A Mutational Study of the Transmembrane Domains of the Yeast Glutathione Transporter, Hgt1p of *Saccharomyces cerevisiae*

A dissertation submitted for the partial fulfilment of
BS-MS dual degree in Science
By

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To the

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April 2014

Certificate of Examination

This is to certify that the dissertation titled "**A MUTATIONAL STUDY OF THE TRANSMEMBRANE DOMAINS OF THE YEAST GLUTATHIONE TRANSPORTER, HGT1P OF *SACCHAROMYCES CEREVISIAE***" submitted by Mr Shambhu Yadav (MS09117) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Samarjit Bhattacharyya Dr. Kausik Chattopadhyay Prof. Anand Kumar Bachhawat (Supervisor)

Declaration

I hereby declare that the matter embodied in this thesis entitled "**A MUTATIONAL STUDY OF THE TRANSMEMBRANE DOMAINS OF THE YEAST GLUTATHIONE TRANSPORTER, HGT1P OF *SACCHAROMYCES CEREVISIAE***" is the result of investigations carried out by me under the supervision of Prof. Anand K Bachhawat at the Department of Biological Sciences, Indian Institute of Science Education and Research (IISER) Mohali.

This work has not been submitted in part or full for a degree, a diploma, or a fellowship to any other university or institute.

In keeping with the general practice of reporting the scientific observations, due acknowledgement has been made wherever the work described is based on the finding of other investigators. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Date: 25/04/2014 Shambhu Yadav

In my capacity as supervisor of the candidate's thesis work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Anand Kumar Bachhawat
(Supervisor)

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Yadav, Rahul choudhary, Amit Ranjan, Ravi Yadav Abhishek Anand and Harsh Katyayan, deserve a special thank for making campus life wonderful.

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Abbreviations

Amino acid

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cysteine
Q	Glutamine
E	Glutamate
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine
F	Phynylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

Chemicals

Amp	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine Triphosphate
dNTPs	2’ –deoxyadenosine 5’- Triphosphate
GSH	Reduced glutathione
HCl	Hydrogen chloride

PEG	Poly Ethylene Glycol
SDS	Sodium Dodecyl Sulphate
TEMED	N,N,N',N' – tetramethylethylenediamine
Tris	[Tris(hydroxymethyl)amino methane
β-ME	β-mercaptoethanol

Techniques

SDS-PAGE	Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis

Others

AAP	Amino Acid Permease
BLAST	Basic Local Alignment Search
DNA	Deoxyribonucleic acid
EUROSCARF	European *S. cerevisiae* archive for functional Analysis
HA	Hemagglutinin
LB	Luria Bertani
OPT	Oligopeptide Transporter
ORF	Open Reading Frame
PT	Peptide Transport
SD	Synthetic Defined
TE	Tris chloride and EDTA
TMD	Ttransmembrane Domain
WT	Wild-type

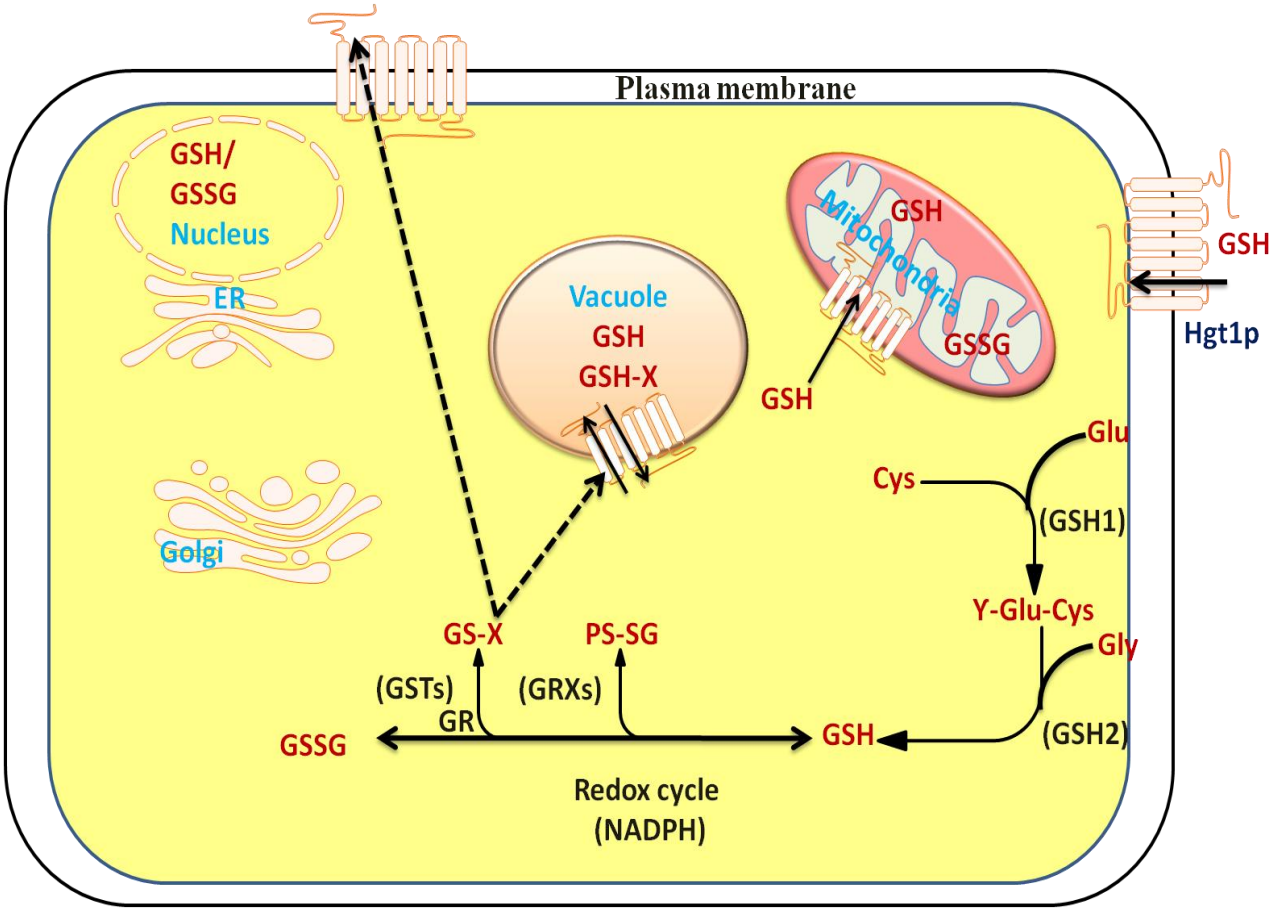
Table of Contents

Abstract

The High affinity glutathione transporter (Hgt1) of Saccharomyces cerevisiae is a 799 amino acid protein, located on the plasma membrane and predicted to have 12 transmembrane domains (TMD). Here we aim to identify residues involved in glutathione binding and transport. In the present study, alanine scanning mutagenesis of 5 transmembrane domains of Hgt1p was attempted to obtain insight into the residue which are involved in substrate binding and translocation. All alanine mutants were analyzed using a plate-based growth assay. This analysis identified W110A (in TMD1), L282A (in TMD5), L429A (in TMD7), Y449A (in TMD7) and W484A (in TMD8) mutants which exhibited severe loss of functional activity. The detailed biochemical characterization of these mutants includes their effect on protein expression levels. It was found that mutants W110A, L282A, Y449A and W484A had a drastic effect on protein level while mutant L429A had no significant effect on protein level and this was likely to be involved in either trafficking or substrate translocation. Multiple sequence alignment of TMD regions of fungal OPT family revealed that the residues W110, L429, Y449 and W484 are conserved. In addition to this, a study was also initiated to identify if residues which are shown to be important for glutathione transport are also important for Leu- Enkephalin transport by Hgt1p.

Chapter 1

Introduction and Review of Literature



1.1 Glutathione

Glutathione (*γ-L-glutamyl-L-cysteinyl-glycine*), is the most abundant thiol (-SH group) containing organic compound in the *Saccharomyce cerevisiae* (budding yeast), as well as many other organisms. Glutathione is synthesized inside the cells by two step reaction as follows:

Step 1: Catalyzed by *γ-glutamylcysteine synthetase* (Gsh1p: encoded by a single gene called GSH1):

$$\text{L- γ-glutamate + L-cysteine + ATP} \xrightarrow[Mg^{2+}]{\textbf{Gsh1p}} \text{L- γ-glutamyl-L-cysteine +ADP}$$

Step 2: Catalyzed by *glutathione synthetase* (Gsh2p: encoded by a single gene called GSH2)

$$\text{L- γ-glutamyl-L-cysteine + glycine +ATP} \xrightarrow[Mg^{2+}]{\textbf{Gsh2p}} \text{Glutathione + ADP}$$

Glutathione biosynthesis is a cytosolic process in animals and yeasts [1a]. However, in plants glutathione biosynthesis occur in partly in plastid and partly in the cytosol [2]. The regulation of glutathione biosynthesis occurs mostly at the first step (GSH1) and the regulation occurs at both the transcriptional and post translational level [3].

Glutathione has two special features- 1) *γ-glutamyl* bond between glutamate and cysteine residues and 2) occurrence of free thiol group of the cysteine residues. This unusual *γ-glutamyl* bond makes glutathione highly resistant to the cellular peptidases and increases stability of glutathione inside the cells [1]. And thiol group provides a redox-center to participate in electron transfer reactions [4] and act as highly reactive nucleophilic center to conjugate with heavy metals (Se,Hg,Cd salts) and other electrophillic groups. Oxidized glutathione is highly water soluble at neutral pH. Due to these unique physiological and chemical stability and high reactivity makes it possible to play many flexible roles in various biological functions under normal and various stress conditions [5].

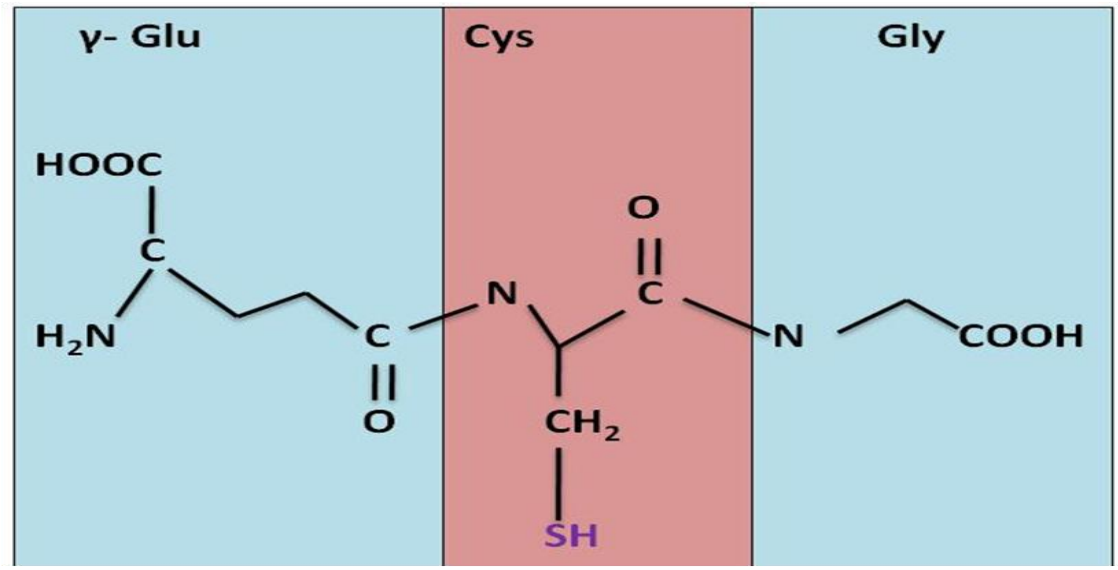


Figure 1: A structural formula of Glutathione. It is an unusual tripeptide of glutamate, cysteine and glycine with N-terminus γ-glutamyl linkage and a –SH group of the cysteine residue.

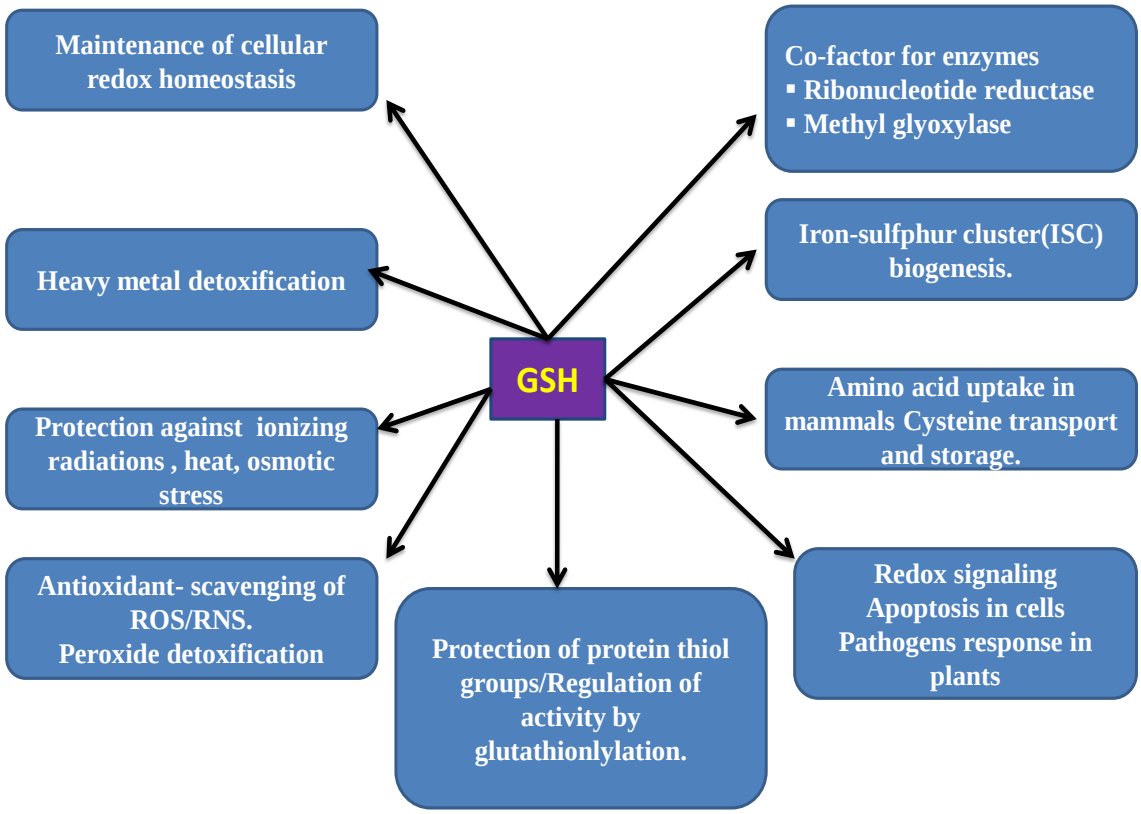


Figure 2: GSH involves in various physiological functions in the cell.

Glutathione has numerous important biological functions in synthesis of protein, metabolism, enzymatic activity, transport and storage of organic sulphur [1], and protection of cells from reactive oxygen species and free radicals, iron sulphur cluster (ISC) biogenesis [6], maintenance of cellular redox homeostasis [1] and various other functions like heavy metal detoxification, protection against ionzing radiation, heat and osmotic stress. In addition, glutathione also acts as co-factor for methyl glyoxylase and dehyroascorbate dehyrogenase especially in plants [7].

Glutathione also has a role in amino acids metabolism. It serves as a source of nitrogen and sulpher in living organisms [8, 9, 10] The essentiality of the glutathione and its fundamental role as redox buffer highlight the significance of the maintenance of GSH homeostasis in the cell. The excess of glutathione inside cells lead to toxicity to the cells [9].

Many GSH functions are connected to reversible redox reactions of the cysteine sulfur group. Like other thiol groups, glutathione can undergo several redox reactions. Oxidized forms of glutathione remarkably include disulfides, either with another glutathione cysteine amino acid residue to produce glutathione disulfide (GSSG) or with a different -SH to form 'mixed disulfides'. In addition it can be converted to more oxidized forms in which the -SH group is converted to sulfenic, sulfinic or sulfonic acids [11]. Inside the cells, the ratio of GSH and GSSG are in equilibrium with each other and, this can be maintained by biosynthesis, degradation, and compartmentalization and utilization in different reactions. (Fig.3)

In budding yeast *Saccharomyces cerevisiae*, glutathione is vital for its growth and survival. In addition to endogenous biosynthesis, cells can also take glutathione from the extracellular environment by glutathione transporters present on plasma membrane.

