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Histidine Kinases and Phosphatases from Yeast to Humans

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Abstract

Phosphohistidine phosphatase (PHPT1) is an enzyme that removes phosphate group from the amino acid histidine. In human it has two splice variants; one resulting in a variant protein and the other in a truncated form. These three proteins were expressed in *Schizosaccharomyces pombe* and subsequently subjected for protein assay. The result from the assay suggested that only the wild type PHPT1 has the protein activity while the other two are functionally inactive. Moreover, in *Candida albicans* histidine kinase (HK) is an important factor for virulency in immunocompromised patients. Since this HK has no homologues in human, it is a promising target for a drug screen. Cloning and sequencing of the histidine kinase is the initial step to this end.

1. Introduction

Histidine kinases are proteins that transfer a phosphate group to the amino acid histidine.

ATP + protein L-histidine ⇌ ADP + protein N-phospho-L-histidine.

On the other hand, histidine phosphatase removes phosphate group from histidine. It is very hard to work with phosphorylated histidine, since this state of the protein is very unstable.

There are two parts of this project. In the first part, the activity of phosphohistidine phosphatase (PHPT1) and its two splice variants (one of these is truncated due to an error in the annotation by one nucleotide) was measured by expressing these proteins in *Schizosaccharomyces. pombe.* The main objective of the second part of the project was to set up a drug screen against *Candida. albicans* Chk1 protein which is one of the main factors for virulency. The first step to do this was by removing the endogenous histidine kinase genes from *S. pombe* and replacing them with the *CHK1* gene. Afterward the transformed *S. pombe* strain will be investigated if it can complement the lack of histidine kinase genes that results in an increased doubling time in rich media. If this works the growth rate can be used to screen for drugs that will inhibit Chk1 protein.

A. Enzymatic activity of Phosphohistidine phosphatase, PHPT1 and two splice variants

1.1 Phosphohistidine phosphatase (PHPT1)

PHPT1 (phosphohistidine phosphatase) was first discovered in 2002 by Pia Ek (Ek et al. 2002). This is the first eukaryotic protein histidine phosphatase that has a structure that has been determined to the resolution of 1.9Å (Busam et al. 2006).

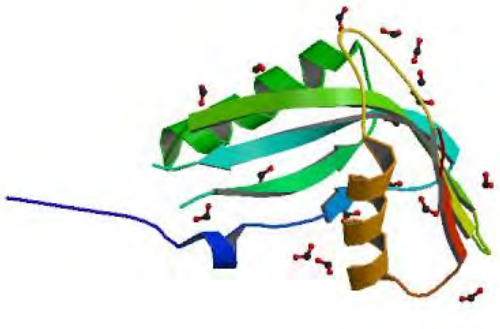

Figure 1: Crystal structure of human phosphohistidine phosphatase. Image from Structural Genomics Consortium. PDB entry 2HW4.

The function of this enzyme is not well characterized yet. A Northern blot analysis indicated that human phosphohistidine phosphatase mRNA is present mainly in heart and skeletal muscle (Ek et al. 2002).

This enzyme is involved in several diseases for example pediatric acute lymphoblastic leukemia. PHPT1 may play a role in human tumor invasion and metastasis through the interference with cytoskeleton reorganization (Xu et al. 2010).

1.2 *Schizosaccharomyces pombe*: a model organism

Schizosaccharomyces pombe is a unicellular organism that is extensively used for different types of researches in Molecular Biology. The organism divides by medial division, so it is called fission yeast. It was first isolated by Lindner in 1893 from East African Mellet beer. Fission yeast is a rod shaped eukaryotic organism with 3-4 μm in diameter and 7-14 μm in length.

The genome of *S. pombe* was sequenced in 2002 (Wood et al. 2002) *S. pombe* has three chromosomes (5.7 Mb, 4.6 Mb and 3.5 Mb) with 4,970 open reading frames. The genome of fission yeast is more like the human genome as compared to budding yeast since for example it contains more introns (approximately 5,000) than *S. cerevisiae* (250). Also in *S. pombe*, the centromere structure, histone modifications are similar to higher eukaryotic organisms (Wood et al. 2002). Fission yeast has a 2-3 hr generation time in rich culture medium. During cell division it grows primarily in length and not in width. When it becomes nearly 14 μm, it starts to divide and produces two 7 μm daughter cells (Egel 2004).

There are three basic strains for *S. pombe* with respect to the organization of the mating-type region, homothallic h^{90}, heterothallic h^{-} and heterothallic h^{+} (Zhao and Lieberman 1995). Strains with h^{-} as well as h^{+} can mate with each other but not with themselves because they express only the minus or the plus mating information, respectively. On the other hand, h^{90} cells contain information for both mating types and can switch between the two types and thus can mate within the strain. Although h^{+} contains the information for the two mating types but cannot switch from one to another.

1.3 Aim of the project

The aim of this project was to study the protein activity of Phosphohistidine phosphatase (PHPT1) and two recently identified splice variants (unpublished). To this end PHPT1 and its two splice variant were cloned into a vector for recombinant expression in bacterial cells. The plan was to make recombinant expression of these three proteins in bacteria and compare their enzymatic activity using an improved as well as effective histidine phosphatase assay that recently was established by the Ek group (Beckman-Sundh et al. 2011). This project also includes subcloning the 2 splice variants from the bacterial vector into the yeast expression vector to allow for recombinant expression in the yeast cell as a host. The enzymatic activity of total protein extract from the yeast cell lysate is compared between yeast cells having an

empty plasmid to yeast cells containing plasmids with the PHPT1 gene or its splice variants.

B. Histidine kinase, Chk1 from *Candida albicans*

1.4 *Candida albicans*

Candida albicans is a commensal organism, which means that it is not harmful in healthy individual but becomes pathogenic in immunocompromised patients with HIV, cancer or people taking immunosuppressive therapy. It is a unicellular, oval-shaped diploid fungus and lives on various mucosal surfaces of the body, including the oral cavity, gastrointestinal tract, and vaginal mucosa (Berman and Sudbery 2002). It is a dimorphic (yeast and hyphae) fungus and can change from one morphological form to the next in different environmental conditions for example in different media. Sometimes a pseudohyphal form can also be found.

The *C. albicans* genome was sequenced by Stanford Genome Technology Center (med.stanford.edu/sgtc/). It contains a diploid genome with 8 pairs of homologous chromosomes. The haploid genome size is nearly 16 Mb and contains a number of retrotransposons (Scherer 2002). The number of ORFs in this organism is estimated to be 6,419. The Candida hyphal form is often found at the tissue invasion area which indicates that this morphology is responsible for the virulence (Odds 1988).

1.5 *CHK1* gene

The cell wall of *C. albicans* is composed of chitin, β-glucan and mannnans (Cheng et al. 2012). Among these polysaccharides β-glucan is more relevant for host-pathogen interaction (Poulain and Jouault 2004), because humans have the C-type lectin-like receptor dectine which is an important pattern recognition receptors (PRRs) for β-glucan. However the yeast has the mechanism to escape the immune system by hiding the β-glucan layer (Netea et al. 2008). *C. albicans* has 3 histidine kinase proteins named as Chk1p, Cos1p, Sln1p which are involved in cell wall biosynthesis and virulence. *CHK1* that encodes Chk1p, is more important for virulence because disruption of this gene changes the cell wall structure and reduces the β-glucan content (Kruppa et al. 2003).

