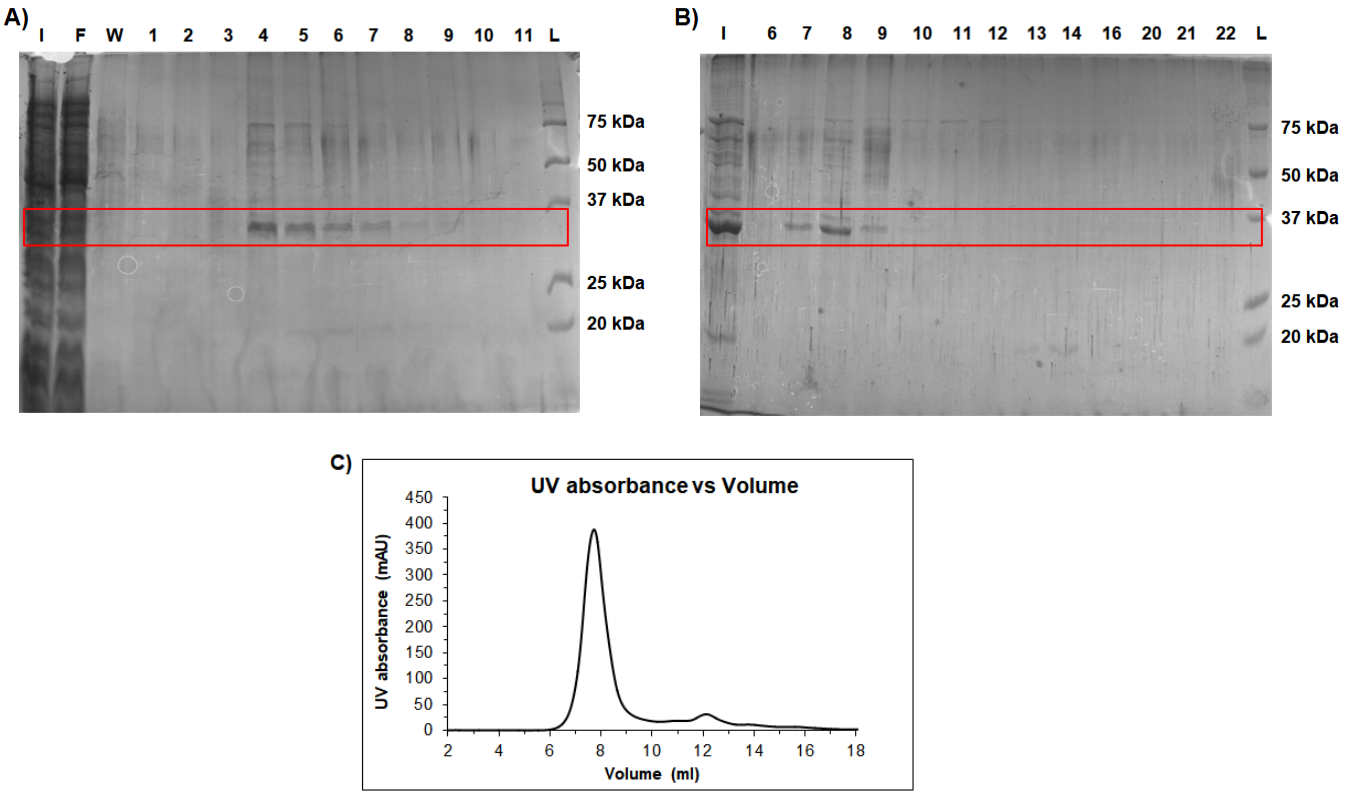


Fig 3.9: Purification of FrzE_CheW_Rec. A) Image of SDS-PAGE gel after strep-tag affinity purification. Lane 1, 2 and 3 are input, flow-through and wash fractions, respectively. The 30 kDa band corresponding to the FrzE_CheW_Rec is present in lane 7 to 11. B) SDS-PAGE gel image after Superdex 200. Lane 1 input, 30 kDa band corresponding to the FrzE_CheW_Rec is present in lane 3 to 5. C) Elution profile after Superdex 200.