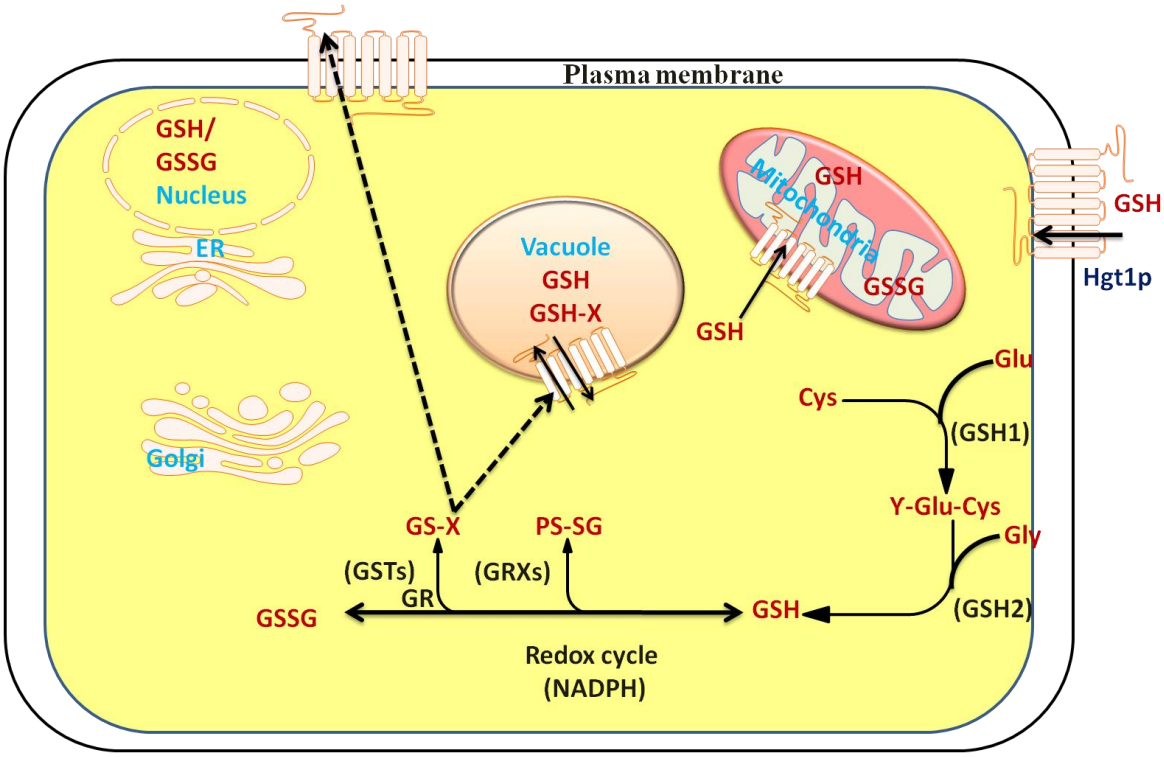


Figure 3: Glutathione homeostasis is regulated by different process in the eukaryotic cells. (a) Synthesis of glutathione by biosynthesis catalyzed by GSH1 and GSH2 enzymes and utilization of glutathione in various reactions. (b) Redox cycling between GSSG and GSH mediated by glutathione reductase (GR), (c) conjugation with different electrophiles or mediated bu glutathione transferase (GSTs) to form GS-conjugates. (d) glutathionlyation of proteins regulated by glutaredoxin (GRXs); (e) compartmentation into different organelles, import or export mediated by transporters. (f) Degradation [1,10].

1.2 Enkephalins

Enkephalins are pentapeptides having opiate activity (pain killer). Enkephalins are found in the thalamus of the mammalian brain and in few region of the spinal cord that transmit pain impulses. In the spinal cord, enkephalins reduce painful sensations by reacting with specific receptor sites on the sensory nerve endings [12]. Enkephalins bind with opiate receptors and reduce pain.

There two type of Enkephalin-

1. Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu)
2. Met-enkephalin((Tyr-Gly-Gly-Phe-Met)

Leu-enkephalin is an endogenous agonist for the receptors that are stimulated by opiate alkaloids. Met-enkephalin is involved in phenomenon associated with modulated pain awareness, regulation of immunological system, regulation of memory and emotional conditions, food and liquid consumption.

Enkephalins are pentapeptides that have been shown to be transported by Hgt1p. In this thesis I have used Leu-Enkephalins as substrates of Hgt1p , not for their biological properties.

1.3 High Affinity Glutathione Transporter (Hgt1p) of Saccharomyce *cerevisiae*

Hgt1p (yeast ORF YJL212C) is high affinity glutathione transporter from the budding yeast *Saccharomyces cerevisiae.* It is 799 amino acids long protein and has 12 predicted transmembrane domains. This is the first glutathione transporter which was discovered as a high affinity glutathione transporter in any system. It is usually found in fungi and plants. Distant homologues are present in bacteria and archaea but not in metazoans and higher animals. It belongs to the oligopeptide transporter family. And no member of this OPT family has yet been structurally characterized. There is no mechanistic or structural information available for any member of OPT family.

Based on genetic and biochemical evidence, our lab has proposed existence of the high affinity glutathione transporter in yeast (*Saccharomyces cerevisiae*) that was encoded by HGT1. Disruption of the gene leads to a complete loss in glutathione

uptake ability of the *Saccharomyce cerevisiae* cells in *gsh1Δ* background. The kinetics study of this transporter over a large range of substrate concentration (0.010mM to 10mM) confirmed the existence of high affinity glutathione transporter (K_m=0.054mM) [13].

In the absence of any other insights on this protein, an alanine scanning mutagenesis of polar and charged residues in the putative transmembrane domains of Hgt1p was initiated, where a total of 22 polar and charged residues were selected [14]. This analysis identified four of the transmembrane domains (TMD1, TMD4, TMD9 and TMD12) and the intracellular loops region (537-568) containing the highly conserved proline rich region as critical for glutathione transport. The residues N124 in TMD1, Q222 in TMD4, and Q526 in TMD9 and K562 in the intracellular loop were found to be directly or indirectly participating in glutathione transport. Q222 in TMD4 and Q526 in TMD9 were found to be required for substrate recognition. A subsequent alanine scanning mutagenesis of TMD9 revealed that in addition to Q526, F523 played a critical role in substrate recognition with Q526. These two key residues were found to be conserved in the OPT members known to function in glutathione uptake [15].

1.4 Approaches used for the structure-function study of High Affinity Glutathione Transporter.

In the absence of crystal structure of characterization of a membrane transporter involves of topology prediction of the protein, identification of domains and amino acid residues that form the substrate binding site and channel for translocation of a particular substrate across the membrane.

1.4.1 Strategies used for the structure-function study of membrane transporter.

A topology model of a membrane protein showing the location of transmembrane domains and its orientation in the membrane gives a basis for structure- function studies.

There are various topology prediction software such as TMHMM 2.0, HMMTOP, TopPred2 and PHDhtm for predicting transmembrane domains and their location in

protein sequences and also the location of N-terminal and C-terminal regions. These softwares use different parameters like physical and chemical properties (hydrophobic index, charge of amino acid residues etc) for prediction purpose.

1.4.2 Mutagenesis based approaches for the structure-function study of membrane proteins.

In vitro mutagenesis and *in vivo* mutagenesis together for functional characterization of mutants is a powerful approach for structure-function characterization of protein. Mutagenesis, either random, site-directed or both are useful for structure- function studies of membrane proteins.

1.4.2.1 Random mutagenesis

Random mutagenesis involves mutation of amino acid residues without any specificity regarding the importance of the residues in the protein functional activity. It can be generated by error prone PCR, chemical mutagenesis, in vivo dissemination of DNA in mutator strains, DNA shuffling and cassette mutagenesis [16].

1.4.2.2 Site-directed mutagenesis

Site-directed mutagenesis is a site specific introduction or alteration of a residue to a different residue.The choice could be based on the protein sequence conservation pattern of the amino acid residues or the property of the residues or based on previous mutation study of a homologue or insights from the crystal structure of the protein or its homologue [17].

1.4.2.2a. Alanine scanning mutagenesis

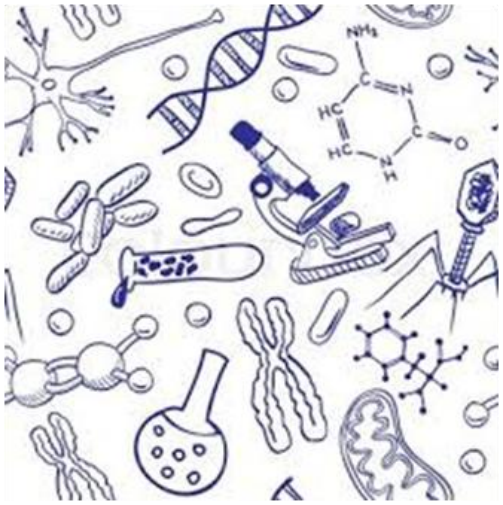
Alanine scanning is a type of site-directed mutagenesis involving systematic replacement of each and every amino acid residue in a transmembrane domain with alanine for functional evaluation of the mutants, to identify the amino acid residues critical for activity of a protein [18]. As alanine has a small and inert side chain, it is a readily acceptable for replacement of original amino acid, without causing any interruption to the structure of protein, especially inside the lipid environment. Hence importance of the primary amino acid residue can be assigned depending on the functional activity of the alanine mutant.

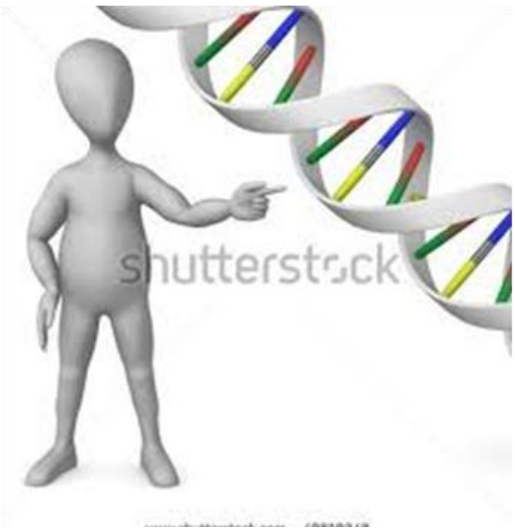
Aim and objective of the present study

The high affinity glutathione transporter (Hgt1) of *Saccharomyces cerevisiae* is a 799 amino acid protein, located on the plasma membrane and predicted to have 12 transmembrane domains (TMD). Here we aim to identify residues involved in glutathione binding and transport. TMDs form the translocation channel and the amino acid residues in the TMDs play a role in binding and translocation of the substrate. So we aimed to do alanine scanning mutagenesis of all the TMDs followed by functional analysis of all the mutants. In the present study, alanine scanning mutagenesis of 5 transmembrane domains (TMD1, TMD5, TMD7, TMD8, and TMD11) of Hgt1p was attempted to obtain insights into the residue which are involved in substrate binding and translocation.

Chapter 2

Materials and Methods





SECTION A: MATERIALS

2.1. CHEMICALS AND REAGENTS

All the chemicals used were obtained from commercial sources and were of analytical grade. Media components, fine chemicals and reagents were purchased from Sigma Aldrich, (St. Louis, USA), HiMedia, (Mumbai, India), Merck. India Ltd (Mumbai, India), USB Corporation (Ohio, USA) or Difco, USA. Oligonucleotides (primers) were designed using Gene Runner software and were purchased from Sigma-Genosys (Bangalore, India), Integrated DNA Technologies (IDT). Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, *Vent* DNA polymerase, *Taq* DNA polymerase and other modifying enzymes), their buffers, dNTPs, DNA and protein molecular weight markers were purchased from New England Biolabs Inc, (Beverly, MA, USA). Gel-extraction kits and plasmid miniprep columns were obtained from QIAGEN or Fermantas. GSH was obtained from Sigma-Aldrich, USA. Nitrocellulose Hybond N^+ membrane (Hybond ECL) and ECL Plus Western Blotting Detection Reagents were purchased from Amersham Biosciences GE health care (U.K.). HA-Tag (6E2) Mouse monoclonal antibody and horse anti-mouse HRP-linked antibody were bought from Cell Signaling (Danvers, MA, USA). Alexa Flour® 488 conjugated goat anti-mouse antibody was obtained from Molecular probes (Eugene, Oregon, USA).

2.2. STRAINS AND PLASMIDS

Escherichia coli DH5α was used as the cloning host. The genotype for the *E. coli* strain and the yeast strains used in the study are given in Table 1. The list of various plasmids used in this study is given in Table 2.

Table 1: List of bacterial and yeast strains used in the study

Strain	Genotype	Source
Escherichia coli strain		
DH5ά	***F' gyr A(Nal) recA1 relA endA1 thi-1 Hsd 17(rk-mk+) gln V44 deoR Δ(lacZYA-argF) U169 [ϕ80d Δ(lacZ) M15]***	***Lab strain***
Yeast strain of S. cerevisiae		
ABC 817	***MATa his3Δ1 leu2Δ met15Δ0 ura3Δ0 hgt1Δ::leu2***	***Lab strain***
ABC1481	***MATa his3Δ1 leu2Δ met15Δ0 ura3Δ0 hgt1Δ::kanMX4***	***Lab strain***

Table 2: List of Plasmids used in the study

Clone No.	Plasmid Name	Descriptions
ABE 443	P416TEF	The CEN-vector bearing URA3 marker and TEF Promoter-MCS-terminator for yeast expression and Amp^r marker for selection in E.coli. (Mumberg, et al., 1995)
ABE 1912	pTEFM1-His-$HGT1^m$-HA	The plasmid contains the HGT1 gene in p416M1-TEF(ABE1888) at BamHI/EcoRI sites. Msc I (BalI) and XbaI sites introduced at position 1366 and 1584 in HGT1, in pTEF-His-HGT1-HA plasmid (ABE1897) (Kaur and Bachhawat. 2009).
ABE 3074	T109A	p416-His-HGT1-HA with T109A mutation in TMD1
ABE 3365	W110A	p416-His-HGT1-HA with W110Amutation in TMD1

ABE 3366	F111A	p416-His-HGT1-HA withF111A mutation in TMD1
ABE 3367	L112A	p416-His-HGT1-HA with L112A mutation in TMD1
ABE 4004	T113A	p416-His-HGT1-HA with T113A mutation in TMD1
ABE 2242	T114A	p416-His-HGT1-HA with T114A mutation in TMD1
ABE 4005	V115A	p416-His-HGT1-HA with V115A mutation in TMD1
ABE 4006	F116A	p416-His-HGT1-HA with F116A mutation in TMD1
ABE 3668	V117A	p416-His-HGT1-HA with V117A mutation in TMD1
ABE 3669	118A	p416-His-HGT1-HA with V118A mutation in TMD1
ABE 3670	119A	p416-His-HGT1-HA with V119A mutation in TMD1
ABE 3671	120A	p416-His-HGT1-HA with F120A mutation in TMD1
ABE 3672	121A	p416-His-HGT1-HA with A121G mutation in TMD1
ABE 3673	122A	p416-His-HGT1-HA with G122Amutation in TMD1
ABE 3674	123A	p416-His-HGT1-HA with V123A mutation in TMD1
ABE 4007	N124A	p416-His-HGT1-HA with N124A mutation in TMD1
ABE 3201	Q125A	p416-His-HGT1-HA with Q125A mutation in TMD1
ABE 3675	F126A	p416-His-HGT1-HA with F126A mutation in TMD1
ABE 4008	F127A	p416-His-HGT1-HA with F127A mutation in TMD1
ABE 3711	F277A	p416-His-HGT1-HA with F277A mutation in TMD5
ABE 3712	F278A	p416-His-HGT1-HA with F278A mutation in TMD5
ABE 3713	L279A	p416-His-HGT1-HA with L279A mutation in TMD5

ABE 3714	I280A	416-His-HGT1-HA with I280A mutation in TMD5
ABE3715	V281A	pP416-His-HGT1-HA with V281A mutation in TMD5
ABE 3716	L282A	p416-His-HGT1-HA with L282A mutation in TMD5
ABE 3716	I283A	p416-His-HGT1-HA with I283A mutation in TMD5
ABE 3718	G284A	p416-His-HGT1-HA with G284A mutation in TMD5
ABE 3075	S285A	p416-His-HGT1-HA with S285A mutation in TMD5
ABE 3955	F286A	p416-His-HGT1-HA with F286A mutation in TMD5
ABE 3956	I287A	p416-His-HGT1-HA with I287A mutation in TMD5
ABE 3719	W288A	p416-His-HGT1V with W288A mutation in TMD5
ABE 3720	Y289A	p416-His-HGT1-HA with Y289A mutation in TMD5
ABE 3721	W290A	p416-His-HGT1-HA with W290A mutation in TMD5
ABE 3722	V291A	p416-His-HGT1-HA with V291A mutation in TMD5
ABE 2852	P292A	p416-His-HGT1-HA with P292A mutation in TMD5
ABE 3723	G293A	p416-His-HGT1-HA with G293A mutation in TMD5
ABE 3724	F294A	p416-His-HGT1-HA with F294A mutation in TMD5
ABE 3725	L295A	p416-His-HGT1-HA with L295A mutation in TMD5
ABE 3726	F296A	p416-His-HGT1-HA with F296A mutation in TMD5
ABE 3662	S427A	p416-His-HGT1-HA with S427A mutation in TMD7
ABE 3840	Y428A	p416-His-HGT1-HA with Y428A mutation in TMD7
ABE 3953	L429A	p416-His-HGT1-HA with L429A mutation in TMD7

ABE 3924	L430A	p416-His-HGT1-HA with L430A mutation in TMD7
ABE 3866	S431A	p416-His-HGT1-HA with S431A mutation in TMD7
ABE 3841	Y432A	p416-His-HGT1-HA with Y432A mutation in TMD7
ABE 3842	A433G	p416-His-HGT1-HA with A433G mutation in TMD7
ABE 3843	L434A	p416-His-HGT1-HA with L434A mutation in TMD7
ABE 2005	N435A	p416-His-HGT1-HA with N435A mutation in TMD7
ABE 3867	F436A	p416-His-HGT1-HA with F436A mutation in TMD7
ABE 3954	A437G	p416-His-HGT1-HA with A437G mutation in TMD7
ABE 3868	A438G	p416-His-HGT1-HA with A438G mutation in TMD7
ABE 3844	V439A	p416-His-HGT1-HA with V439A mutation in TMD7
ABE 3845	I440A	p416-His-HGT1-HA with I440A mutation in TMD7
ABE 3846	A441G	p416-His-HGT1-HA with A441G mutation in TMD7
ABE 4009	V442A	p416-His-HGT1-HA with V442A mutation in TMD7
ABE 4010	F443A	p416-His-HGT1-HA with F443A mutation in TMD7
ABE 4011	V444A	p416-His-HGT1-HA with V444A mutation in TMD7
ABE 2235	H445A	p416-His-HGT1-HA with H445A mutation in TMD7
ABE 4012	C446A	p416-His-HGT1-HA with C446A mutation in TMD7
ABE 4018	I447A	p416-His-HGT1-HA with I447A mutation in TMD7
ABE3847	L448A	p416-His-HGT1-HA with L448A mutation in TMD7
ABE 3848	Y449A	p416-His-HGT1-HA with Y449A mutation in TMD7