1.6 Complementation of histidine kinase gene in *S. pombe*

S. pombe has three histidine kinase genes which encode histidine kinases named as Phk1 (or Mak2), Phk2 (or Mak3) and Phk3 (or Mak1) (Aoyama et al. 2001). The His-to-Asp phosphorelay pathway (an intracellular signal transduction pathway which is ouccured by protein phosphorylation) using these three histidine kinases (Phk1/2/3) plays a crucial role for propagation of the sexual development (Nakamichi et al. 2002). The three histidine kinase genes in *S. pombe* are important to prevent mating when nutrients are available. When the yeast lacks these genes, it can mate and sporulate even when grown on rich media.

1.7 Aim of this project

Although antifungal agents are available, the treatment is complicated due to high host toxicity. The aim of this project was to set up a drug screen against *C. albicans* Chk1 protein using *S. pombe*. First *CHK1* gene was to be amplified and subsequently cloned into a *S. pombe* expression vector. By crossing, an *S. pombe* strain was to be constructed which lacks the three endogenous histidine kinase genes and the *S. pombe* expression vector with *CHK1* gene would be used to transform this strain. After that the transformed strain should be cultured in nutrient rich media and the doubling time determined if the *CHK1* gene can complement the loss of endogenous, *S. pombe* histidine kinase genes we can use the growth rate to screen for drugs that will inhibit Chk1. The drug would give a reduced virulence of *C. albicans*, since hyphal growth will be inhibited.

2. Materials and Methods

2.1 Media

In the present investigation two different yeast species, *S. pombe* and *C. albicans*, and in addition one bacterial strain, *E. coli* were used. Four different media were used to grow these strains, which are enlisted in the appendix.

2.2 Strains

Different strains that were used in this project are listed below (Table 1). *S. pombe* strain PJ121 was used for the transformation with the plasmid that contains PHPT1 and its splice variants. PJ1324 is the *C. albicans* strain that was used for *CHK1* gene amplification. PJ1329 and PJ1142 are two *S. pombe* strains that were used for crossing to produce an *S. pombe* which lacks endogenous histidine kinase genes and has the h^{90} mating type configuration. The bacterial strain *E. coli* DH5α was used for transformation.

Table 1 Genotype of different strains that were used in this project

Strain	Genotype
PJ121	*h^+ ura4-D18 leu1-32 ade6-M216*
PJ1300	PJ121+pREP3X
PJ1338	PJ121+pREP3X+PHPT_wt
PJ1340	PJ121+pREP3X+PHPT_var1
PJ1302	PJ121+pREP3X+PHPT_var2
PJ1329	*h^{90} leu1-32 ura4-D18 ade6-M216*
PJ1142	*h^- leu1-32 ade6-M216 ura4-D18 phk1::ura4$^+$phk2::ura4$^+$ phk3::ura4$^+$*
PJ1324	SC5314 *Candida albicans* wild type2
E. coli (DH5α)	*supE44 ΔlacU169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*

2.3 Bacterial transformation

Competent *E. coli* cells (DH5α) were taken from -80°C and kept on ice for thawing and then the ligation mixture (vector with different PHPT1 construct) was added to the competent cells and mixed well. Subsequently, the reaction tubes were kept on ice for 30 min and after that the tubes were heat shocked at 42°C for 45 sec. Again the tubes were kept on ice for 5 min to reduce damage to the *E. coli* cells. Finally the transformed cells were placed on LB agar plates that contained ampicillin and incubated for at 37°C, overnight.

2.4 Yeast transformation

Yeast transformation was carried out by electroporation using a Bio-Rad gene pulser. The strain PJ121 was grown in 200 ml until it reached to log phase. The cell density

ranging from $5x10^6$ to $1x10^7$ cells/ml as determined by counting in a Burker chamber under the light microscope. The cells were then centrifuged (Heraeus Labofuge 400R) at 4500 rcf at 4°C for 10 min to collect the cells. The supernatant was removed and pellet was resuspended by using 10 ml of ice-cold 1.2 M sorbitol (filter sterilized). In this way the cells were washed 3 times with ice-cold 1.2 M sorbitol. Then 10 ml of sorbitol was used to resuspend the cell. The plasmid DNA that contains the *PHPT1* gene or splice variants (desalted) was added to 200 µl of cells just before adding the mixture into electroporation cuvettes (Merck). After adding the plasmid DNA, it was mixed gently with the cells. Then the cuvettes were put on the Bio-Rad gene pulser and 2.25 KV, 200 Ω, 25 mF was applied. After each pulse 500 µl ice-cold sorbitol was added and mixed well. Finally the cells were plated on selective media PMG-leu with thiamine (PMG-leu+T) and incubated at 30°C. The transformed cells appeared after 3-4 days and cells were restreaked on the same type of plate to obtain single colony isolates.

2.5 Stocking of strains in -80 ºC

Bacteria (*E. coli*)

Bacterial strain with PHPT1 construct was first grown in 3 ml LB (amp) media at 37 ºC overnight and shaken at 200 rpm. The day after 0.8 ml of 50 % glycerol was added to 0.8 ml of bacterial culture and mixed well in a special cryoprotect tube before storing in -80 ºC.

Yeast (*S. pombe*)

Different *S. pombe* strains with the right construct were first grown on a plate containing PMG-leu (+T). Then 1.6 ml 25 % glycerol was taken into special cryoprotect tube followed by addition of a loop of yeast cells scraped of the plates. Cells and glycerol was mixed well and stored in -80 ºC.

2.6 Protein extraction from *S. pombe*

The transformed cells were first grown in 40 ml PMG-leu medium until they reached the culture log phase. When the density of cell was 5 $x10^6$ to 1 $x10^7$ cells/ml (measured by Burker chamber), the cells were collected by centrifugation (Heraeus Labofuge 400R) at 4500 rcf at 4°C for 5 min in a 50 ml Falcon tube. The supernatant was removed and 5 ml PHPT1 buffer (50 mM HEPES-pH7.5, 150 mM NaCl and 1% Tween-20) was added and centrifugation was repeated as above. After removing the supernatant the resuspended pellet was transferred to 1.5 ml ice cold eppendorf tube and centrifuged at 13000 rpm rcf at 4°C for 10 min. The pellet was resuspended by adding 500 µl PHPT1 buffer with protease inhibitor (Roche, protease inhibitor cocktail) and centrifuged at 13000 rcf at 4°C for 1 min. 30 µl of PHPT1 buffer + protease inhibitor was added to the pellet and transferred into pre-chilled safelock 2 ml tubes with 500 µl of glass beads. To break the cells a FAST prep machine at maximum power was set for 20 sec and the process repeated three times. After adding 300 µl of PHPT1 buffer + protease inhibitors, a hole was made in the bottom of the

safelock tube with a needle and a 2 ml tube was attached. Then the two tubes were centrifuged at 700 rcf at 4°C for 5 min. The eluate was collected and centrifuged at 13000 rcf at 4°C for 20 min to separate the pellet and supernatant.