3.8 Purification of FrzE_CheW_CStrep:

Purification of FrzE_CheW from 1 liter culture was performed to confirm the expression of the protein. Strep-tag affinity chromatography was used for the purification. After elution, fractions were checked on 15% SDS-PAGE gel. 16 kDa bands corresponding to the molecular weight of the protein were not observed in any fraction, flow-through, wash and input (Fig 3.10A). Hence, it was confirmed that FrzE_CheW was not expressed.

3.9 Purification of FrzE_Dim_HATPase_N377A:

Strep-tag affinity chromatography was used for the purification of the mutant FrzE_Dim_HATPase_N377A. Fractions were checked on 12% SDS-PAGE gel after the elution. In the SDS-PAGE gel, a faint 35 kDa band corresponding to the molecular weight of the protein was observed along with a band at 20 kD in 4^{th}, 5^{th}, 6^{th}, 7^{th}, 8^{th} and 9^{th} fractions (Fig 3.10B). Bands at low molecular weight could be present if the protein degraded during the purification. This could be due to the instability of protein in given buffer conditions. Hence, further purification with different buffer conditions needs to be done for the purification of FrzE_Dim_HATPase_N377A.

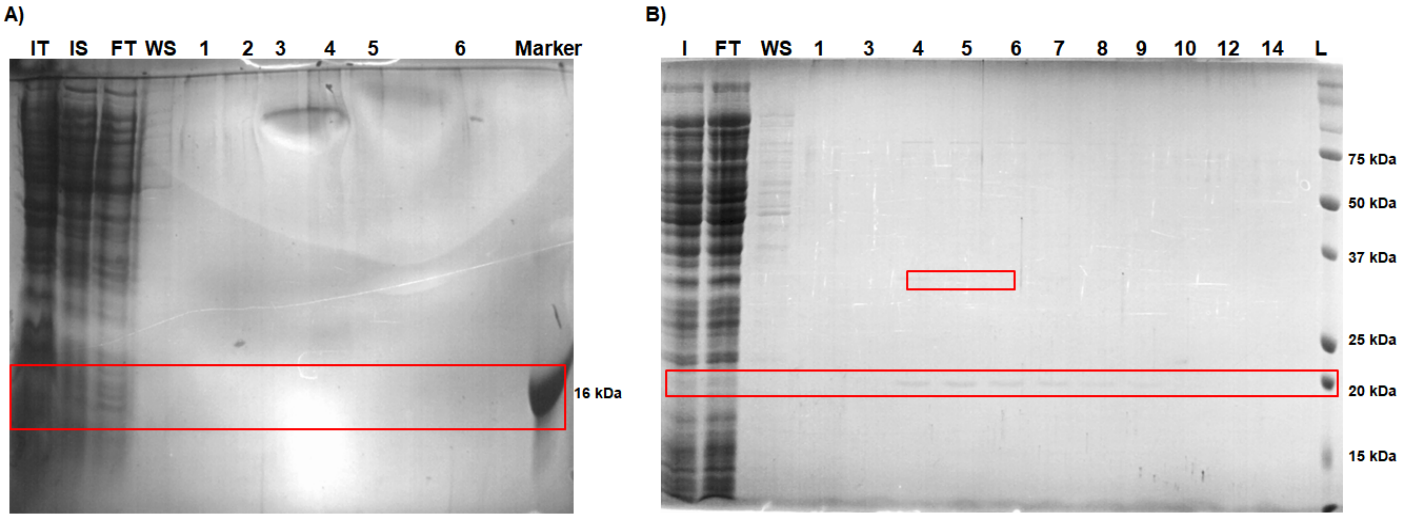


Fig 3.10: A) Image of SDS-PAGE gel after strep-tag affinity purification of FrzE_CheW. Lane 1, 2, 3, 4 and 13 is input total, input soluble, flow-through, wash fractions and 16.8 kDa marker, respectively. 16 kDa band corresponding to the molecular weight of FrzE_CheW is not observed. B) Image of SDS-PAGE gel after strep-tag affinity purification of FrzE_Dim_HATPase_N377A. Lane 1, 2, 3 and 13 is input, flow-through, wash fractions and protein ladder, respectively. Truncated 20 kDa band is present in lane 6 to 11.