ABE 3925	W483A	P416-His-HGT1-HA with W483A mutation in TMD8
ABE 3849	W484A	p416-His-HGT1-HA with W484A mutation in TMD8
ABE 4013	Y485A	p416-His-HGT1-HA with Y485A mutation in TMD8
ABE 3826	L486A	p416-His-HGT1-HA with L486A mutation in TMD8
ABE 3927	L487A	p416-His-HGT1-HA with L487A mutation in TMD8
ABE3928	L488A	p416-His-HGT1-HA with L488A mutation in TMD8
ABE 3203	Q489A	p416-His-HGT1-HA with Q489A mutation in TMD8
ABE 3929	I490A	p416-His-HGT1-HA with I490A mutation in TMD8
ABE 3930	V491A	p416-His-HGT1-HA with V491A mutation in TMD8
ABE 3931	M492A	p416-His-HGT1-HA with M492A mutation in TMD8
ABE 3932	I493A	p416-His-HGT1-HA with I493A mutation in TMD8
ABE 3933	G494A	p416-His-HGT1-HA with G494A mutation in TMD8
ABE 4014	L495A	p416-His-HGT1-HA with L495A mutation in TMD8
ABE 4015	G496A	p416-His-HGT1-HA with G496A mutation in TMD8
ABE 3934	F497A	p416-His-HGT1-HA with F497A mutation in TMD8
ABE 3935	V498A	p416-His-HGT1-HA with V498A mutation in TMD8
ABE 4016	A499G	p416-His-HGT1-HA with A499G mutation in TMD8
ABE 3936	V500A	p416-His-HGT1-HA with V500A mutation in TMD8
ABE 3937	L661A	p416-His-HGT1-HA with L661A mutation in TMD11
ABE 3938	M662A	p416-His-HGT1-HA with M662A mutation in TMD11

ABE 3939	W663A	p416-His-HGT1-HA with W663A mutation in TMD11
ABE 3940	F664A	p416-His-HGT1-HA with F664A mutation in TMD11
ABE 3941	F665A	p416-His-HGT1-HA with F665A mutation in TMD11
ABE 3950	L666A	p416-His-HGT1-HA with L666A mutation in TMD11
ABE 3951	I667A	p416-His-HGT1-HA with I667A mutation in TMD11
ABE 4017	G668A	p416-His-HGT1-HA with G668A mutation in TMD11
ABE 3942	L669A	p416-His-HGT1-HA with L669A mutation in TMD11
ABE 3943	L670A	p416-His-HGT1-HA with L670A mutation in TMD11
ABE 3944	F671A	p416-His-HGT1-HA with F671A mutation in TMD11
ABE 2854	P672A	p416-His-HGT1-HA with P672A mutation in TMD11
ABE 3945	L673A	p416-His-HGT1-HA with L673A mutation in TMD11
ABE 3946	A674G	p416-His-HGT1-HA with A674G mutation in TMD11
ABE 3947	V675A	p416-His-HGT1-HA with V675A mutation in TMD11
ABE 3948	Y676A	p416-His-HGT1-HA with Y676A mutation in TMD11
ABE 3952	A677G	p416-His-HGT1-HA with A677G mutation in TMD11
ABE 3949	V678A	p416-His-HGT1-HA with V678A mutation in TMD11
ABE 3078	Q679A	p416-His-HGT1-HA with Q679A mutation in TMD11

2.3. **OLIGONUCLEOTIDES**

The list of various oligonucleotide primers used in this study is given in Table 3.

2.4. MEDIA

All the media, buffers and stock solutions were prepared using Millipore elix 5 deionized water unless otherwise mentioned. They were sterilized, as recommended, either by autoclaving at 15 lb/inch^2 (psi) pressures at 121ºC for 15 minutes, or by using membrane filters (Advanced Microdevices Pvt. Ltd., India) of pore size 0.2-0.45 µm (for heat labile compounds). Additional amino acid and nutrients were prepared as sterile stock and added as per requirements. Agar was added, if required, at a final concentration of 2.2%. Ampicillin was added at a final concentration of 100 µg/ml.

2.4.1. LB **(Luria–Bertani) Medium**	Yeast extract 5 g/l Tryptone 10 g/l NaCl 10 g/l pH of the above medium was adjusted to 7.0 with 1N NaOH
2.4.2. YPD **(Yeast extract Peptone Dextrose) Medium**	Yeast extract 10 g/l Peptone 20 g/l Dextrose 20 g/l
2.4.3SD **(Synthetic Defined) Medium (per 1000 ml)**	YNB 1.7 g/l (Yeast Nitrogen Base) (without amino acids and ammonium sulphate) $(NH_4)_2 SO_4$ 5 g/l Dextrose 20 g/l Amino acids 50 mg/l (as required) pH was adjusted to 6.0–6.5.

Table1 3: List of Oligonucleotides and their sequences in this study

Oligo Name	Sequence (5'to3')
TMD1 T109A/F	CGTGGTTCTTGACCGCGGTGTTTGTGGTAG
TMD1T109A/R	CTACCACAAACACCGCGGTCAAGAACCACG
TMD1 W110A/F	CCTCAACCACTGGAGAACGGCGTTCTTGACCACGGTGTTTGTGG
TMD1 W110A/R	CAAACACCGTGGTCAAGAACGCCGTTCTCCAGTGGTTGAGGCG
TMD1 F111A /F	CCACTGGAGAACGTGGGCCTTGACCACGGTGTTTGTGG
TMD1 F111A/R	CAAACACCGTGGTCAAGGCCCACGTTCTCCAGTGGTTGAGG
TMD1 L112A/F	CTGGAGAACGTGGTTCGCGACCACGGTGTTTGTGGTAG
TMD1 L112A/R	CCACAAACACCGTGGTCGCGAACCACGTTCTCCAGTGG
TMD1 T113A/F	GAGAACGTGGTTCTTGGCCACGGTGTTTGTGGTAGTTTTC
TMD1 T113A/R	AACTACCACAAACACCGTGGCCAAGAACCACGTTCTCCAGT
TMD1 T114A/F	GAACGTGGTTCTTGACCGCGGTGTTTGTGGTAGTTTTC
TMD1 T114A/R	AACTACCACAAACACCGCGGTCAAGAACCACGTTCTCC
TMD1 V115A/F	GTGGTTCTTGACCACGGCGTTTGTGGTAGTTTTCGCCG
TMD1 V115A/R	GCGAAAACTACCACAAACGCCGTGGTCAAGAACCACGTTC
TMD1 F116A /F	GGTTCTTGACCACGGTGGCTGTGGTAGTTTTCGCCGGTG
TMD1 F116A /R	CCGGCGAAAACTACCACAGCCACCGTGGTCAAGAACCACG
TMD1 V117A /F	CTTGACCACGGTGTTTGCGGTAGTTTTCGCCGGTGTTAATC
TMD1 V117A /R	CACCGGCGAAAACTACCGCAAACACCGTGGTCAAGAACC
TMD1 118A /F	VGACCACGGTGTTTGTGGCAGTTTTCGCCGGTGTTAATC
TMD1 V118A/ R	GATTAACACCGGCGAAAACTGCCACAAACACCGTGGTCAAGAAC
TMD1 V119A /F	CACGGTGTTTGTGGTAGCCTTCGCCGGTGTTAATCAA
TMD1 V119A /R	GATTAACACCGGCGAAGGCTACCACAAACACCGTGGTC
TMD1 F120A /F	GGTGTTTGTGGTAGTTGCCGCCGGTGTTAATCAATTTTTTTC
TMD1 F120A /R	AATTGATTAACACCGGCGGCAACTACCACAAACACCGTGG
TMD1 A121G /F	GTTTGTGGTAGTTTTCGGCGGTGTTAATCAATTTTTTTCC
TMD1 A121G /R	AAAATTGATTAACACCGCCGAAAACTACCACAAACACCG
TMD1 A121G /F	GTTTGTGGTAGTTTTCGGCGGTGTTAATCAATTTTTTTCC
TMD1 G122A /R	GAAAAAAATTGATTAACAGCGGCGAAAACTACCACAAAC
TMD1 V123A/F	GGTAGTTTTCGCCGGTGCCAATCAATTTTTTTCCCTGAG
TMD1 V123A /R	GGGAAAAAAATTGATTGGCACCGGCGAAAACTACCACAAAC
TMD1 N124A/F	GTAGTTTTCGCCGGTGTTGCTCAATTTTTTTCCCTGAG
TMD1 N124A/R	CTCAGGGAAAAAAATTGAGCAACACCGGCGAAAACTAC

TMD1 Q125A/F	TTCGCCGGTGTTAATGCATTTTTTTCCCTGAGATATCC
TMD1 Q125A/R	TATCTCAGGGAAAAAAATGCATTAACACCGGCGAAAACTAC
TMD1 F126A /F	CGCCGGTGTTAATCAAGCTTTTTCCCTGAGATATCCATCG
TMD1 F126A/R	GGATATCTCAGGGAAAAAGCTTGATTAACACCGGCGAAAAC
TMD1 F127A /F	CCGGTGTTAATCAATTTGCTTCCCTGAGATATCCATCGC
TMD1 F127A/R	GATGGATATCTCAGGGAAGCAAATTGATTAACACCGGCG
TMD5 F277A/ F	GACGATGCCCCGTTACAGGGCCTTCTTAATCGTCCTTATCGG
TMD5 F227A /R	GATAAGGACGATTAAGAAGGCCCTGTAACGGGGCATCGTCC
TMD5 F278A /F	CCCGTTACAGGTTCGCCTTAATCGTCCTTATCGGATCG
TMD5 F278A /R	GATAAGGACGATTAAGGCGAACCTGTAACGGGGCATC
TMD5 L279A /F	CCCGTTACAGGTTCTTCGCAATCGTCCTTATCGGATCGTTC
TMD5 L279A /R	CGATCCGATAAGGACGATTGCGAAGAACCTGTAACGGGGC
TMD5 I280A /F	CGTTACAGGTTCTTCTTAGCCGTCCTTATCGGATCGTTCATC
TMD5 I28OA /R	GAACGATCCGATAAGGACGGCTAAGAAGAACCTGTAACGG
TMD5 V281A /F	CAGGTTCTTCTTAATCGCCCTTATCGGATCGTTCATC
TMD5 V281 /R	GAACGATCCGATAAGGGCGATTAAGAAGAACCTGTAAC
TMD5 L282A/ F	GTTCTTCTTAATCGTCGCTATCGGATCGTTCATCTGG
TMD5 L282 /R	GATGAACGATCCGATAGCGACGATTAAGAAGAACC
TMD5 I283A /F	CTTCTTAATCGTCCTTGCCGGATCGTTCATCTGGTATTG
TMD5 I283A /R	CCAGATGAACGATCCGGCAAGGACGATTAAGAAGAAC
TMD5 G284A/ F	CTTAATCGTCCTTATCGCATCGTTCATCTGGTATTGG
TMD5 G284A /R	CCAATACCAGATGAACGATGCGATAAGGACGATTAAG
TMD5S285A/F	AATCGTCCTTATCGGAGCGTTCATCTGGTATTGGGTAC
TMD5S285A/R	CCAATACCAGATGAACGCTCCGATAAGGACGATTAAGAA
TMD5 F286A /F	GTCCTTATCGGATCGGCCATCTGGTATTGGGTACCTG
TMD5 F286A /R	GTACCCAATACCAGATGGCCGATCCGATAAGGACGATTAAG
TMD5 I287A /F	CTTATCGGATCGTTCGCCTGGTATTGGGTACCTGG
TMD5 I287A/ R	CAGGTACCCAATACCAGGCGAACGATCCGATAAGGAC
TMD5 W288A/F	CTTATCGGATCGTTCATCGCGTATTGGGTACCTGGATTCC
TMD5 W288A/R	GAATCCAGGTACCCAATACGCGATGAACGATCCGATAAGG
TMD5 Y289A F/	CGGATCGTTCATCTGGGCTTGGGTACCTGGATTCCTC
TMD5 Y289A /R	GGAATCCAGGTACCCAAGCCCAGATGAACGATCCG
TMD5 W290A/F	GATCGTTCATCTGGTATGCGGTACCTGGATTCCTCTTTAC
TMD5 W290A/R	GAGGAATCCAGGTACCGCATACCAGATGAACGATCC

TMD5 V291A /F	GTTCATCTGGTATTGGGCACCTGGATTCCTCTTTACC
TMD5 V291A /R	GTAAAGAGGAATCCAGGTGCCCAATACCAGATGAACGATC
TMD5 G293A /F	CTGGTATTGGGTACCTGCATTCCTCTTTACCGGTCTG
TMD5 G293A /R	GACCGGTAAAGAGGAATGCAGGTACCCAATACCAGATG
TMD5 F294A /F	GTATTGGGTACCTGGAGCCCTCTTTACCGGTCTGTCC
TMD5 F294A /R	GACAGACCGGTAAAGAGGGCTCCAGGTACCCAATACCAG
TMD5 L295A /F	GTATTGGGTACCTGGATTCGCCTTTACCGGTCTGTCCTATTTC
TMD5 L295A /R	GGACAGACCGGTAAAGGCGAATCCAGGTACCCAATAC
TMD5 F296A /F	GGTACCTGGATTCCTCGCTACCGGTCTGTCCTATTTC
TMD5 F296A /R	GAAATAGGACAGACCGGTAGCGAGGAATCCAGGTACCCAATAC
TMD7 S427A/F	CCGGTATTCGTTCCATTTGCCTATCTTTTGTCGTATGCTTT
TMD7 S427A/R	GCATACGACAAAAGATAGGCAAATGGAACGAATACCGGTGAG
TMD7 Y428A/F	GTATTCGTTCCATTTTCCGCTCTTTTGTCGTATGCTTTAA
TMD7 Y428A/R	AAGCATACGACAAAAGAGCGGAAAATGGAACGAATAC
TMD7 L429A/F	CGTTCCATTTTCCTATGCTTTGTCGTATGCTTTAAATTTTG
TMD7 L429A/R	ATTTAAAGCATACGACAAAGCATAGGAAAATGGAACGAATAC
TMD7 L430A/F	GTTCCATTTTCCTATCTTGCGTCGTATGCTTTAAATTTTGC
TMD7 L430A/R	ATTTAAAGCATACGACGCAAGATAGGAAAATGGAAC
TMD7 S431A/F	CCATTTTCCTATCTTTTGGCGTATGCTTTAAATTTTGCCGC
TMD7 S431A/R	GGCAAAATTTAAAGCATACGCCAAAAGATAGGAAAATGGAA
TMD7 Y432A/F	TCCTATCTTTTGTCGGCTGCTTTAAATTTTGCCGC
TMD7 Y432A/R	CGGCAAAATTTAAAGCAGCCGACAAAAGATAGGAAAATG
TMD7 A433G/F	CTATCTTTTGTCGTATGGTTTAAATTTTGCCGCTGTTATC
TMD7 A433G/R	CAGCGGCAAAATTTAAACCATACGACAAAAGATAGGA
TMD7 L434A/F	CTTTTGTCGTATGCTGCAAATTTTGCCGCTGTTATC
TMD7 L434A/R	GATAACAGCGGCAAAATTTGCAGCATACGACAAAAGATAGG
TMD7 N435A/F	CTTTTGTCGTATGCTTTAGCTTTTGCCGCTGTTATCG
TMD7 N435A/R	CGATAACAGCGGCAAAAGCTAAAGCATACGACAAAAG
TMD7 F436A/F	GTCGTATGCTTTAAATGCTGCCGCTGTTATCGCCGTTT
TMD7 F436A/R	CGGCGATAACAGCGGCAGCATTTAAAGCATACGACAA
TMD7 A437G/F	GTATGCTTTAAATTTTGGCGCTGTTATCGCCGTTTTTG
TMD7 A437G/R	AAACGGCGATAACAGCGCCAAAATTTAAAGCATACGAC
TMD7A438G/F	GTATGCTTTAAATTTTGCCGGTGTTATCGCCGTTTTTGTCCAC
TMD7A438G/R	CAAAAACGGCGATAACACCGGCAAAATTTAAAGCATACG

