



DIPLOMARBEIT

Titel der Diplomarbeit

**Establishment of a high-throughput screen selecting for
thermostable mutants of an *Escherichia coli* dipeptide transporter**

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer.nat.)

Verfasserin / Verfasser: Elisabeth Karin Malle
Matrikel-Nummer: 0201340
Studienrichtung /Studienzweig Molekulare Biologie
(lt. Studienblatt):
Betreuerin / Betreuer: Peggy Stolt-Bergner, Ph.D.

Wien, am 12.02.2009

Table of Contents

Abstract	V
Zusammenfassung	VI
Acknowledgments	VII
List of Figures and Tables	IX
Abbreviations	XI
1. Introduction	1
1.1. Alternating access model of transport	2
1.2. The POT family of transport proteins	6
1.3. X-ray crystallography	8
1.4. Protein thermostability	10
1.5. Outline of the diploma project	12
2. Materials & Methods	13
2.1. Materials	13
2.1.1. General Media, Solutions, Reagents and Enzymes	13
2.1.1.1. Media	13
2.1.1.2. Chemical compounds	13

2.1.1.3. Detergents	14
2.1.1.4. Enzymes	14
2.1.1.5. Antibiotics	14
2.1.1.6. Buffers	15
2.1.2. Bacterial strains	16
2.1.3. Plasmids	17
2.2. Methods	18
2.2.1. Molecular cloning techniques	18
2.2.1.1. Agarose gel electrophoresis	18
2.2.1.2. DNA gel extraction and PCR purification	18
2.2.1.3. Restriction and ligation	18
2.2.1.4. Generation of competent <i>E. coli</i> cells	18
2.2.1.5. Transformation of competent <i>E. coli</i>	19
2.2.1.5. Random mutagenesis	19
2.2.1.7. GFP fusion cloning	23
2.2.2. In vivo assays	26
2.2.2.1. Growth Assay	26
2.2.2.2. In vivo transport assay	26
2.2.2.3. GFP-fluorescence assay	27
2.2.3. Protein expression	27
2.2.3.1. Small-scale high-throughput purification (HTP)	27
2.2.3.2.1. Expression	27
2.2.3.2.2. Purification	27
2.2.3.2. Large-scale purification	28
2.2.3.2.1. Expression	28
2.2.3.2.2. Membrane preparation	29
2.2.3.2.3. Purification	29
2.2.3.2.3.1. Solubilization	29
2.2.3.2.3.2. Immobilized metal affinity chromatography (IMAC)	29

2.2.3.2.3.2. Size exclusion chromatography	31
2.2.4. Protein Analysis	32
2.2.4.1. SDS-polyacrylamid gel electrophoresis (SDS-PAGE)	32
2.2.4.2. Coomassie Staining	33
2.2.4.3. Silver staining	33
2.2.4.4. Western blot	34
2.2.4.5. Determination of protein concentration	34
2.2.4.5.1. Absorbance 260/280 nm	34
2.2.4.5.2. Lowry	34
2.2.4.5.3. Fluoraldehyde assay (Pierce)	34
2.2.4.5.4. 660 nm assay (Pierce)	35
2.2.4.6. Stargazer Screen (Harbinger biotech)	35
3. Results	39
3.1. Optimization of the assays	40
3.1.1. Optimization of the growth assay	40
3.1.2. Optimization of the purification	43
3.2.1.1. Growth and expression	43
3.2.1.2. Lysis	44
3.2.1.3. Purification	48
3.1.3. Establishment and optimisation of the Stargazer thermostability assay	49
3.2. Results of the screen	50
3.2.1. Mutation frequencies	52
3.2.1.1. Library loop 6/7	52
3.2.1.2. Library helix 8/9	52
3.2.2. Growth assay	53
3.2.3. Thermostable mutant candidates	54
3.3. Optimization of the screen	56

3.3.1. Concentration dependence of the Stargazer assay	57
3.3.2. Determination of protein concentration	58
3.3.3. Decreasing the thermostability of the wildtype protein	59
3.3.4. HTP standard curve	60
3.4. Characterization of loss-of-function mutants	62
3.4.1. Localisation to the plasma membrane	62
3.4.1.1. Small-scale membrane preparation	62
3.4.1.2. GFP fluorescence	63
3.4.2. Transport assay	64
3.4.3. Thermostability of loss-of-function mutants	66
4. Discussion	69
5. References	73
Curriculum vitae	79