3.10 Co-expression of FrzE_HPT and FrzE_HATPase:

FrzE_HPT with good individual expression was co-expressed with FrzE_Dim_HATPase to increase the expression of FrzE_Dim_HATPase. The SDS-PAGE gel image of the co-expression showed a 15 kDa band for HPT expression, whereas a faint band corresponding to the FrzE_Dim_HATPase at 35 kDa was observed (Fig 3.11A). There was no significant change in the expression of both proteins after the coexpression. To further confirm that FrzE_Dim_HATPase has expressed, a small scale purification needs to be done.

3.11 Co-expression of FrzE_CheW_Rec and FrzA:

Co-expression of FrzE_CheW_Rec and FrzA was carried out for the expression of FrzA, which was not expressed previously. As FrzE_CheW domain and FrzA are known to interact with each other, co-expressing can lead to the expression of FrzA. FrzE_CheW_Rec was expressed in the SDS-PAGE gel images at 30 kDa but FrzA did not expressed as no bands at 18 kDa were observed (Fig 3.11B).

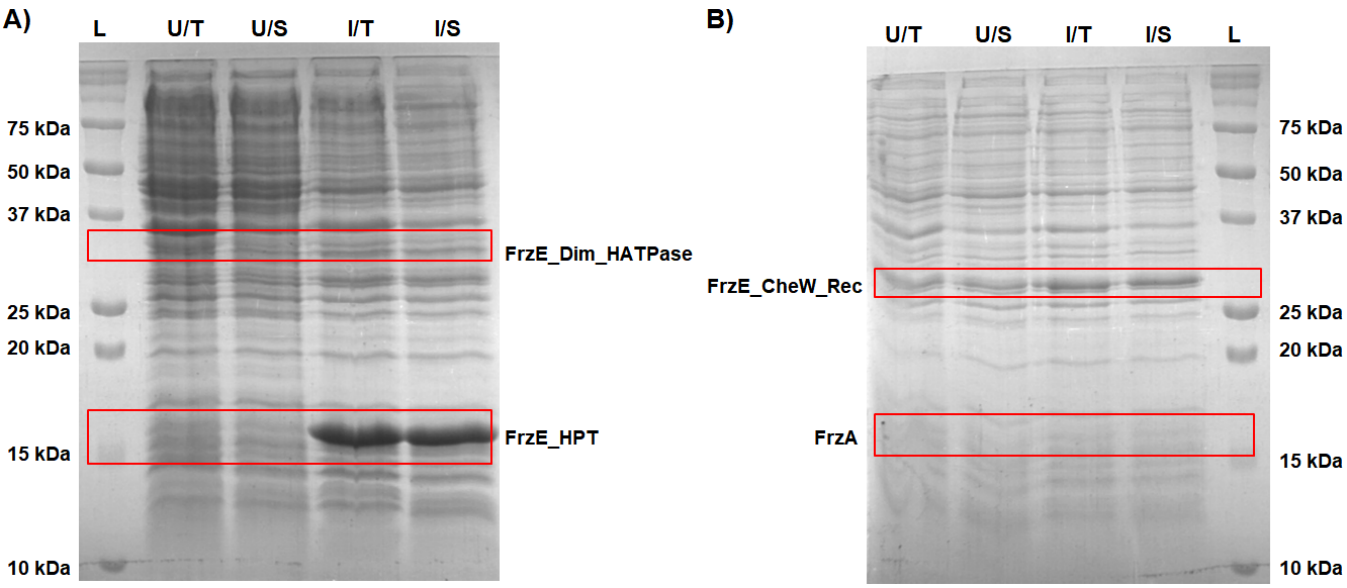


Fig 3.11: A) SDS-PAGE gel image for co-expression of FrzE_Dim_HATPase and FrzE_HPT. FrzE_HPT is expressed in lanes 4 and 5 at 15 kDa. A faint band at 35 kDa corresponding to the molecular weight of FrzE_Dim_HATPase is present in lane 4 and 5. B) SDS-PAGE gel image for co-expression of FrzA and FrzE_CheW_Rec. FrzE_CheW_Rec is expressed in lanes 3 and 4 at 30 kDa. No significant band for FrzA is present at 18 kDa in lanes 3 and 4.