TMD7 V439A/F	CTTTAAATTTTGCCGCTGCTATCGCCGTTTTTGTCCAC
TMD7 V439A/R	GGACAAAAACGGCGATAGCAGCGGCAAAATTTAAAGC
TMD7 I440A/F	AAATTTTGCCGCTGTTGCCGCCGTTTTTGTCCACTGC
TMD I440A/R	GCAACAGCGGCAAAATTTAAAGCATACGACAAAAGATAG
TMD7 A441G/F	TTTTGCCGCTGTTATCGGCGTTTTTGTCCACTGCATC
TMD7 A441G/R	ATGCAGTGGACAAAAACGCCGATAACAGCGGCAAAATTT
TMD7 V442A/F	TTTGCCGCTGTTATCGCCGCTTTTGTCCACTGCATCTTATAC
TMD7 V442A/R	AGATGCAGTGGACAAAAGCGGCGATAACAGCGGCAA
TMD7 F443A/F	CGCTGTTATCGCCGTTGCTGTCCACTGCATCTTATAC
TMD7 F443A/R	ATAAGATGCAGTGGACAGCAACGGCGATAACAGCGGC
TMD7 V444A/F	CTGTTATCGCCGTTTTTGCCCACTGCATCTTATACCACG
TMD7 V444A/R	GGTATAAGATGCAGTGGGCAAAAACGGCGATAACAGC
TMD7 H445A/F	TATCGCCGTTTTTGTCGCCTGCATCTTATACCACGGTA
TMD7 H445A/R	CGTGGTATAAGATGCAGGCGACAAAAACGGCGATAACA
TMD7 C446A/F	ATCGCCGTTTTTGTCGCCTGCATCTTATACCACGGT
TMD7C446A/R	GTGGTATAAGATGCAGGCGACAAAAACGGCGATAAC
TMD7 I447A/F	CGTTTTTGTCCACTGCGCCTTATACCACGGTAAAGAT
TMD7 I447A/R	CTTTACCGTGGTATAAGGCGCAGTGGACAAAAACGGC
TMD7 L448A/F	CGTTTTTGTCCACTGCATCGCATACCACGGTAAAGATATTGTC
TMD7 L448A/R	TATCTTTACCGTGGTATGCGATGCAGTGGACAAAAAC
TMD7 Y449A/F	TGTCCACTGCATCTTAGCCCACGGTAAAGATATTGTC
TMD7 Y449A/R	CAATATCTTTACCGTGGGCTAAGATGCAGTGGACAAA
TMD8 W483A/F	CTATAAGGATTGTCCCGATGCGTGGTATTTACTTTTGCAGATTG
TMD8 W483A/R	GCAAAAGTAAATACCACGCATCGGGACAATCCTTATAG
TMD8 W484A/F	GGATTGTCCCGATTGGGCGTATTTACTTTTGCAGATTG
TMD8 W484A/R	CAATCTGCAAAAGTAAATACGCCCAATCGGGACAATCCTTATAG
TMD8 Y485A/F	GGATTGTCCCGATTGGTGGGCTTTACTTTTGCAGATTGTCATG
TMD8 Y485A/R	CAATCTGCAAAAGTAAAGCCCACCAATCGGGACAATCC
TMD8 L486A/F	GTCCCGATTGGTGGTATGCACTTTTGCAGATTGTCATG
TMD8 L486A/R	CATGACAATCTGCAAAAGTGCATACCACCAATCGGGACAATC
TMD8 L487A/F	CGATTGGTGGTATTTAGCTTTGCAGATTGTCATGATC
TMD8 L487A/R	GATCATGACAATCTGCAAAGCTAAATACCACCAATCGGGAC
TMD8 L488A/F	GATTGGTGGTATTTACTTGCGCAGATTGTCATGATCGGTTTAG
TMD8 L488A/R	CGATCATGACAATCTGCGCAAGTAAATACCACCAATC

TMD8 Q489A/F	GGTGGTATTTACTTTTGCGGATTGTCATGATCGGTTTAG
TMD8 Q489A/R	AACCGATCATGACAATCCGCAAAAGTAAATACCACCAA
TMD8 I490A/F	GTATTTACTTTTGCAGGCTGTCATGATCGGTTTAGG
TMD8 I490A/R	CTAAACCGATCATGACAGCCTGCAAAAGTAAATACCAC
TMD8 V491A/F	GTATTTACTTTTGCAGATTGCCATGATCGGTTTAGGATTTGTAG
TMD8 V491A/R	AATCCTAAACCGATCATGGCAATCTGCAAAAGTAAATAC
TMD8 M492A/F	TACTTTTGCAGATTGTCGCGATCGGTTTAGGATTTGTAG
TMD8 M492A/R	CAAATCCTAAACCGATCGCGACAATCTGCAAAAGTAAATAC
TMD8 I493A/F	CTTTTGCAGATTGTCATGGCCGGTTTAGGATTTGTAGCAG
TMD8 I493A/R	CTACAAATCCTAAACCGGCCATGACAATCTGCAAAAG
TMD8 G494A/F	GCAGATTGTCATGATCGCTTTAGGATTTGTAGCAGTG
TMD8 G494 A/R	CTGCTACAAATCCTAAAGCGATCATGACAATCTGCAAAAG
TMD8 L495A/F	GATTGTCATGATCGGTGCAGGATTTGTAGCAGTGTGC
TMD8 L495A/R	CACACTGCTACAAATCCTGCACCGATCATGACAATCTGC
TMD8 G496A/F	GATTGTCATGATCGGTTTAGCATTTGTAGCAGTGTGCTGTTTC
TMD8 G496A/R	CAGCACACTGCTACAAATGCTAAACCGATCATGACAATC
TMD8 F497A/F	CATGATCGGTTTAGGAGCTGTAGCAGTGTGCTGTTTC
TMD8 F497A/R	GAAACAGCACACTGCTACAGCTCCTAAACCGATCATGACAATC
TMD8 V498A/F	GATCGGTTTAGGATTTGCAGCAGTGTGCTGTTTCGATAC
TMD8 V498A/R	CGAAACAGCACACTGCTGCAAATCCTAAACCGATCATG
TMD8 A499G/F	CGGTTTAGGATTTGTAGGAGTGTGCTGTTTCGATAC
TMD8 A499G/R	GTATCGAAACAGCACACTCCTACAAATCCTAAACCGATC
TMD8 V500A/F	GTTTAGGATTTGTAGCAGCGTGCTGTTTCGATACTAAG
TMD8 V500A/R	CTTAGTATCGAAACAGCACGCTGCTACAAATCCTAAACCG
TMD11 L661A/F	GGCGCATTTATAATCCGGCGATGTGGTTCTTCTTGATTG
TMD11 L661AR/	TCAAGAAGAACCACATCGCCGGATTATAAATGCGCCC
TMD11 M662A/F	CATTTATAATCCGCTGGCGTGGTTCTTCTTGATTGGT
TMD11 M662A/R	CCAATCAAGAAGAACCACGCCAGCGGATTATAAATG
TMD11 W663A/F	TTATAATCCGCTGATGGCGTTCTTCTTGATTGGTTTG
TMD11 W663A/R	CAAACCAATCAAGAAGAACGCCATCAGCGGATTATAAATGC
TMD11 F664A/F	TAATCCGCTGATGTGGGCCTTCTTGATTGGTTTGCTATTC
TMD11F664A/R	GCAAACCAATCAAGAAGGCCCACATCAGCGGATTATAA
TMD11F665A/F	TCCGCTGATGTGGTTCGCCTTGATTGGTTTGCTATTC
TMD11F665A/R	GAATAGCAAACCAATCAAGGCGAACCACATCAGCGGATTA

TMD11 L666A/F	CGCTGATGTGGTTCTTCGCGATTGGTTTGCTATTCCCAC
TMD11 L666A/R	GGAATAGCAAACCAATCGCGAAGAACCACATCAGCGG
TMD11 I667A/F	GATGTGGTTCTTCTTGGCTGGTTTGCTATTCCCACTAG
TMD11 I667A/R	GTGGGAATAGCAAACCAGCCAAGAAGAACCACATCAG
TMD11 G668A/F	GTGGTTCTTCTTGATTGCTTTGCTATTCCCACTAGCC
TMD11 G668A/R	CTAGTGGGAATAGCAAAGCAATCAAGAAGAACCACATC
TMD11 L669A/F	GTTCTTCTTGATTGGTGCGCTATTCCCACTAGCCGTT
TMD11 L669A/R	CGGCTAGTGGGAATAGCGCACCAATCAAGAAGAACCAC
TMD11 L670A/F	TCTTCTTGATTGGTTTGGCATTCCCACTAGCCGTTTATG
TMD11 L670A/R	AAACGGCTAGTGGGAATGCCAAACCAATCAAGAAGAAC
TMD11 F671A/F	CTTGATTGGTTTGCTAGCCCCACTAGCCGTTTATGCT
TMD11 F671A/R	ATAAACGGCTAGTGGGGCTAGCAAACCAATCAAGAAGAAC
TMD11 P672A/F	CTTGATTGGTTTGCTATTCGCACTAGCCGTTTATGCTGTTC
TMD11 P672A/R	GCATAAACGGCTAGTGCGAATAGCAAACCAATCAAGAAG
TMD11 L673A/F	TGGTTTGCTATTCCCAGCAGCCGTTTATGCTGTTCAA
TMD11 L673A/R	AACAGCATAAACGGCTGCTGGGAATAGCAAACCAATC
TMD11 A674G/F	GTTTGCTATTCCCACTAGGCGTTTATGCTGTTCAATGG
TMD11 A674G/R	ATTGAACAGCATAAACGCCTAGTGGGAATAGCAAACCA
TMD11 V675A/F	GCTATTCCCACTAGCCGCTTATGCTGTTCAATGGAAA
TMD11 V675A/R	TCCATTGAACAGCATAAGCGGCTAGTGGGAATAGCAAAC
TMD11 Y676A/F	ATTCCCACTAGCCGTTGCTGCTGTTCAATGGAAATTCC
TMD11 Y676A/R	AATTTCCATTGAACAGCAGCAACGGCTAGTGGGAATAGC
TMD11 A677G/F	CCCACTAGCCGTTTATGGTGTTCAATGGAAATTCCCT
TMD11 A677G/R	GGAATTTCCATTGAACACCATAAACGGCTAGTGGGAA
TMD11 V678A/F	CACTAGCCGTTTATGCTGCTCAATGGAAATTCCCTAAA
TMD11 V678A/R	TAGGGAATTTCCATTGAGCAGCATAAACGGCTAGTGG
TMD11 Q679A/F	AGCCGTTTATGCTGTTGCATGGAAATTCCCTAAATTTAA
TMD11 Q679A/R	AATTTAGGGAATTTCCATGCAACAGCATAAACGGCTAGTGG

2.5. BUFFERS AND STOCK SOLUTIONS

2.5.1. Ampicillin Stock Solution (50 mg/ml)

The required amount of ampicillin (sodium salt) was dissolved in the required volume of deionized water, and it was filter-sterilized using 0.2 μm filter membrane. It was stored at -20°C in a aliquots in micro centrifuge tubes.

2.5.2. GSH Stock Solution (50 mM)

The required amount of glutathione (reduced form) was dissolved in 10 ml of deionized water and was filter-sterilized using 0.2 μm filter membrane. It was stored at -20°C in aliquots.

2.5.3. Methionine Stock Solution (100 mM)

The required amount of methionine was dissolved in 10 ml of deionized water and was filter-sterilized using 0.2 μm filter membrane. It was stored at 4°C.

2.5.4. 50% Glycerol (used for preparing –80°C stocks of *E. coli*)

2.5.5. Alkaline Lysis Buffers (Plasmid DNA preparation from *E. coli*)

a) Solution-I (Resuspension Solution)	50 mM Glucose 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved and stored at 4ºC.
b) Solution-II (Lysis Solution) (freshly prepared)	0.2 N NaOH (freshly diluted from a 10 N stock) 1% SDS (freshly diluted from a 10% stock) Stored at room temperature.
c) Solution-III (Neutralization Solution)	5 M Potassium acetate 60 ml Glacial acetic acid 11.5 ml Deionized water 28.5 ml The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. It was stored at 4ºC.
d) TE Buffer (Tris-EDTA)	10 mM Tris-HCl (pH 8.0).

(pH 8.0)	1 mM EDTA (pH 8.0).
e) TE-RNAse (stock prepared at 10 mg/ml)	Working stock 20 µg/ml in TE Buffer, pH 8.0.
f) PCI (Phenol-chloroform-isoamyl alcohol) Solution (100ml)	Phenol 50 ml [Equilibrated with Tris-HCl (pH 7.6)] Chloroform 48 ml Isoamyl alcohol 2 ml Stored at 4°C in dark brown bottle.

2.5.6. Agarose Gel Electrophoresis Reagents

a) 1× TAE (Tris-acetate-EDTA) Buffer (per 1000 ml) (prepared from 50× TAE stock)	40 mM Tris-acetate. 1mM EDTA (pH 8.0). Autoclaved and stored at room temperature.
b) Orange-G dye (Gel loading dye, 6X)	0.25% orange-G 30% glycerol
c) 0.7-1% Agarose gel in 1× TAE	
d) Ethidium Bromide (10 mg/ml) Stock	Final working concentration used at 0.5 µg/ml.

2.5.7. Solutions for preparation of chemical competent *E. coli* cells [19]

a) SOB	Bactotryptone 20 g Bacto yeast extract 5 g NaCl 0.5 g Above mentioned components were dissolved in 950 ml of water. 10 ml of 250 mM KCl was added and pH was adjusted to 7 with 5N NaOH, volume was made up to 995 ml and autoclaved. Just before use, 5 ml of filter sterilized 2 M $MgCl_2$ was added.
b) SOC	SOB + 20 mM Glucose
c) 10% glycerol	

2.5.8. Yeast Transformation Solutions (*S. cerevisiae*)[20]

a) 0.1 M Lithium acetate in TE (pH 7.5)

b) 50% PEG-3350 in 0.1 M Lithium acetate in TE (pH 7.5).

2.5.9. STES lysis mixture for plasmid / genomic DNA isolation from yeast)

10 mM Tris-HCl (pH 8.0)

1 mM EDTA (pH 8.0)

100 mM NaCl

1% SDS

2% Triton X-100

2.5.10. Solution for Hydroxlyamine mutagenesis [21]

NaOH 90 mg

Hydroxylamine HCl 350 mg

Dissolved in 5 ml water and pH adjusted to 6.5. The solution was made fresh just before use.

2.5.11. Yeast Breaking Buffer (pH 7.5) [22]

a) Yeast whole cell extract preparation.	50 mM Tris-HCl (pH 7.5) 1% Sodium deoxycholate 1% Triton X-100 0.1% SDS 5 mM EDTA 0.05% phenylmethylsulfonyl fluoride (PMSF) Protease inhibitor cocktail (Complete Mini, EDTA free, and Roche Cat #: 11836170001). One table was dissolved 10 ml of the buffer, immediately before use and used for lysis of approximately 12× 50 O.D. of cells.
b) Sorbitol buffer	10 mM Tris-HCl, pH 7.5 300 mM sorbitol, 100 mM NaCl 5 mM $MgCl_2$, 10 mM EDTA and 0.05% phenylmethylsulfonyl fluoride (PMSF)

2.5.12. SDS-PAGE Solutions and Reagents [19]

a) 30% Acrylamide Mix	29.2% (w/v) acrylamide 0.8% (w/v) N, N'- methylenebisacrylamide Filtered before use.
b) Resolving (Lower) Gel Tris Buffer (Stock) (4×) 1.5 M Tris-HCl (pH 8.8)	18.18 g Tris pH adjusted to 8.8 with 6 N HCl and volume made up to 100 ml. (0.4% SDS can be added to the buffer itself).
c) Stacking (Upper) Gel Tris Buffer (Stock) 0.5 M Tris-HCl (pH 6.8)	6.06 g Tris pH adjusted to 6.8 with HCl and volume made up to 100 ml. (0.4% SDS can be added to the buffer itself).
d) 0.4% SDS	
e) TEMED	
f) APS (Ammonium persulphate)	10%
g) Tris-Glycine Gel Running Buffer (Laemmli Buffer) (pH 8.3)	25 mM Tris base 250 mM Glycine (electrophoresis grade) 0.1% SDS
h) 5× Sample Buffer/Gel Loading Buffer (pH 6.8)	0.15 M Tris-HCl (pH 6.8) 5% SDS 25% Glycerol 12.5% β-mercaptoethanol 0.006% Bromophenol blue
i) Gel Staining Solution	40% Methanol 10% Glacial Acetic acid 0.1% Coomassie Brilliant Blue (R250)
j) Gel Destaining Solution	40% Methanol 10% Glacial Acetic acid

Composition of SDS-PAGE Resolving (Lower) Gel (9%) (for 10 ml)

a)	Distilled water	4.35 ml
b)	Resolving (Lower) Gel Tris Buffer (Stock) (4×) 1.5 M Tris-HCl (pH 8.8) with 0.4% SDS	2.5 ml
c)	30% Acrylamide Mix	3.35 ml
d)	10% APS (Ammonium persulphate)	0.05 ml
e)	TEMED (N, N, N', N'-Tetramethylethylenediamine)	0.01 ml

Composition of SDS-PAGE Stacking (Upper) Gel (4%) (For 10 ml)

a)	Distilled water	6.1 ml
b)	Stacking (Upper) Gel Tris Buffer (Stock) 0.5 M Tris-HCl (pH 6.8) with 0.4% SDS	2.5 ml
c)	30% Acrylamide Mix	1.3 ml
d)	10% APS (Ammonium persulphate)	0.05 ml
e)	TEMED (N, N, N', N'-Tetramethylethylenediamine)	0.01 ml

2.5.13. Immunoblotting (Western Blotting) Reagents [19]

a) Transfer Buffer (pH 9.2)	48 mM Tris base 39 mM Glycine 0.037% SDS 20% Methanol
b) Stripping Buffer	62.5 mM Tris-HCl, pH 6.7 100 mM β-mercaptoethanol 2% SDS
c) Tris-Buffered Saline (TBS)	150 mM NaCl 20 mM Tris-HCl (pH 7.5) (Made as 10× stock and kept at 4°C)
d) Washing Buffer TBS-Tween 20 (TBST) Buffer	0.1% Tween 20 in TBS (pH7.5)
e) Blocking Agent	5% Skim milk in TBST.
f) Ponceau S Staining Solution	0.5% Ponceau S 1% Glacial Acetic acid

2.5.14. Immunofluorescence Reagents

a) Potassium phosphate buffer (100 mM, pH 6.5)	
b) Polylysine stock (1% w/v in sterile water) (10×)	Diluted ten times in 1× PBS just before use. (For coating the cover slips, a 10-15 µl of the working stock of polylysine is dropped on each and a stack is prepared. Kept in a moist chamber at 4°C overnight. Coated coverslips washed in sterile water and dried and used upto a month).
c) Fixative buffer	4% paraformaldehyde prepared in 100 mM potassium phosphate buffer (pH 6.5) 4 g of paraformaldehyde was dissolved in 50 ml of sterile water and kept on magnetic

	stirrer with heating around 60°C. To this 50 ml of 200 mM phosphate buffer (pH 6.5) is added. pH adjusted to around 6.5 with NaOH and allowed to dissolve. Kept in aliquots at -20°C. (May precipitates out after one-two months, in which case a fresh solution should be made).
d) Sorbitol Buffer	1.2 M sorbitol in 0.1 M phosphate buffer
e) 50 mg/ml Zymolase (Seikagaku, Tokyo, Japan)	50 mg/ml stock prepared in 50% glycerol in sorbitol buffer and kept at -20°C (up to 6 months).
f) Phosphate-Buffer Saline (PBS)	40 mM K_2HPO_4 10 mM KH_2PO_4 pH 7.4 150 mM NaCl. Prepared as 10× stock. pH of stock comes around 6.7.
g) Permeabilization buffer	0.4% Triton X-100 in PBS
h) Blocking/ antibody dilution buffer	1% BSA (w/v) in PBS
i) Mowiol mounting medium	To 6 ml of glycerol add 2.4 g of Mowiol 4-88 (Polyvinyl alcohol, Fluka) and 6 ml of water add and keep at room temperature for 2 hours. Add 12 ml of 200 mM Tris-HCl, pH 8.5 and incubate for 53°C overnight to 16 hours, till Mowiol dissolves. To this add paraphenylene diamine at 2.4 mg/ml. Dissolve by vortexing. Golden brown when freshly made, but turns dark brown upon light exposure and should not be used.