Abstract

All cells are separated from their environment by semipermeable membranes. This division demands the presence of systems with which cells can communicate with their surroundings. Integral membrane proteins carry out essential biological functions including signal transduction, metabolite and ion homeostasis and energy production. Secondary transporters are one kind of membrane protein that facilitate uptake of nutrients into a cell. Furthermore, they transport a vast number of drugs. Determining the structure of a biological macromolecule is a crucial step towards the understanding of its function and mode of operation. Very few X-ray structures of secondary transporters are available since crystallization of membrane proteins is a highly challenging endeavour. A novel approach to facilitating crystal growth is to enhance the thermal stability of a protein. Here, an easy and fast high-throughput screen was developed in order to identify thermostable variants of the *Escherichia coli* dipeptide transporter YdgR. In a first step, the screen distinguishes between active transporters and loss-of-function mutants. Second, the mutant transporter proteins are high-throughput purified, and their thermal stability is determined by static light scattering. Functional and structural studies on both the wildtype and mutant proteins should yield a more detailed insight into the mechanism of peptide transport. This promising strategy should be widely applicable to other proteins, and could be a very useful tool to boost the number of solved membrane protein structures.

Zusammenfassung

Jede lebende Zelle ist von einer semipermeablen Membran umgeben, die sie von ihrer Umwelt abgrenzt. Diese Abgrenzung verlangt das Vorhandensein von Systemen, durch die Zellen mit ihrer Umgebung kommunizieren können. Essentielle biologische Funktionen wie zum Beispiel Signaltransduktion, Metaboliten- und Ionenhomöostase und Energieproduktion werden von integralen Membranproteinen ausgeführt. Eine spezielle Art von Membranproteinen sind sogenannte sekundäre Transporter, die für die Aufnahme von Nährstoffen in die Zelle verantwortlich sind. Zusätzlich transportieren diese Proteine eine Vielzahl von Wirkstoffen. Ein wichtiger Schritt, um die Funktion und die Mechanismen eines biologischen Makromoleküls besser zu verstehen, ist die Aufklärung der Proteinstruktur. Da die Kristallisation von Membranproteinen sehr anspruchsvoll und mühsam ist, gibt es nur sehr wenige Röntgenstrukturen von sekundären Transportern. Eine neuartige Verfahrensweise, in der die thermische Stabilität eines Proteins erhöht wird, erleichtert die Kristallisation. In diesem Projekt wurde ein einfacher und schneller „high-throughput“ Screen entwickelt, um thermostabilere Mutanten des *Escherichia coli* Dipeptidtransporters YdgR zu identifizieren. Der Screen selbst besteht aus zwei Schritten. Der erste trennt funktionelle Transportermutanten von inaktiven Mutanten, im zweiten Schritt werden die mutierten Proteine aufgereinigt, und deren Stabilität wird mittels static light scattering (statische Lichtstreuung) bestimmt. Funktionale und strukturelle Studien an Wildtyp- und Mutantenproteinen sollen einen detaillierteren Einblick in den Mechanismus des Peptidtransports geben. Die hier entwickelte Strategie ist ein vielversprechendes Werkzeug, welches auch an anderen Proteinen anwendbar ist, und somit helfen könnte, die Anzahl an gelösten Membranproteinstrukturen zu erweitern.

Acknowledgments

This work would not be possible without the support and aid of many people.

I would like to thank ...

- my boss Peggy Stolt-Bergner for her help, patience, ideas, support and a great project, giving me the chance to work independently and being a great supervisor.
- my whole lab for being friends, for a great working atmosphere, the fun, a healthy share of craziness (long live the brain bug), valuable and other discussions, endless coffee/tea parties, delicious cakes, non-smoker smoking breaks and near-death experiences:
 - Bettina, the coffee-queen (now tea-queen, I guess). You are the best bench-neighbor ever.
 - Barbara, who is always in a good mood, cheering us all up.
 - Thomas, for helping me out with a lot of work, and for fitting in so well in our little henhouse.
 - Romana, for saving our honor in table-soccer and being an enrichment for our team. (I still want to hear a Karaoke song.)
 - Fritz, for already mentioned near-death experiences and the worst muscle ache in my whole life.
- the IMP/IMBA service departments, especially Gotthold and his team for their efforts and the analysis of many, many mutants.
- the media and washing kitchen people, who saved me a lot of exasperation and working time.
- Ron Yeung from Harbinger Biotech for helping me conquer the Stargazer.

- my former biology and chemistry teachers Peter Holub and Franz Scherr for awakening and supporting my interest in science.
- my study colleagues Bernhard, Barbara, Conny, Dany and Simone for being so supportive, sharing their ideas, help and notes and for worthwhile trips and sushi parties.
- my friends for a great time here in Vienna, for being patient and interested and accepting my massive lack of free-time during my studies.
 - Bernhard, again, for being not only a great study colleague but also a good friend and travel companion.
 - David & Corinna, for being good friends. I miss you.
 - Christof, thanks for pre-listening to many of my seminar talks (even if you didn't understand them), your friendship and support.
 - Mikael, for listening to my work talk and complaints, delicious dinners, countless massages after a long day of work, for being there for me and for (almost) never being grumpy, for your love and care.
- my awesome family, for your love, support (financial as well as mental), acknowledgment, and your trust in me.