3.12 Circular Dichroism Spectroscopy for FrzE_Rec:

As FrzE_Rec protein was eluted in the void using Superdex 75 and SEC650 column, it was necessary to confirm that FrzE_Rec was not misfolded and forming aggregate. CD spectrum profile (Fig 3.12) showed that 65 % alpha-helix, 25% beta-strand and 10% disordered region was present in the protein. This data was similar to the secondary structure prediction from PSIPRED. Hence, it was confirmed that the protein FrzE_Rec was present with the secondary structures.

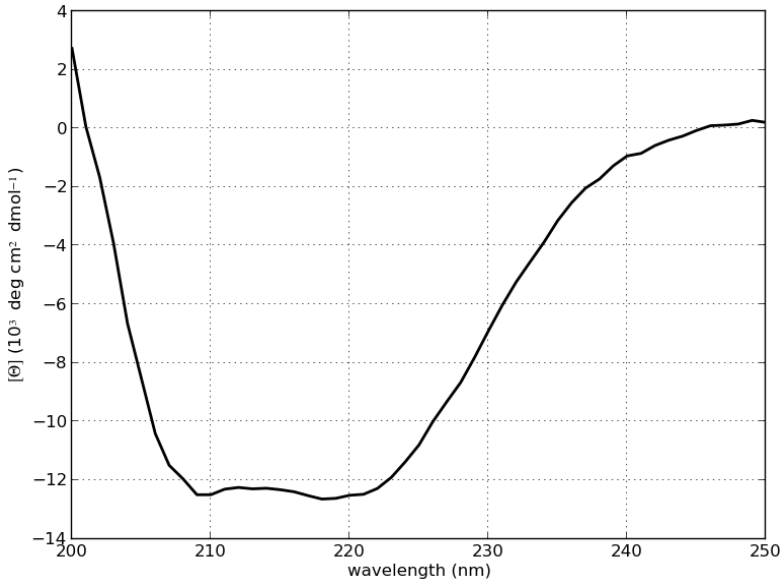


Fig 3.12: CD spectroscopy plot for FrzE_Rec.

3.13 Thermal shift assay for FrzE_Rec:

Protein stability and presence of properly folded tertiary structure were checked by thermal shift assay. In the fluorescence vs temperature plot (Fig 3.13A), high fluorescence was observed at low temperatures which decreased with the temperature. This was the case for all the buffer conditions. Also, the plot $-(dF)/dT$ vs temperature showed negative derivatives (Fig 3.13B). Hence, FrzE_Rec was probably misfolded.