SECTION B: METHODS

2.1 Growth and maintenance of bacteria and yeast strains

The *Eschericha coli* strains DH5α was routinely grown at LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The *S. cerevisiae* strains were regularly maintained on YPD medium and grown at 28-30°C. The yeast transformants were selected and maintained on SD medium with supplement as per requirements.

2.2 Recombinant DNA methodology (restriction digestion, ligation, transformation of *E. coli.* PCR amplification, etc)

All the molecular techniques used in the study for manipulation of DNA, protein, bacteria and yeast were according to standard protocols [19,23] or as per manufacturers' protocol, unless specifically mentioned.

2.3 Construction of Site-directed mutants of *HGT1*

The double tagged *HGT1* (tagged with hexa-histidine epitope at the N-terminus and HA tag at the C-terminus) was cloned downstream of the TEF promoter at *Bam*HI and *Eco*RI site, of a modified p416TEF vector (p416M1-TEF, ABE1888), in which all the restrictions sites flanking the *Bam*HI and *Eco*RI restriction sites have been removed [25]. Two silent mutations were introduced to generate the restriction sites *Msc*I (1366) and *Xba*I (1584) in the wild-type *HGT1* gene (Kaur & Bachhawat, 2009). This modified gene product pTEFM1-His-HGT1^{M}-HA (ABE 1912) was used as a template for site-directed mutagenesis for creation of different site-directed mutants of Hgt1p using splice overlap extension strategy. The different mutagenic oligonucleotides pairs used for generation of these mutants are given in Table 2.3.

2.4 *Invitro* Hydroxylamine mutagenesis

The protocol for *in vitro* mutagenesis was adopted from Rose and Fink, (1987). 10 μg plasmid DNA was dissolved in 0.5 ml of Hydroxylamine solution (90 mg NaOH, 350 mg

hydroxylamine HCl in 5 ml water, pH around 6.5. freshly made up before use). This mixture was incubated at 37°C for 20 hrs and the DNA was purified using Qiagen column. The pool of mutagenized plasmid was directly transformed into the appropriate yeast strain.

2.5. Transformation of yeast

The transformation of *S. cerevisiae* strains was carried out by lithium acetate method [21]. *S. cerevisiae* cultures were grown in YPD at 30°C with shaking for 16-24 hrs and then reinoculated in fresh YPD to an initial OD_{600} of 0.1, cells were allowed to grow at 30°C for 4-5 hrs with shaking. Cells were harvested at 6000 rpm for 5 min, then were washed with sterile water followed by subsequent wash with 0.1 M lithium acetate solution (prepared in TE, pH 7.5) and were finally resuspended in the same solution. Cells were incubated at 30°C for 30 min with shaking. The cells were spun down, suspended in 0.1 M lithium acetate solution to a cell density of 1×10^9 cells/ml and divided into 100µl aliquots. Approximately 50 µg (5 µl of 10 mg/ml stock solution) of heat denatured, salmon sperm carrier DNA, followed by 0.3 µg- 0.7µg of plasmid/DNA fragment were added to each aliquot and whole cell suspension was incubated at 30°C for 30 min. After the incubation, 0.3 ml of 50% PEG 3350 (prepared in 0.1 M lithium acetate, pH 7.5) was added to each tube, mixed well and again kept at 30°C for 45 min. The cell suspensions were subjected to heat shock at 42°C for 10 min. and the cells were allowed to cool to room temperature. The cells were pelleted down at 7000 rpm for 3 min. The cell pellet was resuspended in sterile water and appropriate volume of cell suspension was plated on selection plates.

2.6. Isolation of plasmid from yeast

Selected yeast transformants were inoculated in 3 ml of selection medium and the cultures were incubated at 30°C with shaking for 18-20 hrs. After the incubation, the cells were harvested at 8,500 rpm for 5 min at room temperature and the pellets were suspended in 200 µl of STES lysis solution. Equal amounts of sterile, acid-washed glass beads (425 to 600 µm, Cat# G8772, Sigma) were added and the cell suspensions were vortexed vigorously for 1 min at room temperature. The lysed suspensions were then

treated with phenol-chloroform adding 200 µl of phenol-chloroform solution and then vortexing for 1-2 min at room temperature. The lysates were spin down at 12,000 rpm for 5 min at RT and the aqueous phase was collected in a fresh microfuge tube. 2 µl of this DNA was transformed in *E. coli* and transformants were selected on LB plates (containing ampicillin). The *E. coli* transformants were then grown to isolate plasmids and verified by re-transformation into yeast.

2.7 Isolation of genomic DNA from yeast

Genomic DNA from *S. cerevisiae* strains was isolated as described by using the glass bead lysis method and the STES lysis buffer [21].

2.8 Growth assay by dilution spotting

For growth assay, the different *S. cerevisiae* strains carrying the plasmid were grown overnight in minimal medium without uracil and reinoculated in fresh medium to an OD_{600} of 0.1 and grown for 6 hours. The exponential phase cells were harvested washed with water and resuspended in water to an OD_{600} of 0.2. These were serially diluted to 1:10, 1:100 and 1:1000. 10 µl of these cell resuspensions were spotted on minimal medium containing different concentrations of glutathione, and methionine, as sole sulphur source. The plates were incubated at 30ºC for 3 to 4 days and photographs taken.

For evaluation of the functional activity of Hgt1p mutants on plate dual-complementation-toxicity assay was used. The yeast strain *met15Δ hgt1Δ* (ABC 817) was transformed with a single copy, centromere vector expressing wild type or different *HGT1* mutants expressed downstream of the TEF promoter and analyzed by dilution spotting. pTEF-HGT1 complement the *met15Δ hgt1Δ* at low glutathione concentration but is toxic at higher concentration.

2.9 Yeast whole cell extract preparation

Total crude cell extract were prepared as described previously with certain modifications [22]. Briefly, overnight cultures of transformants grown in minimal media containing methionine and other supplements without uracil, were reinoculated at OD_{600} of 0.1 in 50 ml of fresh medium and grown to exponential phase (OD_{600} of ~0.6 to 0.8). Cells were harvested by centrifugation and resuspended in yeast breaking buffer for whole cell

extract preparation containing protease inhibitors. Acid-washed glass beads (425 to 600 µm) were added to the cell suspension and cells were lysed by vigorous vortexing. The resulting homogenate was centrifuged for 10 min at 13,000 rpm and the supernatant fraction was collected.

2.10 Protein estimation

Protein estimation of the samples was done by Bradford Assay [24] using Bradford reagent as per the manufacturer's instructions. The protein samples were diluted 20 times and 2.5µl of the diluted sample was used for protein estimation, using BSA (Albumin, Fraction V, USB Cat# 70195) as standard. Protein estimations were done in Triplicates.

2.11 Protein electrophoresis and western blotting

The western blot analysis was done using a modified protocol of the standard western blot [25]. Equal amount of protein samples (20 µg) were resolved by 9% SDS–polyacrylamide gel electrophoresis and electroblotted onto Hybond ECL nitrocellulose membrane (Amersham Bioscience) in a mini transblot apparatus (Bio-Rad) at 120V for 1 hrs using Tris-glycine transfer buffer. Immediately after the transfer, the membrane was incubated at 55°C for 15 min in the stripping buffer. After blocking the membrane for 1 h at room temperature in 5% skim milk in TBST buffer, it was probed with mouse monoclonal anti-HA primary antibody (Cell signaling) at a dilution of 1:2,000 in the TBST buffer for 4 hrs at room temperature. After 4×10 minutes washing of the blot with TBST, the membrane blot was incubated for 1 h in horse anti-mouse (horseradish peroxidase-conjugated) antibody at a dilution of 1:2,500 in the TBST buffer. The signal was detected with an ECL plus Western detection kit as per the manufacturer's instruction.

Densitometry analysis of the unsaturated band signals was performed using the Scion Image software to quantify the protein expression levels in different mutants. The resulting signal intensity was normalized with respect to the band surface area (in square pixels) and expressed in arbitrary units. The relative protein expression levels in the mutant Hgt1p were represented as percentage expression relative to wild-type Hgt1p.

2.12 Sequence analysis

The ORF sequences (*HGT1*) as well as the corresponding proteins sequences were retrieved from Saccharomyces genome database (SGD).

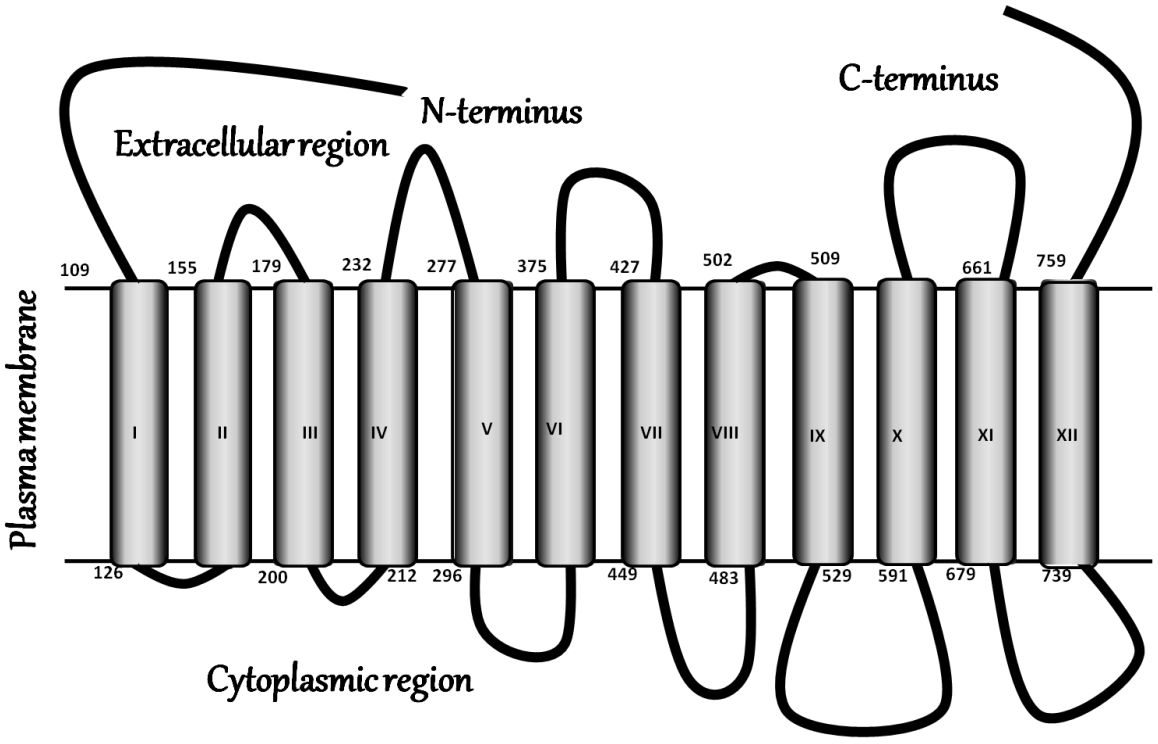
The Hgt1p sequence was used as query sequence in BLAST search of protein database from Entrez at NCBI website, (http://www.ncbi.nlm.nih.gov/) to retrieve a list of homologues of Hgt1p.

For the phylogenetic analysis, multiple sequence alignment of the protein sequences was generated using CLUSTAL X program using default parameters [29] and the MEGA 4 software was used to construct the phylogenetic tree of the family [28].

The Gene Runner (version 3.05) software was used for restriction analysis and oligo analysis. The Webcutter 2.0 was used to find novel restriction sites that could be generated in the *HGT1* gene by introducing silent mutations in the coding sequence of the gene (http://rna.lundberg.gu.se/cutter2/).

Chapter 3

Alanine Scanning of Transmembrane Domain 1, 5, 7,8,11 of Hgt1p



Results and Discussion

Very little information is available on mechanistic or structural aspect of members of the OPT family. The most widely studied OPT member is Hgt1p. Explorations of role of cysteines and polar/charged amino acid residues in the transmembrane domains of Hgt1p have revealed that the TMD1, 4, and 9 and the intracellular loop region 537-568 are crucial for substrate translocation [30]. In this chapter, I have carried out alanine scanning of all the amino acid residues (alanine replaced by glycine) on selected transmembrane domains of Hgt1p to identify the role of these TMDs. A rationally design topology model is a requirement for probing the role of amino acids in the transmembrane domains of Hgt1p. Previously, Becker and co-workers predicted a topology model of Hgt1p with 12 transmembrane domain and extracellular N-termini and C-termini, using topology prediction software in combination with multiple sequence alignment of functionally characterized members of family [30]. We have used this model in our analysis.

Hgt1p has 254 amino acid residues in 12 transmembrane domains. We have selected 5 transmembrane domain's amino acid residues for alanine scanning mutagenesis in this study. The mutants were functionally characterized using the plate based dual complementation-cum-toxicity assay.

3.1 Alanine scanning mutagenesis of Transmembrane Domain 1,5,7,8 and 11

The predicted transmembrane domain 1 of Hgt1p contains 19 amino acid residues, TMD5 contains 20 amino acid residues, TMD7 contains 23 amino acid residues, TMD8 contains 20 amino acid residues, and TMD11 contains 19 amino acid residues [30, 31]. To assess the contribution of these individual residues in glutathione transport, each of all non-alanine residues were mutated to alanine by site-directed mutagenesis. In addition, all the alanine residues were mutated to glycine, to examine if the side chain of this alanine residue might be functionally important. Each of the mutants was subjected to an initial functional characterization using the previously designed sensitive plate assay, known as dual complementation cum-toxicity assay [30]. The assay is based on the dual behavior of HGT1 expressed under the strong constitutive TEF

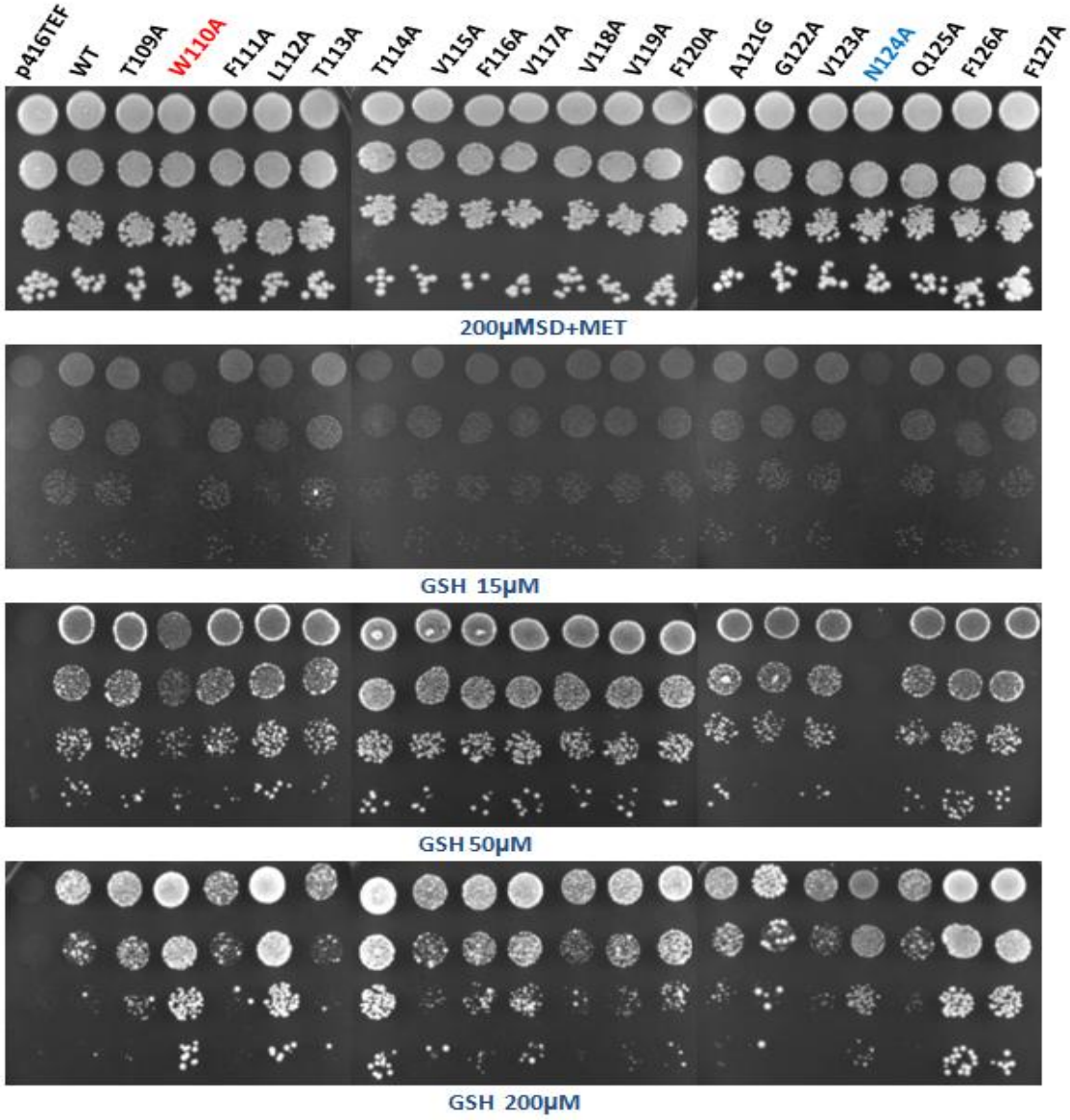


Figure 4: Functional characterization of alanine mutants transmembrane domain 1 of Hgt1p. Hgt1p and the different alanine mutants of TMD1 of Hgt1p expressed under the TEF promoter, and corresponding vector (p416TEF) were transformed into strain ABC817 and analyzed by the dual complementation-com- toxicity assay by dilution spotting on minimal media containing different concentration of glutathione. Transformants were grown in minimal medium containing methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002. OD_{600} of cells. 10 µl of these dilutions were spotted on minimal medium containing different concentrations of glutathione. The photograph was taken after 3 days of incubation at 30˚C.