2.7 Measuring the protein concentration

A BCA (Bicinchoninic acid) assay kit (Pierce® BCA Protein Assay Kit, Thermo Scientific) was used for measuring the protein concentration. Eight tubes were marked as 1-8 and in 1 tube 50 µl of protein extract was added in 100 µl of BCA stock solution (2 mg/ml). From this tube 50 µl was transferred to tube number 2 and then from 2 to tube 3. This process was continued until the 7th tube. Tube 8 only contained PHPT1 buffer. 10 µl from each tubes of the serial dilution was taken into 3 wells of the plate and reagent A and reagent B were mixed in 50:1 ratio. Afterwards 200 µl of A+B reagent was taken into all wells either with standard serial dilution or sample. Then the plate was incubated at 37ºC for 10-15 min and protein concentration was measured at 560 nm in a spectrophotometer.

2.8 Western Blot

For making the SDS-PAGE gel, first separation gel was prepared by adding the compounds in the right order (composition is given in appendix) and after solidification the gel was then placed on the gel holder (Bio-Rad) and filled with 1 X running buffer up to mark. After loading sample, 150 V was applied to run the gel until the dye was about to near the bottom. Subsequently by using transfer cassette, transfer was carried out on membrane. The cassette was then placed in transfer tray and filled with 1 X transfer buffer and transfer was conducted for 60 min room temperature at 100V. When the transfer was completed the membrane was blocked by blocking buffer overnight in the cold room. Rabbit antibody against PHPT1 protein (from Pia Ek) was used as primary antibody and diluted 100 times with blocking buffer. After incubation for 1.5 h with slow agitation at room temperature, the antibody solution was removed by washing with wash buffer (2 X quick, 1 X 5 min with agitation, 1 X 15 min with agitation, 1 X 5 min with agitation 2 X quick). Horseradish peroxidase conjugated anti-rabbit antibody from donkey was used as the secondary antibody and diluted 5000 times by using blocking buffer and subsequently incubated for 1 h at room temperature, followed by washing the Ab with wash buffer (2 X quick, 1 X 5 min with agitation, 2 X 15 min with agitation, 1 X 5 min with agitation 2x quick). The membrane was then transferred to 1 X PBS without Tween. Finally the membrane was soaked in ECL solution and analyzed by using Bio-Rad gel doc.

2.9 Measurement of PHPT1 protein activity

The activity of PHPT1 and its two splice variants was obtained from Pia Ek at the department. For this purpose phosphohistidine was used as a substrate.

2.10 Purification of total genomic DNA from *Candida albicans*

For isolation of genomic DNA from *Candida albicans*, E. Z. N. A. Genomic DNA Isolation kits, Spin protocol was followed. Cells were first grown in YEA medium to 6×10^5 cells/ml -1×10^7 cells/ ml and then 3 ml culture was centrifuged at 4000 rcf for 10 min at room temperature. Cells were resuspended in 480 µl Buffer and 40 µl lyticase solution and incubated at 30ºC for 60 min and followed by centrifugation for 3 min at 2000 rcf at room temperature. Cells were resuspended in 200 µl Buffer and vortexed for 5 min after adding approximately 50 µl of glass beads. 20 µl Proteinase solution was added and vortexed to mix well. Then 2.5 µl RNaseA was added to samples and incubated at room temperature for 5 min and supernatant was collected by centrifugation at 10000 rcf for 5 min. After the addition of 220 µl Buffer the tube was vortexed at maximal speed for 15 seconds followed by an incubated at 65ºC for 10 minutes. Subsequently 220 µl absolute ethanol was added and mixed thoroughly by vortexing at max speed for 20 seconds. The sample was transferred to HiBind^R DNA mini column and centrifuged at 10000 rcf for 1 min. The column was placed into a second 2 ml tube and washed by adding 500 µl Buffer and centrifuged at 10000 rcf for 1 min. Again the column was placed into the same collection tube and wash by adding 700 µl DNA Wash Buffer centrifuged at 10000 rcf for 1 min and this step was repeated The column was then placed into 1.5 ml microfuge tube and 60 µl of preheated (65ºC) Elution Buffer was added in the column and incubated at 3-5 minutes at room temperature. For the DNA elution, the column was centrifuged at 10000 rcf for 1 min.

2.11 Amplification of the *CHK1* gene

The DNA was first isolated from *C. albicans* and diluted by a 100 X factor. Phusion enzyme (Fermentas) was used for this PCR and mixing was done on ice. Five µl of DNA was mixed with 45 µl of PCR mix (as given in appendix). First the block of PCR was heat up at 98ºC and the CHK1 PCR program (as given in appendix) was used for the amplification of the DNA.

2.12 Cloning of the *CHK1* gene into TOPO vector

First QIAquick Gel Extraction Kit (QIAGEN®) was used to purify the PCR DNA from an excised gel slice. Afterward, for the purpose of cloning, the addition of A-overhang to the PCR product was undertaken (10 µl PCR product; 1.2 µl PCR Buffer; 1.2 µl $MgCl_2$; 0.5 µl 10 mM dATP, 0.1 µl Taq polymerase). The mixture was placed on a 72 ºC heating block for 15 min and then put on ice. Then cloning was carried out by making ligation reaction (4 µl PCR product with A-overhang, 1 µl salt solution, 0.5 µl of TOPO 2.1 vector). After incubation at room temperature for 20 min, transformation was carried out by adding 2 µl ligation mix to one tube of OneShot competent *E. coli*. The cells were incubated on ice for 10 minutes and after heat shock for 30 sec at 42 ºC placed on ice. The cells were grown at 37 ºC, 200 rpm for 1 h after adding 250 µl of liquid LB (amp) medium. The cells were then spread onto pre-warmed LB+amp plate with added X-gal (60 mg/plate) and IPTG 40 µl/plate).

2.13 Yeast crossing

Two *S. pombe* strains, PJ1142 and PJ1329 were used for crossing. First the PJ1142 was streaked on PMG total media as a straight line. Then the second strain was used to streak over the previous one. The plate was then incubated for 3 days at 30°C. Then Gulsulase enzyme (filter sterilized) was used to dissolve spore ascus and other cells that did not form spore. This was done by taking a small amount of cells from the PMG plate that was mixed with 500 µl of the enzyme followed by an overnight incubation at 30°C. After incubation the spores were checked for removal of ascus as well as vegetative cells under light microscope. The spores were then plated on YEA and AA-ura plate.

2.14 Colony PCR to detect mak 1/2/3 deletions

For colony PCR, fresh cells (plate not older than 1 week) was taken by a toothpick and dissolved in 100 µl of water. Finnzymes' Phire™ Hot Start DNA Polymerase enzyme was used for this PCR and the reaction was done on ice. 5 µl of cells was mixed with 45 µl of PCR master mix. First the block of PCR was heated up at to 94ºC and the JTAG PCR program (as given in appendix) was used for colony PCR.

2.15 Sequencing

For sequencing the samples were sent to Eurofins MWG Operon (Ebersberg, Germany). The volume of DNA that was sent for sequencing was 15 µl with concentrations of 50-100 ng/µl for plasmid DNA. The pCR®2.1-TOPO® (Invitrogen) plasmid with different inserts were used for sequencing with different primers. The sequencing result was analysed by using the software CLC Main Workbench 6.