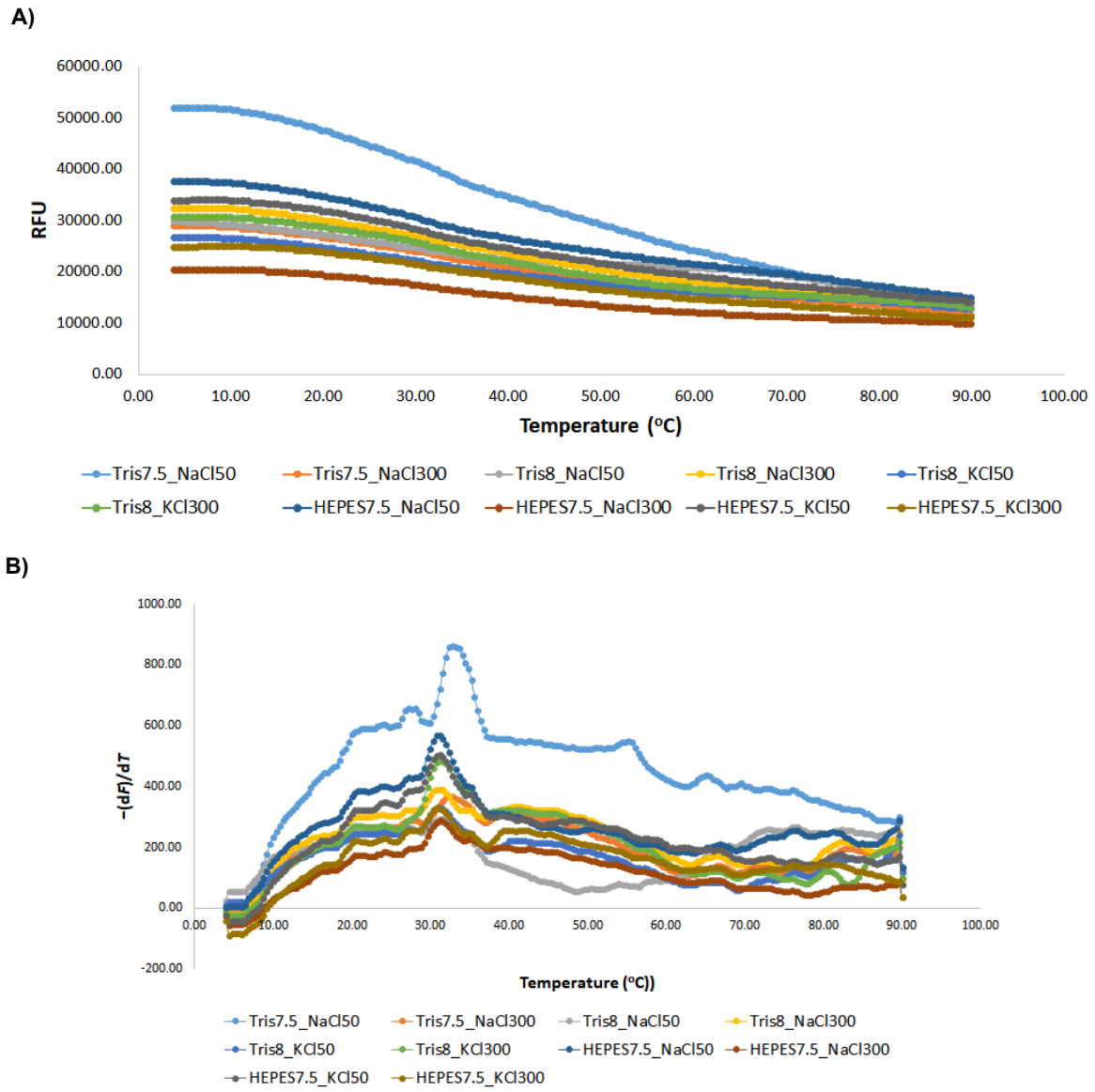


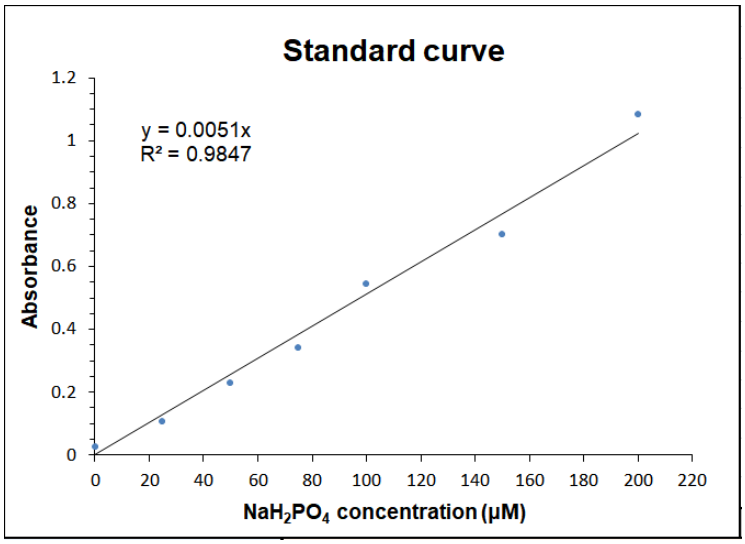
Fig. 3.13: Thermal shift assay for FrzE_Rec. A) Fluorescence vs Temperature plot. B) $(-(dF)/dT)$ vs Temperature plot.