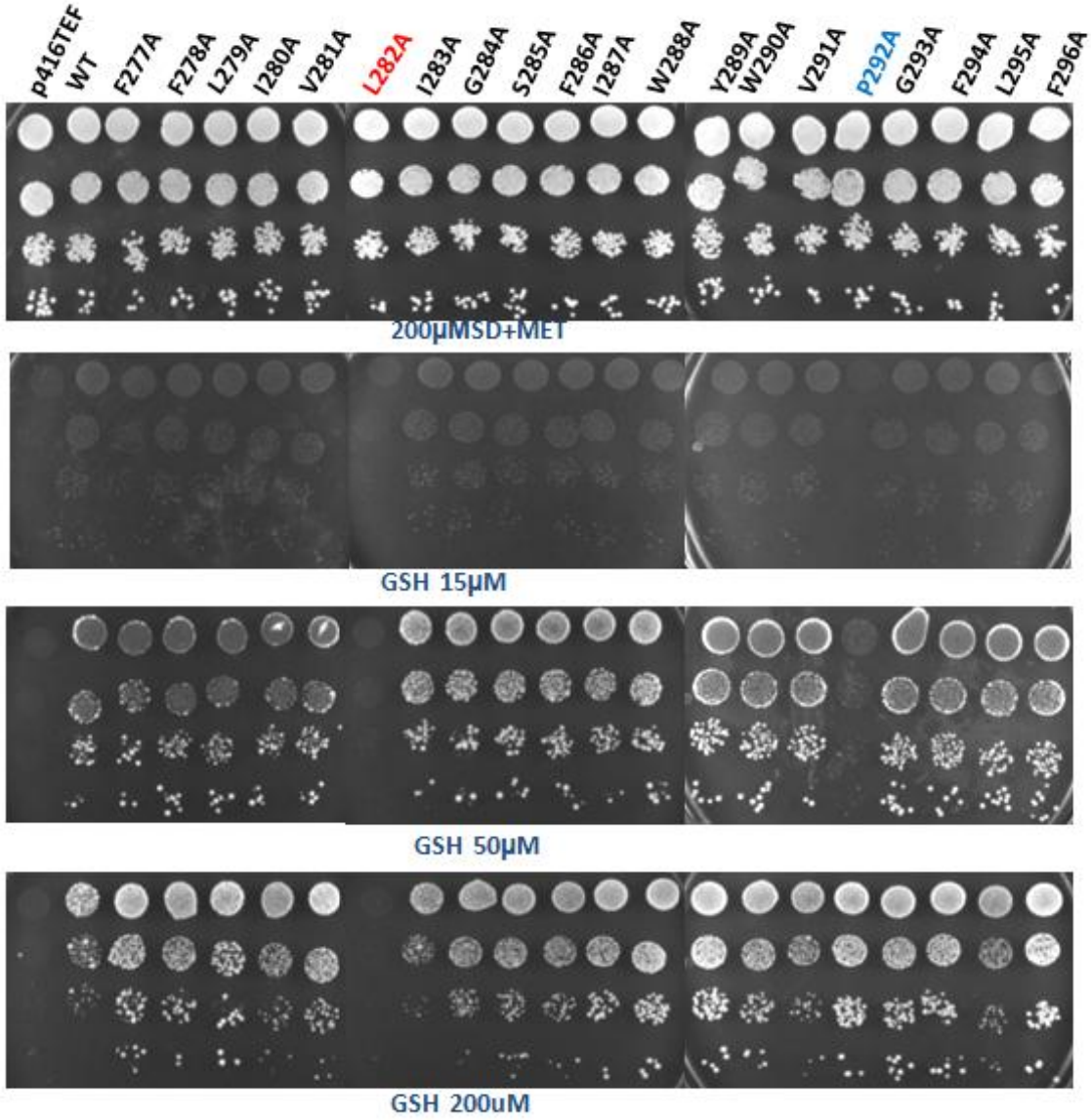


Figure 5: Functional characterization of alanine mutants transmembrane domain 1 of Hgt1p. Functional characterization of alanine mutants transmembrane domain 5 of Hgt1p. Hgt1p and the different alanine mutants of TMD5 of Hgt1p expressed under the TEF promoter, and corresponding vector (p416TEF) were transformed into strain ABC817 and analyzed by the dual complementation-com- toxicity assay by dilution spotting on minimal media containing different concentration of glutathione. Transformants were grown in minimal medium containing methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002. OD_{600} of cells. 10 µl of these dilutions were spotted on minimal medium containing different concentrations of glutathione. The photograph was taken after 3 days of incubation at 30˚C.

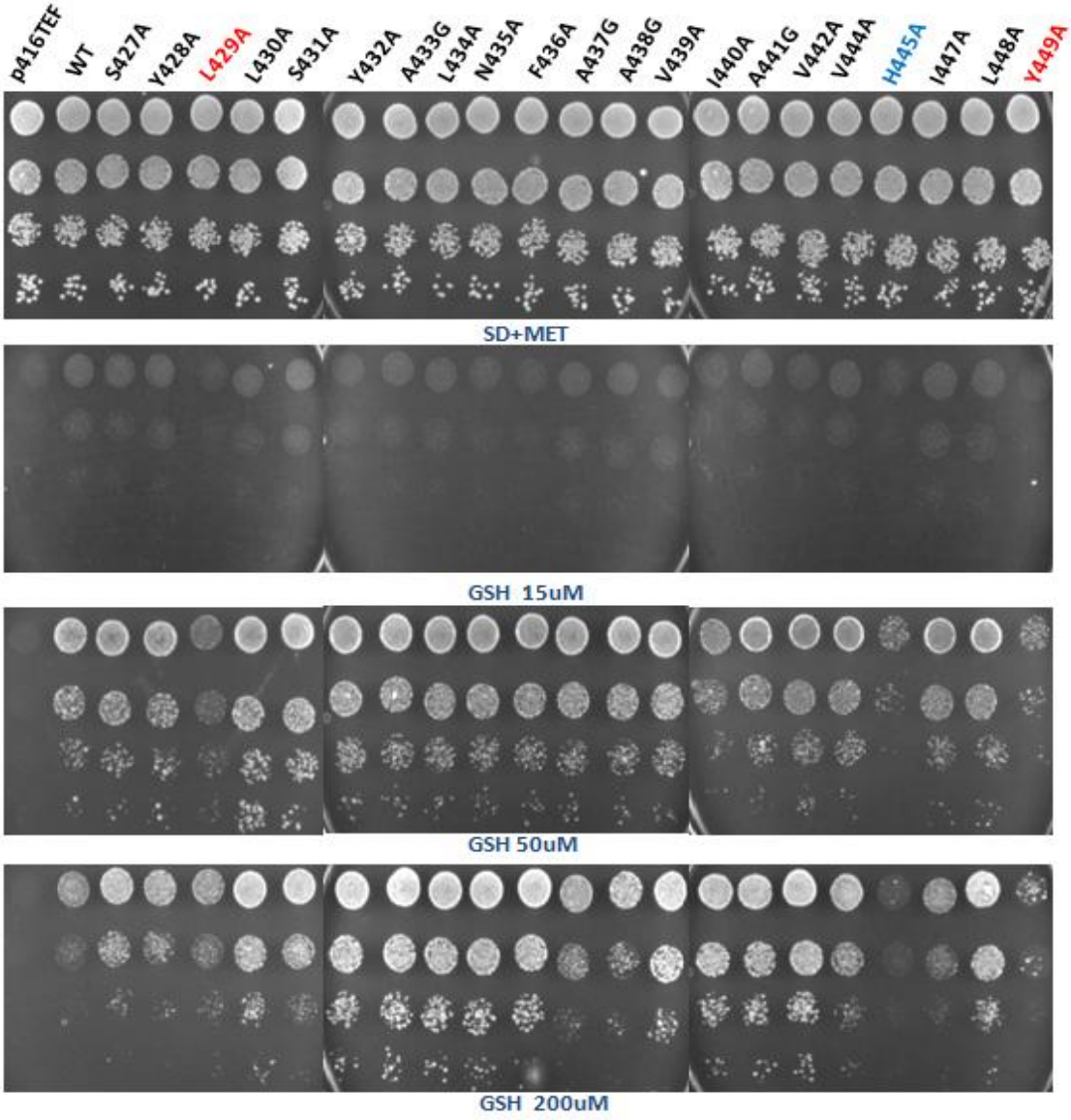


Figure 6: Functional characterization of alanine mutants transmembrane domain 7 of Hgt1p**.** Hgt1p and the different alanine mutants of TMD7 of Hgt1p expressed under the TEF promoter, and corresponding vector (p416TEF) were transformed into strain ABC817 and analyzed by the dual complementation-com- toxicity assay by dilution spotting on minimal media containing different concentration of glutathione. Transformants were grown in minimal medium containing methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002. OD_{600} of cells. 10 µl of these dilutions were spotted on minimal medium containing different concentrations of glutathione. The photograph was taken after 3 days of incubation at 30˚C.

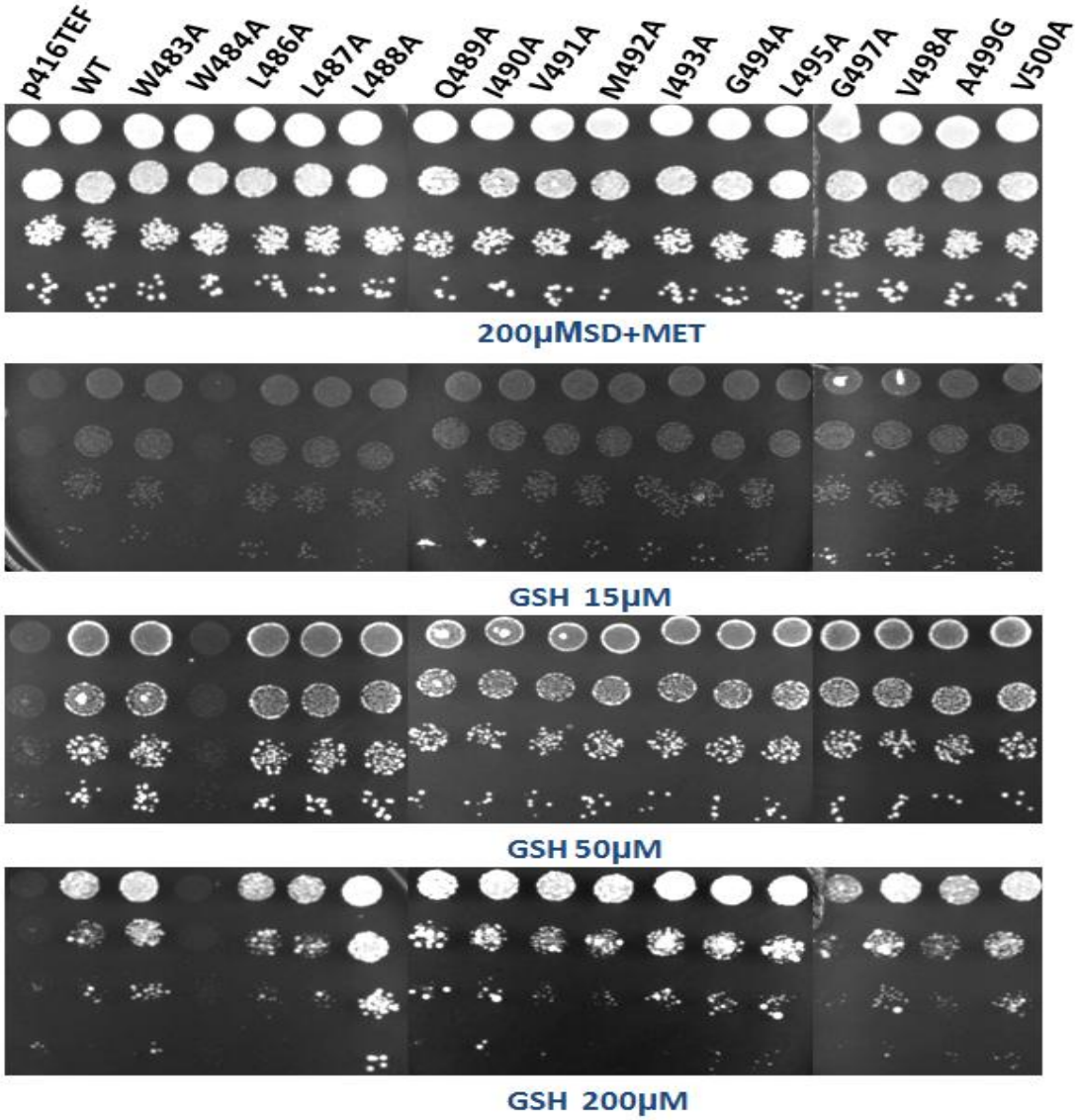


Figure 7: Functional characterization of alanine mutants transmembrane domain 8 of Hgt1p**.** Hgt1p and the different alanine mutants of TMD8 of Hgt1p expressed under the TEF promoter, and corresponding vector (p416TEF) were transformed into strain ABC817 and analyzed by the dual complementation-com- toxicity assay by dilution spotting on minimal media containing different concentration of glutathione. Transformants were grown in minimal medium containing methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002. OD_{600} of cells. 10 µl of these dilutions were spotted on minimal medium containing different concentrations of glutathione. The photograph was taken after 3 days of incubation at 30˚C.

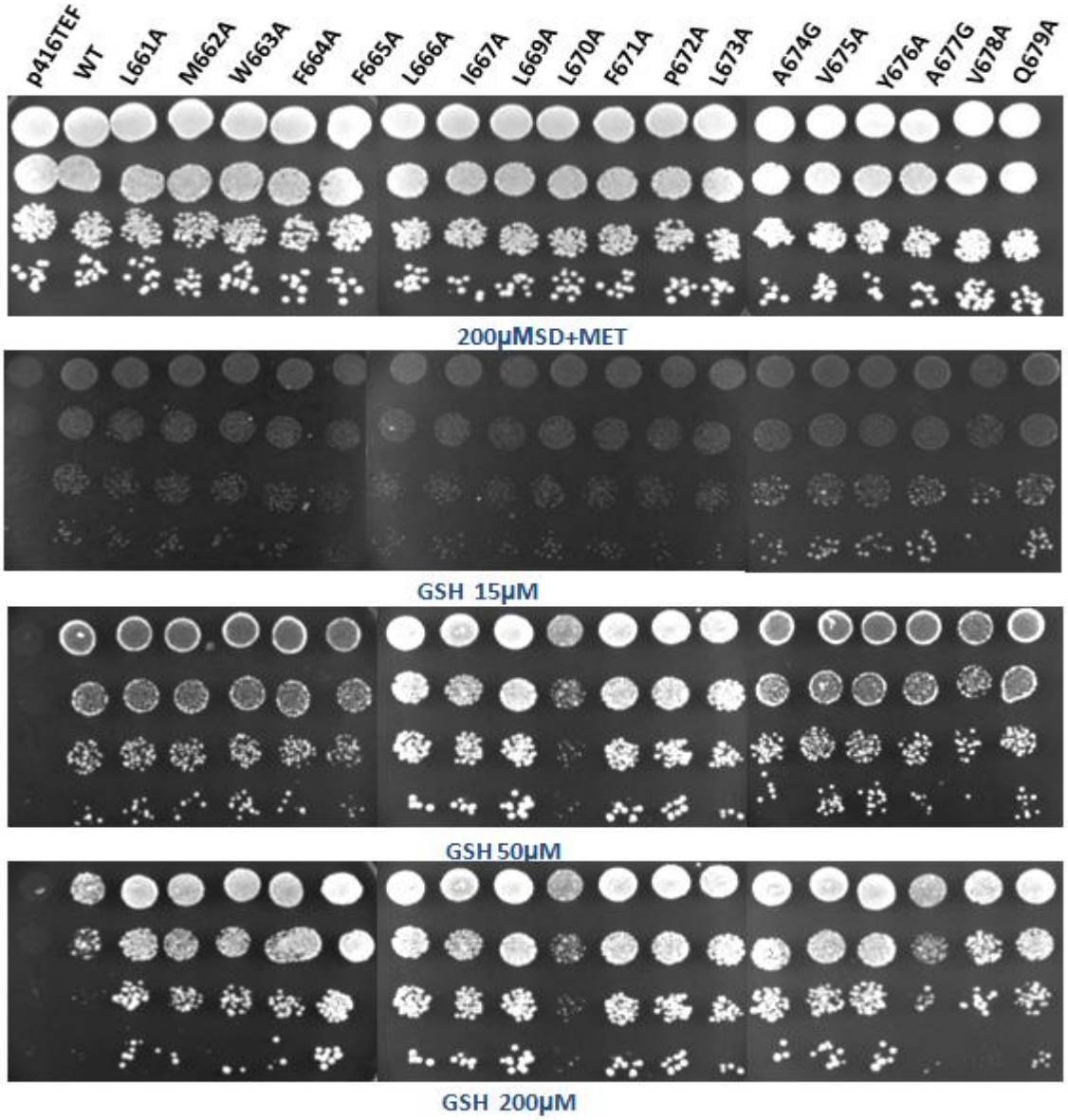


Figure 8: Functional characterization of alanine mutants transmembrane domain 11 of Hgt1p. Hgt1p and the different alanine mutants of TMD11 of Hgt1p expressed under the TEF promoter, and corresponding vector (p416TEF) were transformed into strain ABC817 and analyzed by the dual complementation-com- toxicity assay by dilution spotting on minimal media containing different concentration of glutathione. Transformants were grown in minimal medium containing methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002. OD_{600} of cells. 10 µl of these dilutions were spotted on minimal medium containing different concentrations of glutathione. The photograph was taken after 3 days of incubation at 30˚C.