3. Results

A. Study of *PHPT1*

3.1 Construction of *PHPT1* gene and its splice variants in yeast vector

The vector pREP3X contains a strong yeast promoter *nmt* and was used for insertion of *PHPT1* and its splice variants. (Figure 2). This vector and constructed plasmid were subsequently used for transformation of *S. pombe* strain PJ121.

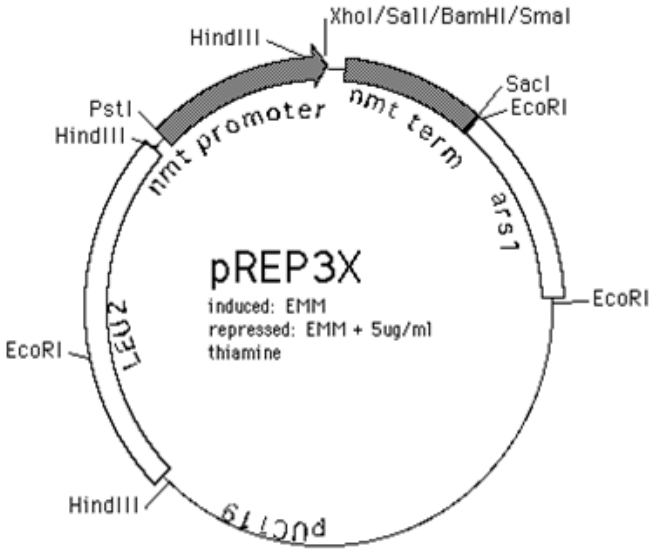

Figure 2: Fission yeast expression vector pREP3X

Source: Pombnet, Forsburg lab (http://www-bcf.usc.edu/~forsburg/)

Initially two yeast vectors pREP3X and pREP81X were used for the insertion of *PHPT1* gene to see which one can express well in *S. pombe*. Between these vectorsthe former one has a strong promoter and the latter one has a weaker promoter. Before the gene insertion these two vectors were treated with SAP (shrimp alkaline phosphatase) to prevent relegation of vector. The inserts were obtained by cutting pPB83 (*PHPT1*_wt) and pPB84 (*PHPT1*_var1) with *BamHI* and gel purification of the inserts. Figure 3 shows the gel picture to compare the vector and insert before the ligation reaction.

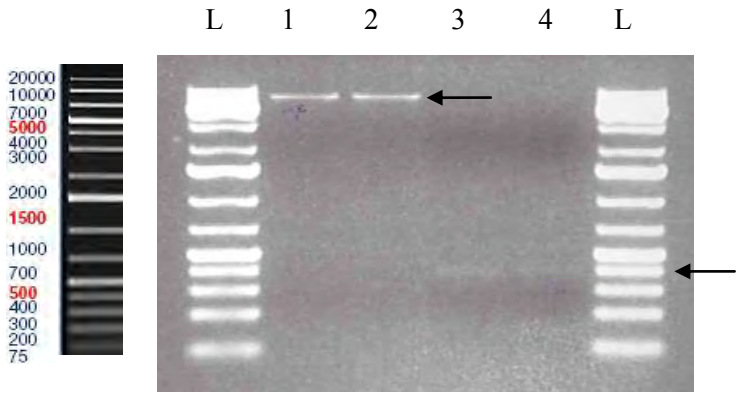

Figure 3: Gel picture of *BamHI* digested Yeast vector and *PHPT1* gene. L= Gene Ruler^{TM} 1kb plus DNA ladder. Lane 1 and 2 are pREP3X and pREP81X *BamHI* digested and SAP treated yeast vectors, respectively. Lane 3 and 4 are *BamHI* digested and gel purified inserts from pPB83 (*PHPT1*_wt) and pPB84 (*PHPT1*_var1). The arrows correspond to 1.0kb (left) and 400bp (right).

After the ligation reaction, competent *E. coli* (DH5α) was used for transformation and LAamp plates were used to select transformants. Plasmid were purified from the transformed colonies and digested with *BamHI* to check whether the *PHPT1* gene was inserted into the vectors or not. Figure 4 shows the digestion of plasmid from transformed bacteria with *PstI*, to check the the right orientation. Here bacterial expression vector pET11a was also used, from where the *PHPT1* gene and its variants were cloned into pREP3X vector. Digestion with *PstI* generated different length of restriction fragment based on the orientation, smaller fragment when *PHPT1* gene was in right orientation and larger fragment when in wrong orientation (Figure 5).

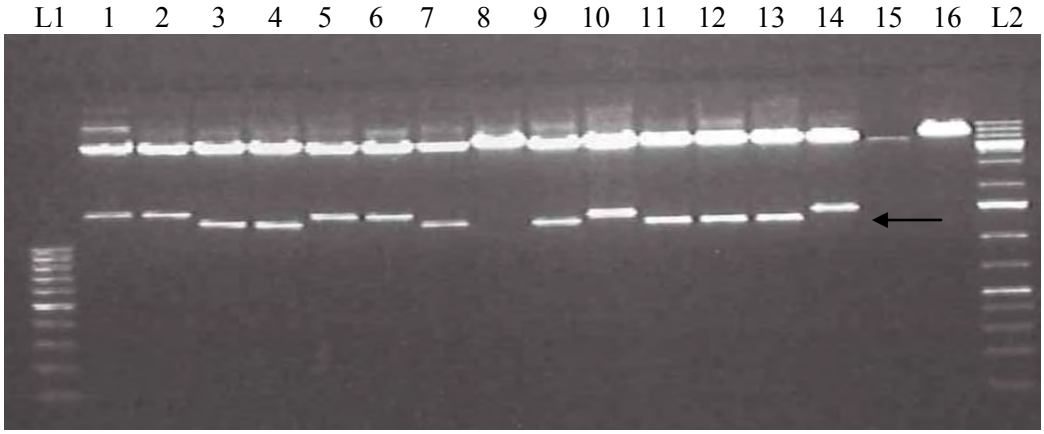

Figure 4: Digestion of plasmid from transformed *E. coli* with *PstI*. L1 100bp and L2 1kb plus Gene Ruler^{TM} DNA ladder. Digestion that produced smaller fragments is in right orientation which is shown in 3, 4, 7, 9, 11, 12, and 13. Lane 15 and 16 shows empty vector pET11a and pREP3X.