3.12 Malachite assay for FrzE_Dim_HATPase and FrzE_ΔRec:

Upon receiving the signal, the FrzE_Dim_HATPase domain hydrolyzes ATP releasing the free phosphoryl group. This phosphoryl group is transferred to the histidine residue present in the FrzE_HPT domain. In absence of the FrzE_Rec domain, FrzE_ΔRec will remain phosphorylated and the free phosphoryl group will not be present in the reaction. Hence, a malachite assay was performed to check the ATPase activity of FrzE_Dim_HATPase and FrzE_ΔRec. The absorbance of the reaction at 630 nm was measured at 20 min intervals after stopping the reaction by EDTA addition. Upon addition of the malachite green solution, the reactions for FrzE_Dim_HATPase turned green with increasing intensity at each time point due to the complex formation between malachite green, ammonium molybdate and the phosphoryl group. Intensity increase was less or almost negligible for FrzE_ΔRec. The concentration of free phosphate was calculated for each reaction and was plotted with respect to the time. From the plot, it was observed that the concentration of the free phosphate was increasing exponentially for reactions containing FrzE_Dim_HATPase (Fig 3.14B). There was no significant increase observed for reactions containing FrzE_ΔRec as compared to FrzE_Dim_HATPase (Fig 3.14B). k_{cat} value was calculated to be 8.14 s^{-1} and 0.98 s^{-1} for FrzE_HATPase and FrzE_ΔRec, respectively.

From this assay, FrzE_HATPase was found to be ATPase active, whereas FrzE_ΔRec did not show significant ATPase activity. Higher activity for FrzE_HATPase can also be because of the impurities present in the protein. Hence, assays with ATPase dead mutant needs to be performed to further confirm the ATPase activity of FrzE_HATPase and FrzE_ΔRec.

A)



B)

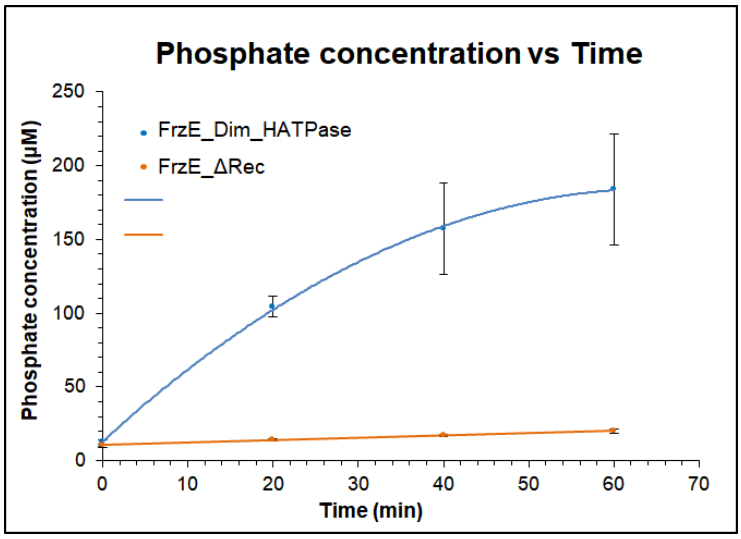


Fig. 3.14: Malachite assay for FrzE_Dim_HATPase and FrzE_ΔRec. A) Standard curve. B) Plot of free time vs phosphate concentration. Blue line is for FrzE_Dim_HATPase and the orange line for FrzE_ΔRec. Error bars are showing standard deviation for tree trails.

CHAPTER 4
CONCLUSION AND FUTURE PROSPECTS

4.1 FrzE_Dim_HATPase and FrzE_Dim_HATPase_N377A:

The FrzE_Dim_HATPase domain was cloned into the pHis17 vector and purified. After the Strep-tag affinity and size-exclusion chromatography purification, protein still had impurities and was present in the void. Hence, the purification needs to be standardized. As the protein expression of FrzE_Dim_HATPase was less, it was co-expressed with FrzE_HPT, since FrzE_Dim_HATPase interacts with FrzE_HPT. The proteins that interact with each other or form complexes can increase the stability of the proteins within the cell and hence co-expression of proteins can lead to the increased expression or solubility than when expressed alone. After co-expressing FrzE_Dim_HATPase and FrzE_HPT, no significant increase in the expression was observed.

The malachite assay was performed for the protein to check and quantify the ATPase activity. FrzE_Dim_HATPase protein was found to be catalytically active with k_{cat} 8.14 s^{-1}. This assay was performed along with FrzE_ΔRec which remains phosphorylated since the FrzE_Rec domain is not present. FrzE_ΔRec did not show any significant ATPase activity as compared to the FrzE_Dim_HATPase with k_{cat} value 0.98 s^{-1}. As impurities were present in the protein, a similar assay needs to be performed with the ATPase dead mutant to check if FrzE_Dim_HATPase protein is contributing to the total ATPase activity and protein present in the impurities are not contributing to the activity.