promoter in a *met15Δhgt1Δ* strain. The *met15Δhgt1Δ* strain is organic sulphur auxotroph owing to *met15Δ*, and also deficient in glutathione uptake owing to *hgt1Δ*. At low glutathione concentration (15μM), HGT1 expressed under TEF promoter complements the growth defect while at higher glutathione concentration (50μM or higher), HGT1 expressed under TEF promoter show toxicity owing to excess accumulation of glutathione [8]. Thus the p416TEF plasmid bearing the different mutants of these TMDs (or WT) was individually transformed into the *met15Δhgt1Δ* strain and mutants were analyzed for their ability to confer complementation and toxicity to the cells over a range of glutathione concentrations (fig. 4 to 8) the different mutations exhibited a differential effect on the ability of the cells to grow on glutathione at low and high concentrations as compared to the wild-type Hgt1p. Hence, based on their growth behavior in the dual complementation-cum-toxicity assay the mutants were characterized into 4 groups. The 'Severe effect,' group included those in which complementation was defective at 15μM of glutathione concentration. The 'Moderate effect' group included those in which complementation was observed at 15μM, but toxicity was not seen at higher glutathione concentration. The 'Mild effect' group included those in which complementation was observed at 15μM, but shows limited toxicity at higher glutathione concentrations. The 'minor effect' group comprises mutants in which complementation was observed at 15μM, and toxicity was seen at higher concentration of glutathione (mutants very similar to wild-type protein) (table 4)

Based on this functional dual complementation-com-toxicity assay, in transmembrane domain 1 only W110A and N124A (N124A studied earlier) showed a severe defect in functionality, and residues L112A, T114A, F120A, F126A and F127A showed a moderate effect on functional activity of Hgt1p. 6 Mutants had a mild effect (F116A, V117A, V118A, V119A, A121G, and G122A), remaining mutants (T109A, F111A, T113A, V115A, V123A, and Q125A) had no or insignificant effect comparable to wild-type.In transmembrane domain 5 only L282A and P292A (P292A studied earlier) showed a severe defect in functionality, and residues Y289A and W290A showed a moderate effect on functional activity of Hgt1p. 1 Mutant had a mild effect (I287A), remaining mutants

Table 4: Grouping of alanine mutants of Hgt1p based upon their effect on the functional activity of the transporter using dual complementation-cum-toxicity assay.

The mutants were analyzed for the functional activity using the plate based assay categorized into 4 groups based upon their activity in terms of their ability to complement ABC 817 (*met15Δ hgt1p Δ*) at low concentration of glutathione and cause toxicity to the cells at glutathione higher then 50µM

Mutant (TMD)	Complementation (15µM GSH)	Toxicity (>50µM GSH)
WT-Hgt1p	+	+
SEVERE EFFECT (Complementation defective)		
W110A(TMD1)	-	-
N124A(TMD1)	-	-
L282A(TMD5)	-	-
P292A(TMD5)	-	-
L429A(TMD7)	-	-
H445A(TMD7)	-	-
Y449A(TMD7)	-	-
W484A(TMD8)	-	-
MODERATE EFFECT (No toxicity)		
L112A(TMD1)	+	-
T114A(TMD1)	+	-
F120A(TMD1)	+	-
F126A(TMD1)	+	-
F127A(TMD1)	+	-
Y289A(TMD5)	+	-
W290A(TMD5)	+	-
A438G(TMD7)	+	-
V439A(TMD7)	+	-
I447A(TMD7)	+	-
L448A(TMD7)	+	-
L488A(TMD8)	+	-
L666A(TMD11)	+	-
I667A(TMD11)	+	-
L669A(TMD11)	+	-
L670A(TMD11)	+	-
F671A(TMD11)	+	-
P672A(TMD11)	+	-
L673A(TMD11)	+	-

MILD EFFECT (Mild toxicity at high GSH concentration)		
F116A(TMD1)	+	+/-
V117A(TMD1)	+	+/-
V118A(TMD1)	+	+/-
V119A(TMD1)	+	+/-
A121G(TMD1)	+	+/-
G122A(TMD1)	+	+/-
I287A(TMD5)	+	+/-
L430A(TMD7)	+	+/-
S431A(TMD7)	+	+/-
Y432A(TMD7)	+	+/-
A433G(TMD7)	+	+/-
L434A(TMD7)	+	+/-
N435A (TMD7)	+	+/-
F436A(TMD7)	+	+/-
A437G(TMD7)	+	+/-
I440A(TMD7)	+	+/-
A441G(TMD7)	+	+/-
V442A(TMD7)	+	+/-
V444A(TMD7)	+	+/-
W483A(TMD8)	+	+/-
L486A(TMD8)	+	+/-
L487A(TM8D)	+	+/-
Q489A(TMD8)	+	+/-
I490A(TMD8)	+	+/-
V491A(TMD8)	+	+/-
M492A(TMD8)	+	+/-
I493A(TMD8)	+	+/-
G494A(TMD8)	+	+/-
L495A(TMD8)	+	+/-
F497A(TMD8)	+	+/-
V498A(TMD8)	+	+/-
A499G(TMD8)	+	+/-
V500A(TMD8)	+	+/-
V675A(TMD11)	+	+/-
Y676A(TMD11)	+	+/-
V678A(TMD11)	+	+/-
Q679A(TMD11)	+	+/-
NO EFFECT (severe toxicity)		
T109A(TMD1)	+	+
F111A(TMD1)	+	+
T113A(TMD1)	+	+

V115A(TMD1)	+	+
V123A(TMD1)	+	+
Q125A(TMD1)	+	+
F277A(TMD5)	+	+
F278A(TMD5)	+	+
L279A(TMD5)	+	+
I280A(TMD5)	+	+
V281A(TMD5)	+	+
I283A(TMD5)	+	+
G284A(TMD5)	+	+
F286A(TMD5)	+	+
W288A(TMD5)	+	+
V291A(TMD5)	+	+
P292A(TMD5)	+	+
G293A(TMD5)	+	+
F294A(TMD5)	+	+
L295A(TMD5)	+	+
F296A(TMD5)	+	+
S427A(TMD7)	+	+
Y428A(TMD7)	+	+
L661A(TMD11)	+	+
M662A(TMD11)	+	+
W663A(TMD11)	+	+
F664A(TMD11)	+	+
F665A(TMD11)	+	+

(F277A, F278A, L279A, I280A, V281A, I283A, G284A, F286A, V291A, G293A, F294A, L295A, and F296A) had no or insignificant effect comparable to wild-type.

In transmembrane domain 7 only L429A, H445A, (H445A studied earlier), and Y449A showed a severe defect in functionality, and residues A438G, V439A, I447A, and L448A showed a moderate effect on functional activity of Hgt1p. 12 Mutants had a mild effect (L430A, S431A, Y432A, A433G, L434A, N435A, F436A, A437G, I440A, A441G, V442, and V444A), remaining mutants (S427A, and Y428A) had no or insignificant effect comparable to wild-type.

In transmembrane domain 8 only W484A showed a severe defect in functionality and residue L488A showed a moderate effect on functional activity of Hgt1p. 14 Mutants had a mild effect (W483A, L486A, L487A, Q489A, I490A, V491A, M492A, I493A, G494A, L495A, F497A, V498A, A499A, and V500A), remaining mutants.

And in transmembrane domain 11 no residue showed a severe defect in functionality, and residues L666A, I667A, L669A, L670A F671A, P672A, and L673A showed a moderate effect on functional activity of Hgt1p. 4 Mutants had a mild effect (V675A, Y676A, V678A, and Q6789A), remaining mutants (L661A, M662A, W663A, F664A, and F665A) had no or insignificant effect comparable to wild-type.

3.2. Evaluation of protein expression levels of Transmembrane Domain 1,5,7,8 and 11

The loss of functional activity of the alanine mutants might be due to decreased in expression level of Hgt1p protein. To determine if the cause for the loss of functional activity Hg1p is because of decrease in expression level, the expression levels of the mutants defective in GSH transport was measured by western blotting. Equal amount of the crude protein extracts were prepared from the *met15Δhgt1Δ* strain (ABC817) transformed with plasmid bearing the alanine mutants of TMD1, 5, 7, 8, and 11of Hgt1p. Protein extracts were loaded onto the SDS-PAGE and elecroblotted to the membrane and probed with anti-HA monoclonal antibody. A 85 kDa band corresponding to the wild-type Hgt1p was observed for all the severely defective mutants. The mutants showed differences in levels of protein expression (fig.16) and

densitometric analysis of the band signal was performed to quantitate the differences. The protein expression levels of Q142A, L373A, Y374A, and L429 were 80 to 95 percent compare to the wild-type protein levels. And W110A, L282A Y449, and W484A showed a significant fall in protein expression levels and it is probable that the decreased protein levels in these mutants could responsible for the loss in functionality for these, but not for the other mutants.

3.3. Analysis of conservation pattern of severely defective mutants residues of TMD1, 5, 7, and 8 of Hgt1p by multiple sequence alignment of OPT family members.

The OPT family contains two distinct clades, the peptide transporting (PT) clade and the yellow stripe (YS) clade. Hgt1p belong to PT clade. Multiple sequence alignment of these 19 PT family members revealed that W110 in TMD1, L282 in TMD5, L429 and Y449 in TMD7 were present in the all 19 PT family (except W484 in TMD 8 is showed severe defective on plate based assay but not conserved at all) (fig. 9 and 10).

An examination of helix wheel projection of these transmembrane domain show that these residues tends to cluster with the other hydrophilic residues, therefore also towards the cytoplasmic end of the transmembrane domains (fig. 11, 12, 13, 14, and 15).

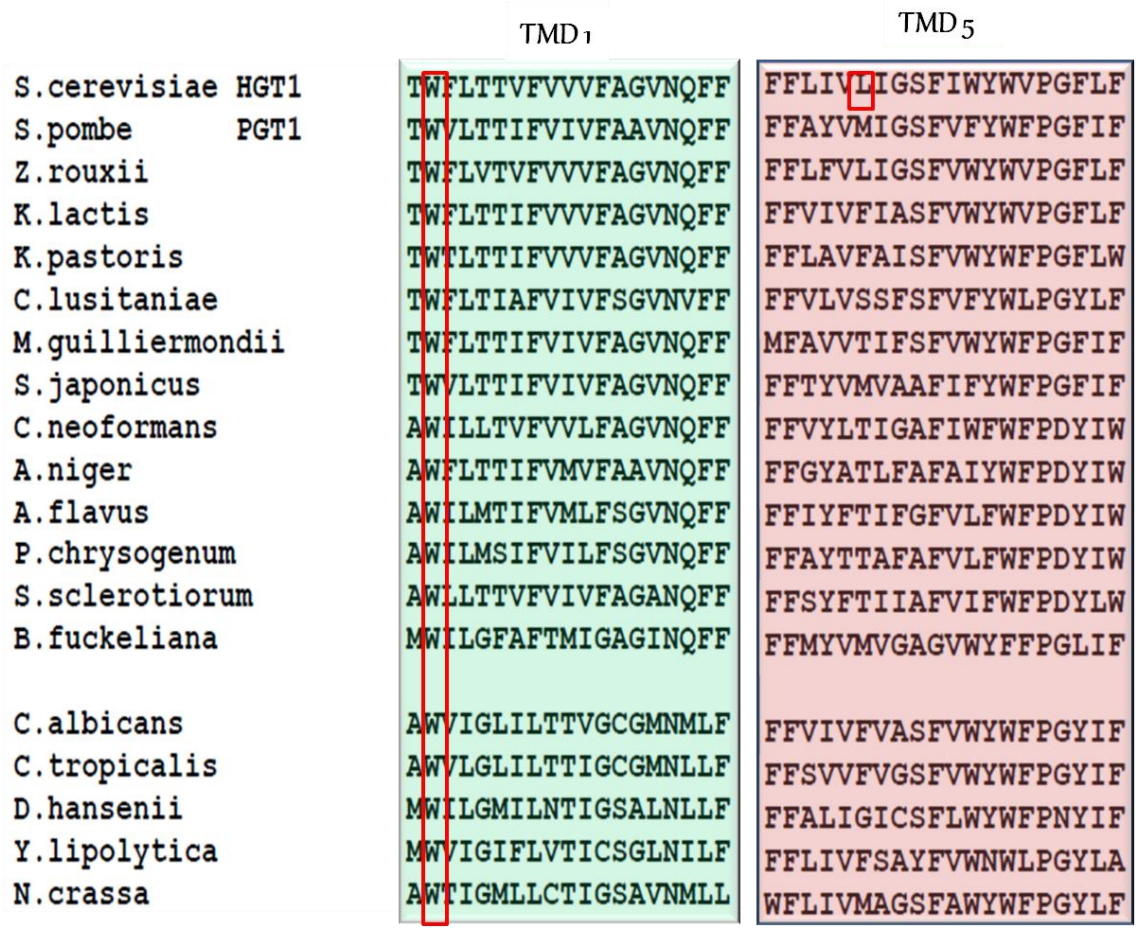


Figure 09 : Multiple sequence alignment for the protein sequences of the PT members of the OPT family. Sequences of *S.cerevisiae* Hgt1, *K.lactis* (XP_00265150.1), *P.guilliermondii* (XP_00148681.1), *S.pombe* Pgt1, *S.japonicus* (XP_002172910.1), *C.neoformans* (XP_772672.1), *A.niger* (XP_001397394.1), *A.Flavous* (XP_002381590.1), *P.chrysogenum* (XP_002567631.1), *S.sclerotiorum* (XP_001585829.1), *B.fuckeliana* (XP_001547017.1), *C. albicans* Opt1, *C tropicalis* (XP_002546105.1), *D.hansenii* (XP_458419.1), *Y.lopolytica* (XP_502145.1), *A.nidulans* (XP_664792.1) and *N.crassa* (XP_964577.1). Were retrieved from the Entrez at NCBI website and aligned using the CLUSTAL W program. The sequence alignment has been edited to show only the residues corresponding to W110 in TMD1 and L282 in TMD5.

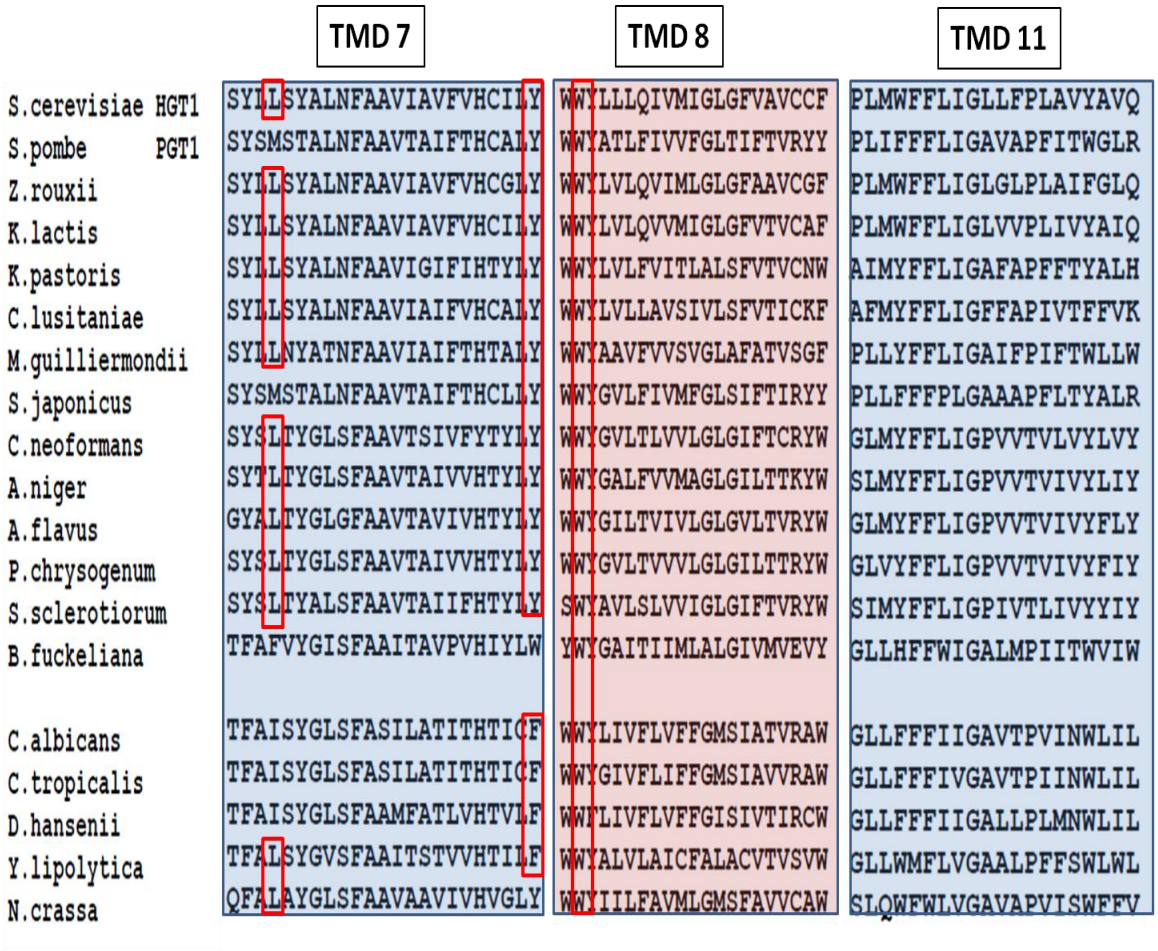


Figure 10 : Multiple sequence alignment for the protein sequences of the PT members of the OPT family. Sequences of *S.cerevisiae* Hgt1, *K.lactis* (XP_00265150.1), *P.guilliermondii* (XP_00148681.1), *S.pombe* Pgt1, *S.japonicus* (XP_002172910.1), *C.neoformans* (XP_772672.1), *A.niger* (XP_001397394.1), *A.Flavous* (XP_002381590.1), *P.chrysogenum* (XP_002567631.1), *S.sclerotiorum* (XP_001585829.1), *B.fuckeliana* (XP_001547017.1), *C. albicans* Opt1, *C tropicalis* (XP_002546105.1), *D.hansenii* (XP_458419.1), *Y.lopolytica* (XP_502145.1), *A.nidulans* (XP_664792.1) and *N.crassa* (XP_964577.1). Were retrieved from the Entrez at NCBI website and aligned using the CLUSTAL W program. The sequence alignment has been edited to show only the residues corresponding to L429 in TMD7, Y449 in TMD7 and W484 in TMD8.