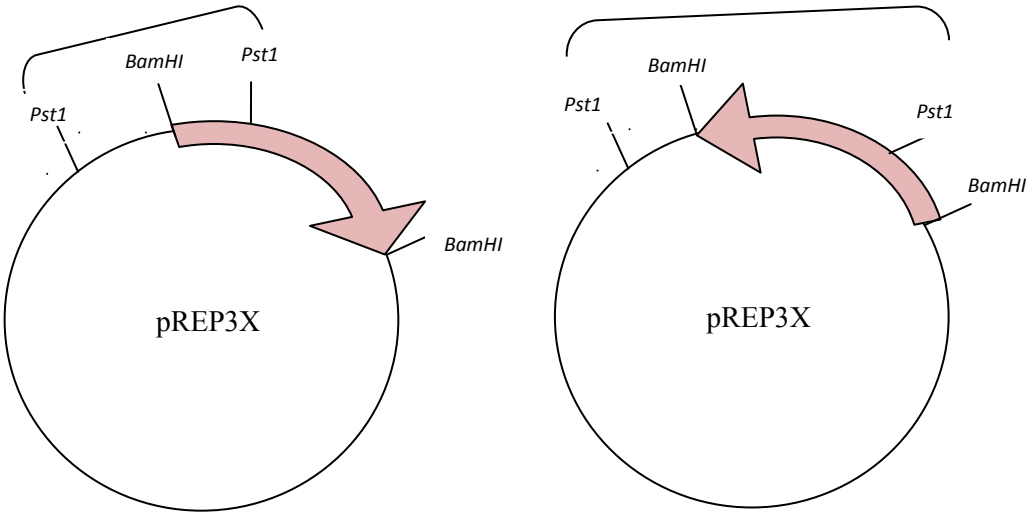

Figure 5: *PstI* digested fragment in right (left) and wrong (right) orientation.

The plasmids with the right orientation of the *PHPT1* gene are given in following table.

Table 2: Plasmid with *PHPT1* gene and its splice variants

Name of plasmid	**Vector construct**
pYA1	pREP3X+ pPB83 (*PHPT1*_wt)
pYA2	pREP81X+ pPB83 (*PHPT1*_wt)
pYA3	pREP3X+ pPB84 (*PHPT1*_var1)
*pPB81	pREP3X+ pPB85 (*PHPT1*_var2)
*pPB82	pREP81X+ pPB85 (*PHPT1*_var2)

(*The last two vectors were constructed by my supervisor, Pernilla Bjerling).

3.2 *S. pombe* transformation and measurement of the activity of PHPT1 protein and its variant

S. pombe strain PJ121 was transformed with the three plasmids pYA1 (*PHPT1*_wt), pYA3 (*PHPT1*_var1), pPB81 (*PHPT1*_var2) and an empty vector. From these transformants, protein was extracted, and the phosphatase activity of protein was measured by Pia Ek (Ek et al. 2011).

Table 3: Protein activity (from Pia Ek)

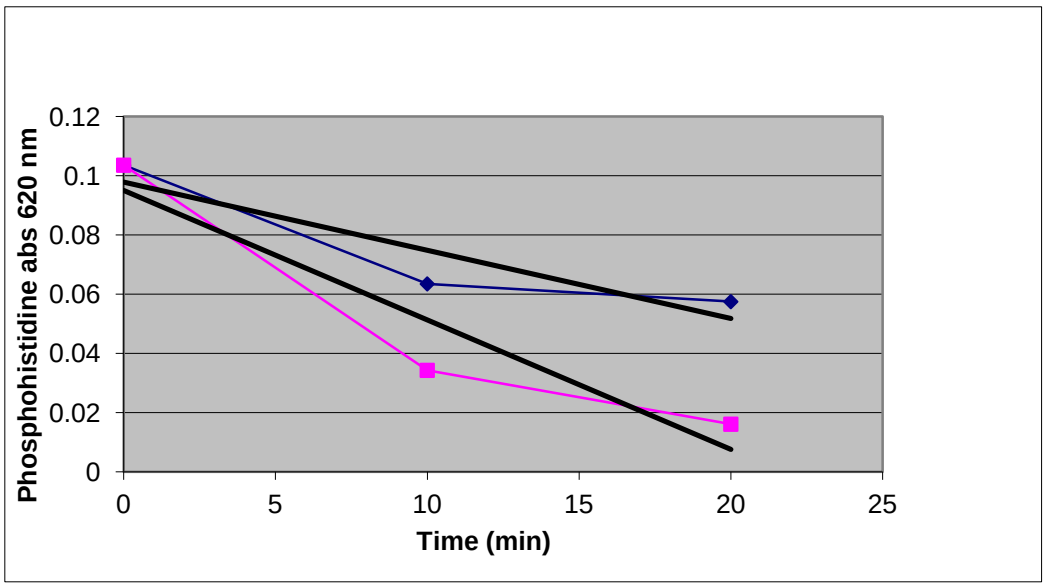

Figure 6: Measurement of the activity of PHPT1 protein and its two splice variants. Blue line indicates the background activity in the negative control while pink line indicates the activity with wild type PHPT1 protein. No activity was seen for the splice variants or the truncated PHPT1 protein.

Phosphohistidine was used as substrate for measuring the activity of PHPT1 protein and its two splice variants at 620 nm absorbance (Beckman-Sundh et al. 2011). PHPT1 protein removes the phosphate group from the phosphohistidine in the peptide. Only extracts with wild type PHPT1 protein showed activity and it was 420 pmol/min/µl extract (Figure 6). The protein concentration from wild type variant PHPT1 (unpurified) was measured using the Bradford method (Bradford 1976) and the specific activity was calculated to 4200 pmol/min/mg protein extract.

3.3 Detection of PHPT1 protein using Western blot

The concentration of total protein that was extracted from *S. pombe* was measured by using BSA assay kit and the result is given in Table 4.

Table 4: Concentration of different protein extract (wavelength 560 nm)

Conc. of protein (mg/ml)							
Supernatant				**Pellet**			
PJ1300	PJ1302	PJ1338	PJ1340	PJ1300	PJ1302	PJ1338	PJ1340
5.2	5.5	5.1	5.8	3.8	3.7	3.3	4.2

After measuring the protein concentration, proteins were separated using SDS-PAGE and different PHPT1 variants were detected by Western blot. In Figure 7, it could be observed that only the wild type PHPT1 protein expression could be detected (lane 3 and lane 7).

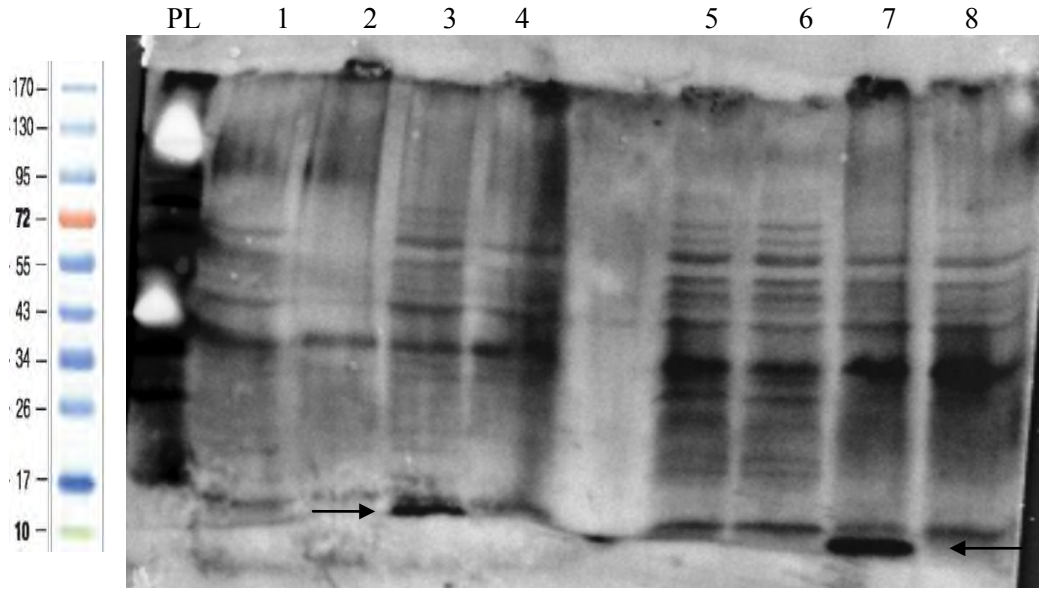

Figure 7: Western blot of PHPT1 protein and its splice variants. PL= prestained protein ladder (Fermentas). 1-4 represents the protein from supernatant of PJ1300, PJ1302, PJ1338, PJ1340, respectively. 5-8 depicts the protein from pellet with same order. The arrows correspond to the size of PHPT1 protein *i.e.* 14kD.