An ATPase dead mutant FrzE_Dim_HATPase_N377A was cloned into the pHis17 vector. Asparagine is a conserved residue found in the HATPse family and plays the role in stabilizing the magnesium ion during the ATPase activity. From the analysis (Meenakshi PV, MS thesis, May 2022), Asn377 in FrzE_Dim_HATPase was found to be conserved. Hence, Asp377 was mutated to alanine to stop the ATPase activity. Protein

purification for FrzE_Dim_HATPase_N377A was carried out but after the strep-tag purification truncated protein was obtained. This could be due to the degradation of the protein during the purification in given buffer conditions. Further purification trials are needed to confirm this and purification needs to be standardized to obtain the stable form of the protein.

4.2 FrzE_CheW and FrzE_CheW_Rec:

The FrzE_CheW domain was cloned into the pHis17 vector with C-terminal strep tag, N-terminal His-tag and C-terminal His-tag. Protein was not expressed in any of the clones. This could be due to the incomplete domain which could lead to the degradation of the protein within the cell resulting in no expression. So, a clone with CheW and Rec domain together was cloned into the pHis17 vector, to get the entire FrzE_CheW protein. FrzE_CheW_Rec protein was expressed and purification of the protein was carried out. Impurities were present in the purified protein and it eluted into the void. Hence, the purification needs to be standardized.

Since, FrzE_CheW interacts with FrzA, a co-expression of FrzA and FrzE_CheW_Rec was carried out for the expression of FrzA. FrzE_CheW_Rec was selected instead of FrzE_CheW as FrzE_CheW was not expressed. But, there was not expression of FrzA.

As a future prospect, mutational studies can be done to identify the residues of FrzE_CheW that interact with FrzA.

4.3 FrzE_Rec:

The FrzE_Rec domain was cloned into the pHis17 vector and purified. The purified protein contained impurities and was present in the void even after passing through the SEC650 column, which could be due to aggregate formation from misfolded protein. Hence, CD Spectroscopy and thermal shift assay were performed to check the stability and secondary and tertiary structure of the protein. CD spectroscopy data showed that the secondary structure was present in the protein. But from the thermal shift assay, it can be said that protein was probably misfolded. To obtain the stable form of the protein, purification needs to be standardized. Malachite assay along with FrzE_Dim_HATPase and FrzE_ΔRec can be performed for the stable FrzE_Rec protein.

Table 4.1 summarizes the work done for the FrzE_Dim_HATPase, FrzE_CheW and FrzE_Rec domain.

Table 4.1: Summary table

Constructs	Protein expression	Purification	Details
pHis17_FrzE_Dim_ HATPase_ CStrep	Bl21-Al, 18C/12hr, 0.2% Ara	poor yield with impurities, void in SUP200	Malachite assay, Co-expression with FrzE_HPT
pHis17_FrzE_ CheW_CStrep	No expression	Strep-tag affinity purification with no yield	FrzE_CheW_Rec domain cloned to obtain FrzE_CheW
pHis17_FrzE_ CheW_NHis	No expression	-	-
pHis17_FrzE_ CheW_CHis	No expression	-	-
pHis17_FrzE_Rec_ CStrep	Bl21-Al, 18C/12hr, 0.2% Ara	poor yield with impurities, void in SUP75 and SEC650	CD Spectroscopy, Thermal shift assay
pHis17_FrzE_ CheW_Rec_ CStrep	Bl21-Al, 18C/12hr, 0.2% Ara	Good yield with impurities, void in SUP200	Co-expression with FrzA
pHis17_FrzE_Dim_ HATPase_N377A_CStrep	Bl21-Al, 18C/12hr, 0.2% Ara	Truncated protein after affinity purification	-
pHis17_FrzE_HPT_NHis (Kan)	Co-expression, Bl21-Al, 18C/12hr, 0.2% Ara	-	Co-expression with FrzE_Dim_HAPTase
pHis17_FrzA_CHis (Kan)	No-expression	-	Co-expression with FrzE_CheW_Rec

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