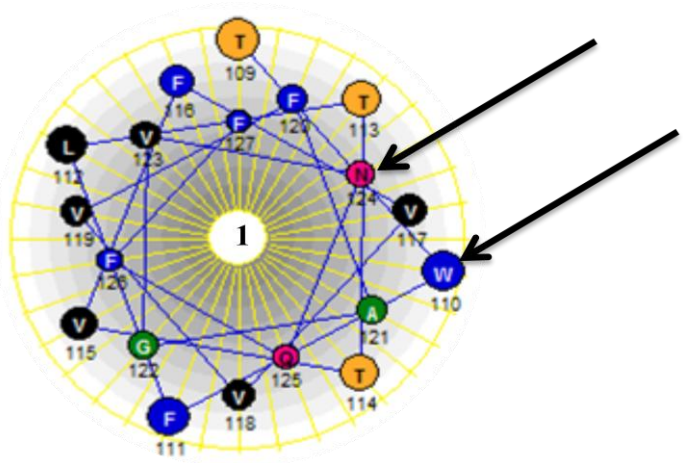


Figure 11: Helical wheel representation of the helix 1 of Hgt1p viewed from thee xoplasmic surface of the membrane. Amino acid representation by Single code . The arrow point to the residue where alanine substitution resulted in functionally severe defective. Helical wheel model of the transmembrane helix 1 of Hgt1p was constructed using the DNAstar

Software.

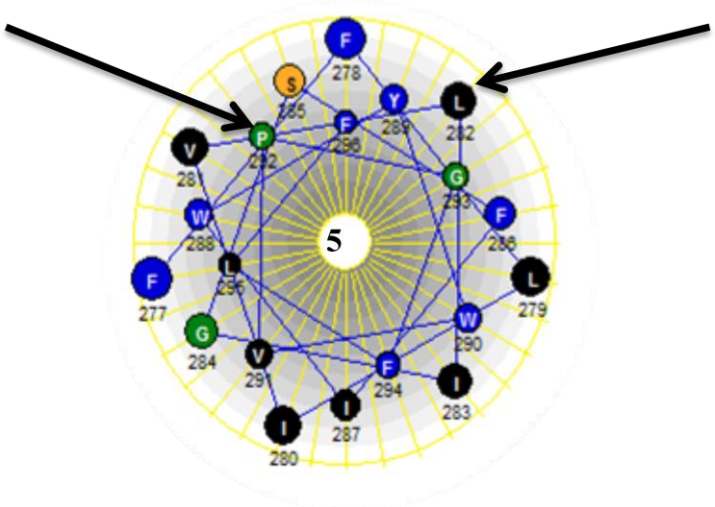


Figure 12: Helical wheel representation of the helix 5 of Hgt1p viewed from thee xoplasmic surface of the membrane. Amino acid representation by Single code . The arrow point to the residue where alanine substitution resulted in functionally severe defective. Helical wheel model of the transmembrane helix 5 of Hgt1p was constructed using the DNAstar
Software.

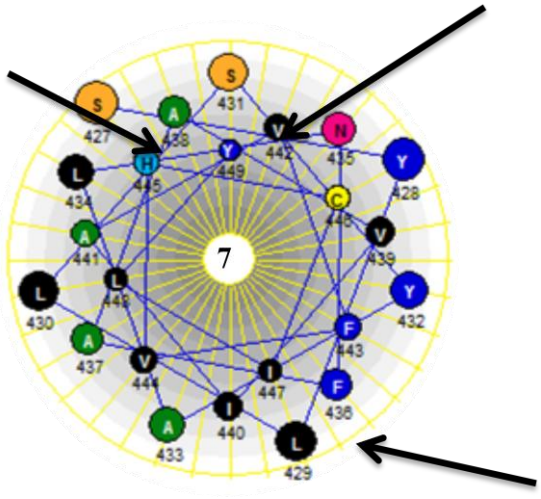


Figure 13: Helical wheel representation of the helix 7 of Hgt1p viewed from thee xoplasmic surface of the membrane. Amino acid representation by Single code. The arrow point to the residue where alanine substitution resulted in functionally severe defective. Helical wheel model of the transmembrane helix 7 of Hgt1p was constructed using the DNAstar
Software.

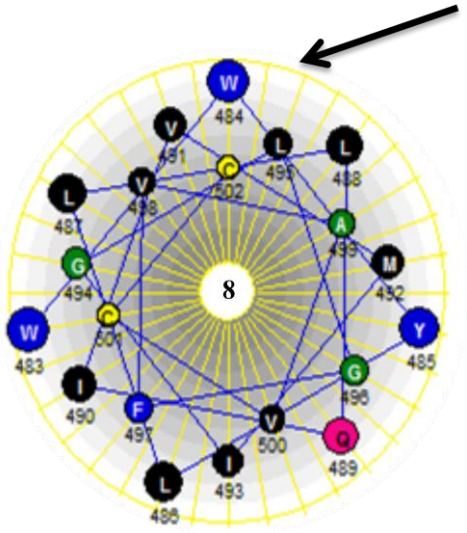


Figure 14: Helical wheel representation of the helix 8 of Hgt1p viewed from thee xoplasmic surface of the membrane. Amino acid representation by Single code. The arrow point to the residue where alanine substitution resulted in functionally severe

defective. Helical wheel model of the transmembrane helix 8 of Hgt1p was constructed using the DNAstar
Software.

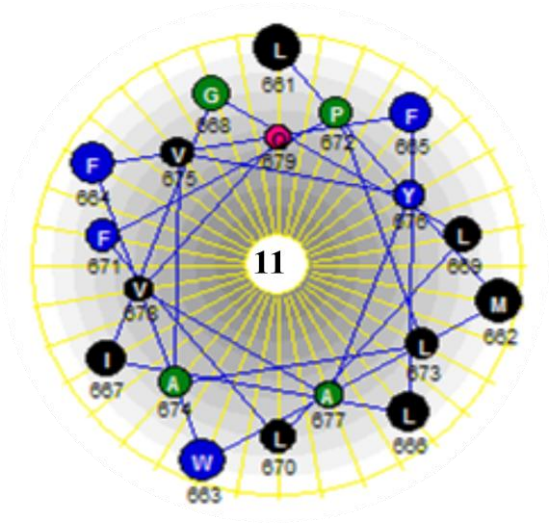


Figure 15: Helical wheel representation of the helix 11 of Hgt1p viewed from the exoplasmic surface of the membrane. Amino acid representation by single code. Helical wheel model of the transmembrane helix 11 of Hgt1p was constructed using the DNAstar Software.

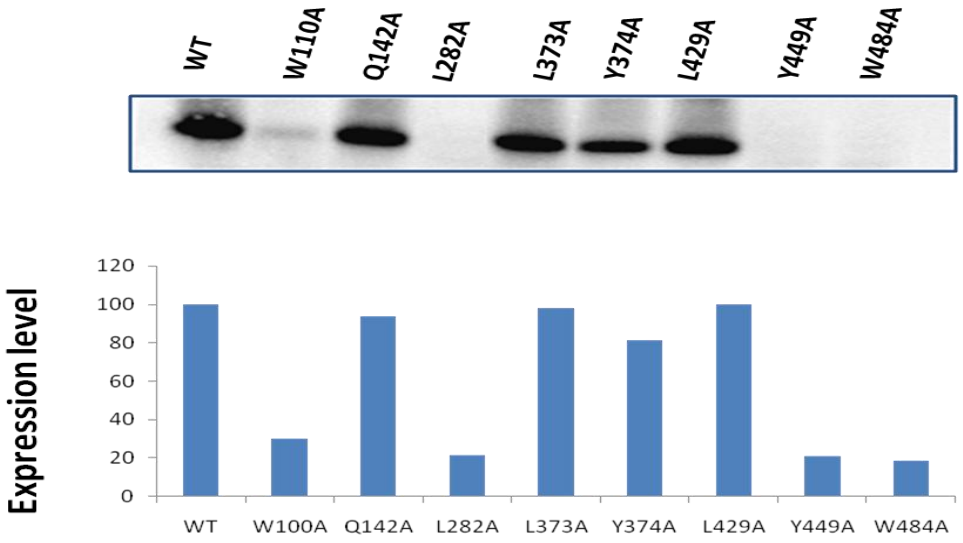


Figure 16: Quantification of the total protein expression levels of the different TMD1, 5, 7, and 8 mutants of Hgt1p. Extracts prepared from the *met15 Δhgt1Δ strain* transformed with plasmid bearing the alanine mutants of Hgt1p were subjected to western blot analysis. The total protein was quantified by densitometry analysis of protein bands. The data is expressed as percentage protein expression normalized to wild-type expression level. A representative blot is shown in the inset. Equal loading of proteins (20µg) in each well of the gel was also visually monitored by coomassie blue staining (data not shown).

3.5. Substrate specificity for Leucine–Enkephalin transport in *Saccharomyces cerevisiae.*

Hauser et al (JBC 2000) showed that Hgt1p expressed under a constitutive promoter could rescue the growth of leucine and methione auxotrophic yeast strains, when grown on the endogenous opioids Leu–Enk and Met-Enk, as the sole source of leucine and methione respectively, Km for enkephalin was higher (310µM) as compared to GSH (50µM).

Here we have checked if residues which are shown to be important for glutathione transport (Q222A, Q526A, and F523A) are also important for pentapeptide (Leu-Enkephalin) transport by Hgt1p (fig. 18) We found that the residues which are important for GSH transport are also required for Leu-enkpephalin utilization. This suggests that these residues participate in the transport of Leu-enkaphalin by Hgt1p.

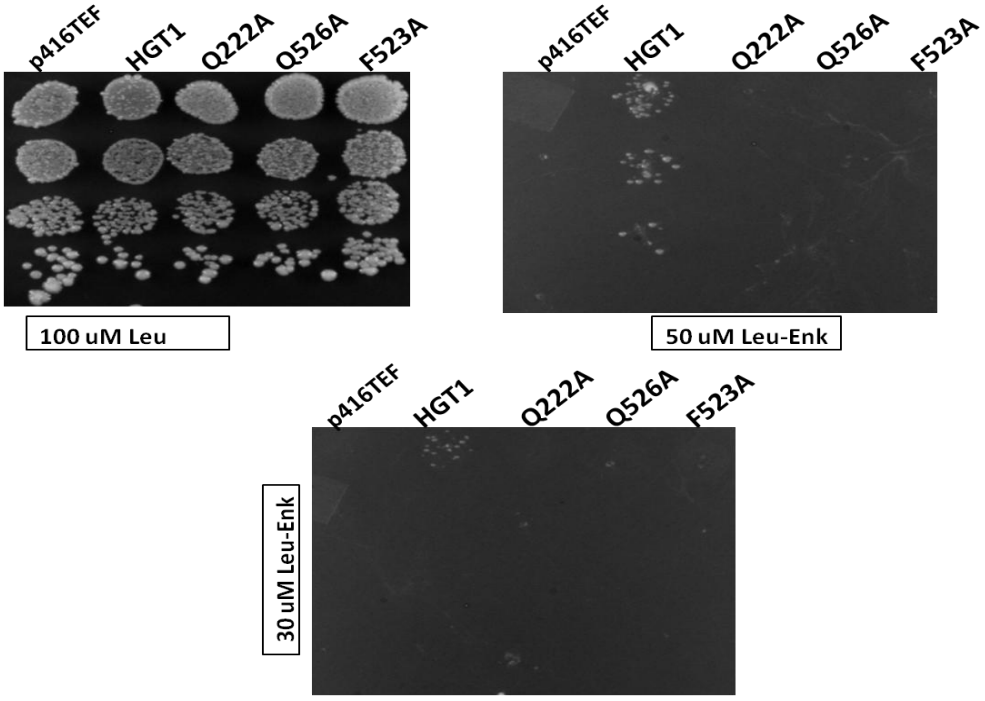


Figure 17: Functional evaluation of mutants defective in glutathione transport for growth on Leu-enkephalin. Vector (p416TEF) were transformed into strain ABC1481 (leucine auxotroph) and analyzed by for growth by dilution spotting on minimal media containing different concentration of Leu-Enk. Transformants were grown in minimal medium containing methionine, harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002. OD_{600} of cells. 10 µl of these dilutions were spotted on minimal medium containing different concentrations of Leu-Enk. The photograph was taken after 3 days of incubation at 30˚C.

3.6. Summary & Conclusions

Glutathione (*γ-L-glutamyl-L-cysteinyl-glycine*), is the most abundant thiol (-SH group) containing organic compound in the *Saccharomyce cerevisiae* (budding yeast), as well as many other organisms.

Hgt1p, the high affinity glutathione transporter in *Sacharomyces cerevisiae* is the first high affinity glutathione-specific transporter to be cloned and characterized for any system so far. It belongs to a novel family of transporter proteins the Oligopeptide Transporter family, which is restricted to plants, fungi and some remote homologues in bacteria. Till date no member of this family has been structurally characterized. In this study, efforts were made to carry out structure function study of Hgt1p using genetic and biochemical methods.

Alanine scanning mutagenesis of transmembrane domains (TMD1, 5, 7, 8, and 11) of Hgt1p revealed that in W110A in TMD1, L282A and P292A (P292A studied earlier) in TMD5, L429A and Y449A in TMD7 and W484A in TMD8 upon mutation to alanine led to significant reduction in the functional activity of Hgt1p. The exact reasons for this loss in activity are under investigation.

In addition to this, a study was also initiated to identify if residues which are shown to be important for glutathione transport are also important for Leu- Enkephalin transport by Hgt1p.

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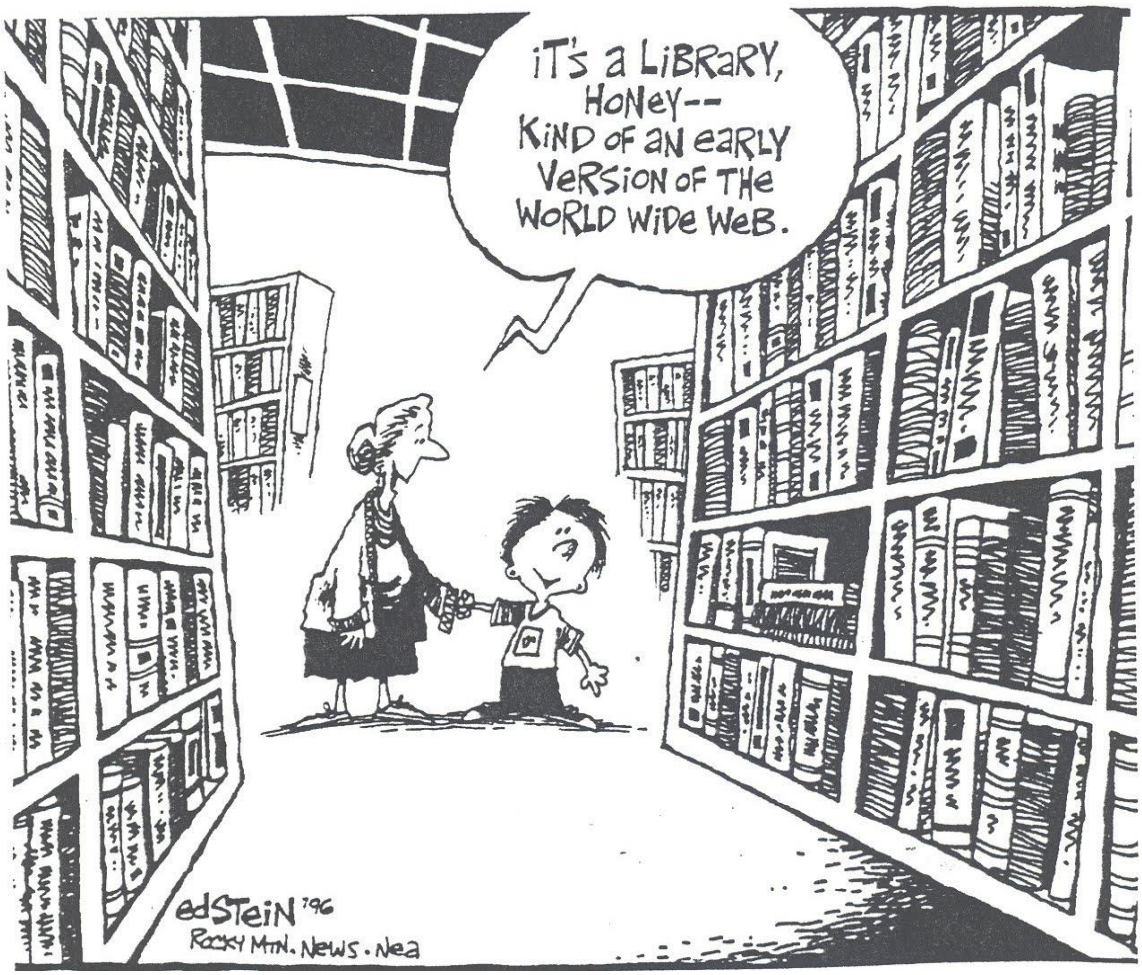

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