The primary antibody has a recognition site for the wild type (Shown in green colour for pYA1 in following protein sequence, Figure 8). That's why in the Western blot only the wild type could be detected. The splice variants, however does not contain the epitope and thus could not be detected in the Western blot.

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pPB81          MAVADLALIPDVDIDSDGVFKYVLIRVHSAPRSGAPAAESKEIVRGYKWA
pYA1           MAVADLALIPDVDIDSDGVFKYVLIRVHSAPRSGAPAAESKEIVRGYKWA
pYA3           MAVADLALIPDVDIDSDGVFKYVLIRVHSAPRSGAPAAESKEIVRGYKWA
               **************************************************

pPB81          EYHADIYDKVSGDMQKQGCDCECLGGGRISHQSQDKKIHVYGYSMVSRSP
pYA1           EYHADIYDKVSGDMQKQGCDCECLGGGRISHQSQDKKIHVYGYSMAYGPA
pYA3           EYHADIYDKVSGDMQKQGCDCECLGGGRISHQSQDKKIHVYGYSMAYGPA
               ******************************************.  ..

pPB81          VPPCRRPQYQLRGPPEPAALTRGPS
pYA1           QHAISTEKIKAKYPDYEVTWANDGY
pYA3           QHAIST------------------
```

Figure 8: CLUSTAL W (1.83) multiple sequence alignment

B. *Candida albicans CHK1* gene study

DNA from *Candida albicans* strain, PJ1324 was extracted and used for amplification of the CHK1gene. For the PCR reaction (as given in appendix: CHK1 PCR), the gene specific primers were tested for optimum annealing temperature by using gradient PCR. Annealing temperature was set between 65ºC - 72.5 ºC and the bands can be seen in Figure 9. Gradient temperature of 65 ºC - 67.6ºC shows good PCR amplification, so for the subsequent experiment 66.5 ºC was chosen. The PCR product (*CHK1* gene) was resolved and used for cloning into the TOPO 2.1 vector.

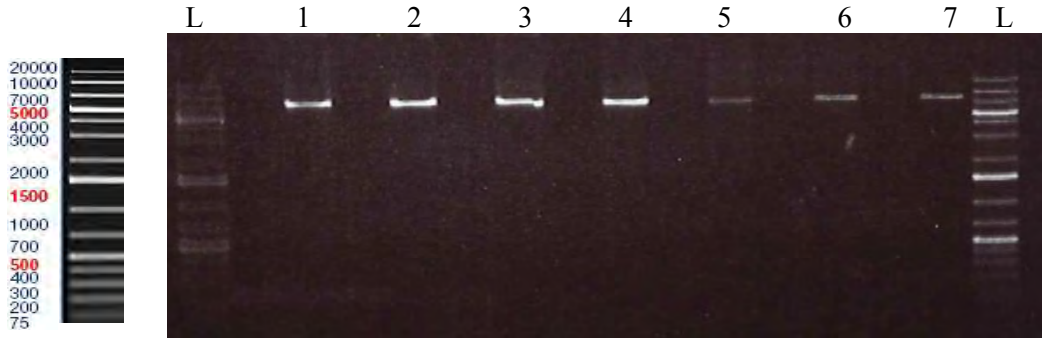

Figure 9: Gradient PCR of primer F53 and F54. L=1kb plus Gene Ruler^{TM} DNA ladder. The 1, 2, 3, 4, 5, 6, 7 indicates the gradient temperature 65, 65.7, 66.5, 67.6, 69, 70.7 and 72.2 ºC, respectively. The negative controls for each temperature are on the left side of corresponding temperature.

3.4 Sequencing of *CHK1* gene

The purified PCR product (*CHK1* gene) was cloned into TOP10 competent *E.coli* cells and plasmids from the white colonies on X-gal plates were subjected to digestion with *XhoI* enzyme. This digestion resulted in two DNA fragments and the larger one (nearly 7000 bp) is *CHK1* gene (Figure 10).

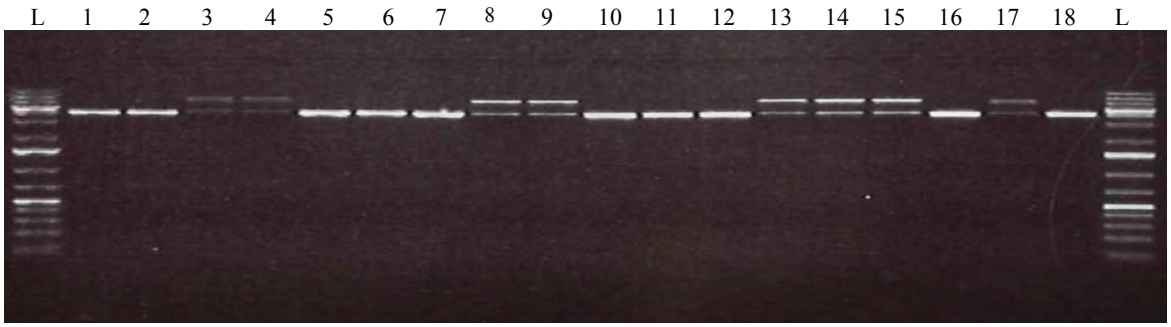

Figure 10: *XhoI* digestation of transformed TOP10 clone with *CHK1*gene. Number 3, 4, 8, 9, 13, 14, 15, 17 are the digested positive plasmids with an insert.

Some of the positive samples were sent for sequencing. Almost all colonies with uni 21 primer showed no mutation whereas with rev 29 primer showed 1 or 2 base pair changes in codon (Figure 11). Till now at least one clone has been sequenced without any mutation, 1-1810 nucleotide from forward and 7416-5640 nucleotide from reverse side.

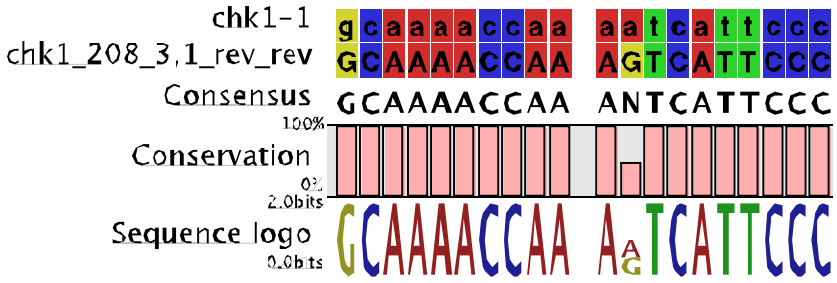

Figure 11: Mutation in *CHK1*gene with rev 29 primer. The software CLC Main Work bench 6 was used for analyzing the sequences.

3.5 Crossing of yeast strain

Two *S. pombe* strains PJ1142 and PJ1329 were crossed to construct an *S. pombe* strain that lacks all of its endogenous histidine kinase genes and has the ability to switch mating-type. After crossing a number of colonies in the h^{90} mating configuration was obtained (confirmed by checking for spores after growth on low nutrient plates under light microscope). The colonies were then used for colony PCR (Figure 12). Here F50, F51, F52 were used as forward primer and as a reverse primer A3 was used. However, no PCR product was obtained with these primers.

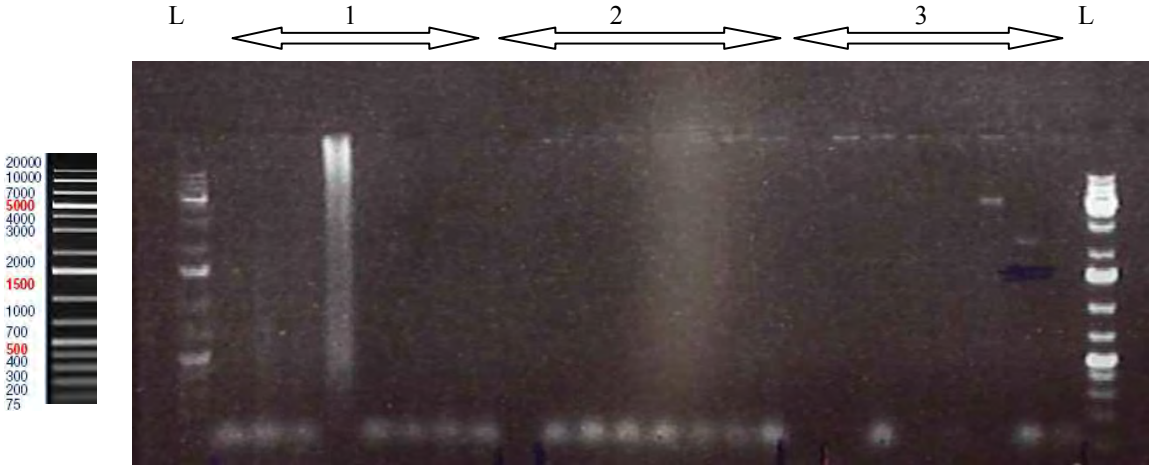

Figure 12: Colony PCR of sporulating colony of crossing between PJ1142 and PJ1329. L=1kb plus Gene Ruler^{TM} DNA ladder. 1, 2 and 3 represents the PCR of 7 different colonies with F50, F51 and F52 primer, respectively. There are some unspecific bands with F52 primer.

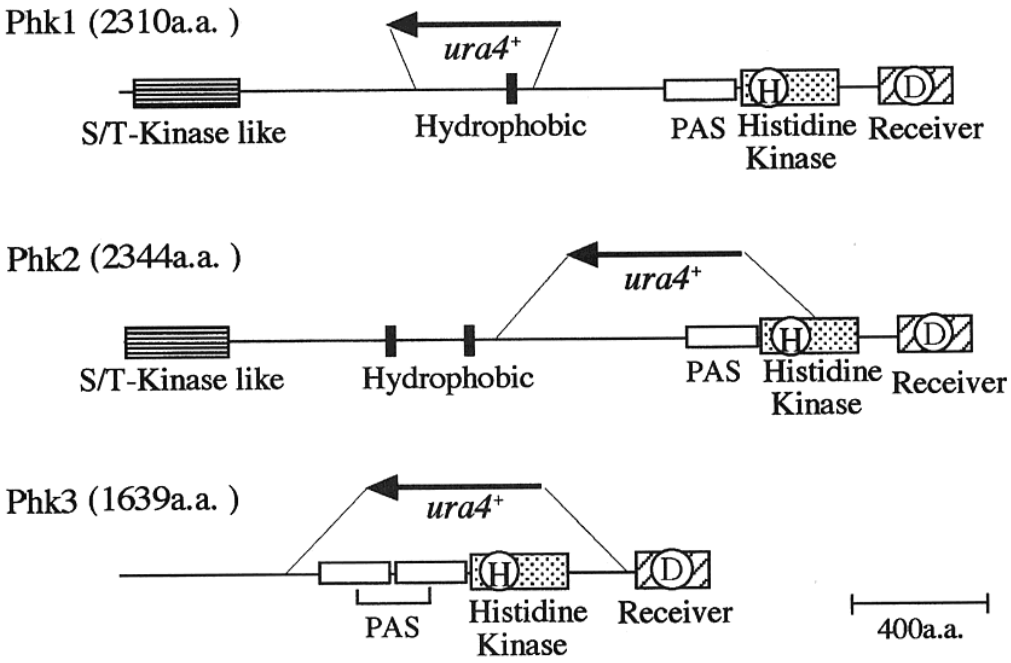

Figure 13: Three histidine kinases of *S. pombe* Phk1, (Mak2), Phk2 (Mak3), Phk3 (Mak1). Source: Aoyama et al 2001.

S. pombe has three histidine kinase genes; *mak1*$^+$, *mak2*$^+$ and *mak3*$^+$ which are different in sizes i. e. 4917, 6930, and 7032 bp, respectively. In strain PJ1142 some regions of the genes are replaced by *ura4*$^+$ marker for deletion construction of these genes (Figure 13). In a PCR reaction to detect whether *mak1*$^+$, *mak2*$^+$ and *mak3*$^+$ are deleted, the band size should be around 2500, 3500 and 4500 bp, respectively. Gene specific primers were used as forward primer while *ura4*$^+$ was used as reverse primer.

4. Discussion

4.1 Activity of *PHPT1* gene

This work aimed to look at the activity of *PHPT1* gene and its splice variants. To this end the wild type *PHPT1* gene and its two splice variants were successfully cloned from bacterial vector pET11a to yeast expression vector pREP3X. Initially pREP81X was also used as a yeast vector because pREP3X has a strong promoter and expression of recombinant proteins sometimes have deleterious effect on cell. However, it was shown that high expression levels of PHPT1 from the pREP3X vector did not affect the cell survivability.

After transformation, we could observe colonies on minimal media, which indicated that the transformation was successful. The *S. pombe* cells were allowed to grow in media without thiamine, to allow for expression of the proteins. Using Western blot it was shown that only the wild type PHPT1 protein could be detected. The activity of PHPT1 protein and splice variants 1 and 2 were measured by Pia Ek (Department of IMBIM). She used phosphohistidine as a substrate for these enzymes and got positive result only for the wild type. So this means that the splice variants of PHPT1 cannot remove phosphate group from the substrate, or they are not properly expressed in the yeast. Later it was discovered that the two splice variants are different. One is in terms of protein variant and the other one is truncated form. The latter might not even be a biological relevant splice variant but perhaps a result of an error in the annotation by one nucleotide.

4.2 *CHK1* gene

The *C. albicans* histidine kinase Chk1 is one of the main virulence factors for creating disease in immunusupressed patients and the main objective of this second project is to find a potential drug against this protein. Amplification of the *CHK1* gene was tried by using gradient PCR, and the optimal annealing temperature was found to be 66.5ºC.

Crossing between PJ1142 and PJ1329 was carried out and checked by colony PCR to check if the spores have the deleted construct of *mak1*, *mak2* and *mak3*. But no band was found on the PCR gel. Then different parameters were changed, for example the PCR was tried with different PCR kit- EXPAND, Phusion. Again different magnesium concentration was also used, but no band was found on gel upon changing all these parameters. The expected size of PCR product of *mak1::ura4*$^+$, *mak2::ura4*$^+$ or *mak3::ura4*$^+$ are around 2500, 3500 and 4500 bp, respectively, which are long. In PCR reaction it is much more difficult to get a long PCR product compared to a short. If the DNA from the *S. pombe* after crossing was purified rather than using the whole cell, it will perhaps solve the problem with PCR.

4.3 *CHK1* gene sequencing

The clone with *CHK1* gene plasmid miniprep was sequenced by using different forward and reverse primers. *CHK1* is a 7416 kb and so far 1-1810 nucleotide from forward and 7416-5640 nucleotide from reverse side was sequenced.

4.4 Future directions

The whole *CHK1* gene will be sequenced before cloning into *S. pombe* expression vector to see if there is any mutation in this gene. By crossing, *S. pombe* strain will be constructed that lacks all endogenous histidine kinase genes as well as has the h^{90} mating configuration. The sequenced *CHK1* gene will subsequently be transformed into this *S. pombe* strain and checked if it can restore the doubling time in nutrient rich media and can complement the lack of endogenous histidine kinase genes. If this will work then the growth rate can be used to screen for drugs against CHK1 protein.

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7. Appendix

Media

YEA (1 litre)

5 g Yeast extract
2 g Casamino acids
0.1 g Adenine
0.1 g Uracil
20 g Agar
150 ml 20 % Glucose

The Glucose and the media were first autoclaved separately and then mixed.

LB/LA (1 litre)

10 g NaCl
5 g Yeast extract
10 g Tryptone
(20 g agar)

The media were sterilized by autoclaving and for some batches ampicillin was added to a concentration of 100 µg / ml.

PMG-total (Minimal media) (1 litre)

3 g KH phthalate, SigmaUltra
2.76 g $NA_2HPO_4.2H_2O$
3.74 g L-Glutamic acid

0.25 g L- Leucine
0.1 g Adenine
0.1 g Uracil
0.1 g Histidine
0.1 ml Mineral stock (10000 X)
1 ml Vitamin stock (1000 X)
20 ml Salt stocks (50 X)
20 gm/l agar

First the bottle was filled up with 700 ml distilled water and dissolved the reagents. Before addition of agar pH was adjusted at 5.6. Then it was sterilized by autoclaving and 20% D (+) Glucose-Monohydrate (MERCK) (Separately sterilized) was added with it.

PMG-Leu (selective minimal media)

This media was prepared as PMG by excluding leucine and histidine amino acids.

Solutions

Buffers for Western blot

10% SDS

100 g of SDS was mixed with 900 ml of H_2O and let to dissolve. The volume was then adjusted to 1 L with H_2O.

10% APS

1 g of APS was dissolved in 10 ml of H_2O and kept in freeze by using 1 ml of aliquots.

Blocking buffer

2 g of BSA was dissolved in 40 ml of wash buffer or 2.5 g of milk powder was dissolved in 50 ml of wash buffer.

Wash buffer

5 ml of 20% Tween-20 was diluted in 995 ml 1 X PBS

Running buffer (10 X)

288 g of glycine, 60.4 g of Tris and 20 g of SDS was dissolved in 1.8 L of H_2O and then the volume was set for 2 L.

Transfer buffer (10 X)

288 g of glycine, 60.4 g of Tris was dissolved in 1.8 L of H_2O and the volume was adjusted to 2 L.

For making 1 X transfer buffer 100 ml of 10 X transfer buffer was taken and mixed with 100 ml of methanol and 800 ml of H_2O.

Resolving gel 10%, 20 ml

H_2O	7.9
30% acrylamide mix	6.7
1.5 M Tris (pH 8.8)	5.0
10% SDS	0.2
10% ammonium persulfate	0.2
TEMED	0.008

Staking gel 5%, 4 ml

H_2O	2.7
30% acrylamide mix	0.67
0.5 M Tris (pH 6.8)	0.5
10% SDS	0.04
10% ammonium persulfate	0.04
TEMED	0.004

Primers

Primers for PCR reactions:

Primer Name	Primer Sequences
F50 (mak1_upst_F)	5’ AGG TAA GTG ATT TCA ATA CTT G 3’
F51 (mak2_upst_F)	5’ TGT CGT ATT AAG GAT AAT TAT TG 3’
F52 (mak3_upst_F)	5’ AAT TGA AGT TCA ATA ACT TAT AAC 3’
F53 (CaChk1_F)	5’CTC GAG ATG TCT ATG AAC TTT TTT AAT TCA AG3’
F54 (CaChk1_R)	5’ CTC GAG TTA CAT ATT CAC TGT TTC CCC TAC3’
F60 (mak1_F_New)	5’ CAG CAC AGT ATA GTG TAG TGA CTT G3’
F61 (mak2_F_New)	5’ TCA GAT ACC AAA CTG CGT AG 3’
F62 (mak3_F_New)	5’ AGA GAC ATG ATT GTC CGA TC 3’
A3 (UraR)	5’TTC GAC AAC AGG ATT ACG AC 3’

Primers for sequencing

Primer Name	Primer Sequences
M13 (uni_21)	5’TGA CCG GCA GCA AAA TGT3’
M13 (rev_29**)**	5’CAG GAA ACA GCT ATG ACC3’
F67 (Chk1_878_uni)	5’GAT AGT GAG GCT ATC ACC3’
Chk1_70_rev	5’ GTT GTG CCA ATC CCT TC 3’

Colony PCR (JTAG)

5 µl Template (cell solution)

10 µl Buffer 5 X (1.5 mM $MgCl_2$)

2.5 µl $MgCl_2$ 850 mM)

1 µl dNTP (10mM total)

0.3 µl primer- F50/F51/F52 (100 µM)

0.3 µl primer- A3 (100 µM)

1 µl Phire polymerase

30.6 µl dH_20

---
50 µl

94 °C	2 min		
94 °C	15 s		
47 °C	30 s		X10
68 °C	4 min		
94 °C	15 s		
47 °C	30 s		X20
68 °C	4 min	dt 15 s	

(This means 15 s are added to each cycle)

4 °C

CHK1 PCR

5 µl Template (CHK1 DNA)

10 µl Buffer 5 X

1 µl dNTP (10mM total)

1.5 µl $MgCl_2$ (50mM)

0.3 µl F53 primer (100 µM)

0.3 µl F54 primer (100 µM)

1 µl Phusion polymerase

31.6 µl dH_20

---

50 µl

98 °C	1 min
98 °C	0.15 s
66.5 °C	0.30 s
72 °C	4 min
72 °C	7 min

4 °C

Total cycle 25