And most of all, I want to thank my mother for believing in me and encouraging me to pursue my goals. Without you, my studies and this work would not be possible. I love you.

List of Figures and Tables

	page
Figure 1. The alternating access model of transport.	3
Figure 2. Conformational changes in MFS family members upon substrate uptake.	4
Figure 3. Overall structure of LacY.	5
Figure 4. Detergents.	9
Figure 5. Schematic representation of the pCS19 vector.	17
Figure 6. Principle of GeneMorph (Stratagene) random mutagenesis.	20
Figure 7. Targeted regions of YdgR.	21
Figure 8. The In-Fusion cloning protocol.	25
Figure 9. Coomassie stained SDS-gel of immobilized metal affinity chromatography (IMAC).	30
Figure 10. Gel filtration of IMAC purified YdgR.	31
Figure 11. The Stargazer.	36
Figure 12. The phosphonopeptide alafosfalin.	40
Figure 13. Growth assay with alafosfalin.	42
Figure 14. Expression of YdgR-GFP in different cell lines.	43
Figure 15. Optimization of lysis.	45
Figure 16. Gel filtration chromatograms of YdgR purified in FOS-12/DDM.	47
Figure 17. High-throughput purification of YdgR.	48
Figure 18. Aggregation temperature curves from a Stargazer experiment.	50
Figure 19. Scheme of the complete thermostability screening protocol.	51
Figure 20. Topological representation of YdgR from Helix 5 to Helix 10 with loss-of-function mutations.	54
Figure 21. Aggregation temperatures of purified thermostable candidates.	56
Figure 22. Concentration dependency of aggregation temperatures.	58
Figure 23. Stabilization and destabilization of YdgR.	58
Figure 24. High-throughput purification standard curve.	61
Figure 25. Membrane expression of L-O-F mutants.	63
Figure 26. GFP-fluorescence of membrane expressed L-O-F mutants.	64

Figure 27.	In-vivo transport assay.	66
Figure 28.	Aggregation temperatures of purified L-O-F mutants.	67
Figure 29.	Stabilizing effects of substrate addition.	68
Table 1.	Alignment of conserved motifs of the SLC15 peptide transporter family.	6
Table 2.	Bacterial strains with respective genotypes.	16
Table 3.	Primer pairs used in the first PCR reaction.	22
Table 4.	Mutagenic PCR reaction cycles.	22
Table 5.	Primer pairs for cloning of a C-terminal GFP-tag.	24
Table 6.	Reaction cycle of GFP cloning.	25
Table 7.	FOS-12/DDM detergent ratios and concentrations.	46
Table 8.	Optimization of the HTP expression and purification.	49
Table 9.	Increase of aggregation temperatures in different functional mutants.	55

List of abbreviations

%	percent
°C	degree Celsius
β-Ala-Lys-AMCA	N-7-amino-4-methylcoumarin-3-acetic acid labeled alanyl-lysine
β-AR	β-1 adrenergic receptor
β-D-OG	β-D-octylglucoside
β-ME	β-mercaptoethanol
μg	microgram
μl	microliter
3'	three prime end of a nucleic acid
5'	five prime end of a nucleic acid
<i>A. aeolicus</i>	<i>Aquifex aeolicus</i>
A-A	H-Ala-Ala-OH (alanyl-alanine)
ABC transporter	ATP binding cassette transporter
ACE inhibitor	angiotensin-converting enzyme inhibitor
AgNO ₃	silver nitrate
alafos	alafosfalin, L-alanyl-L-1-aminoethylphosphonic acid
AMCA	N-7-amino-4-methylcoumarin-3-acetic acid
APS	ammoniumperoxodisulfate
ATP	adenosine triphosphate
BSA	bovine serum albumin
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CaCl ₂ *2 H ₂ O	calcium chloride dihydrate
C _i	inward-facing conformation
Cl ⁻	chloride anion
cmc	critical micelle concentration
C _o	outward-facing conformation

C-terminus	carboxy-terminus
Cu^+	monovalent copper ion
Cu^{2+}	divalent copper ion
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DDM	n-dodecyl- β -D-maltoside
dGTP	deoxyguanosine triphosphate
dH ₂ O	deionized water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotides (mixture of dATP, dTTP, dGTP, dCTP)
DppA	dipeptide permease from <i>S. typhimurium</i>
DSC	differential scanning calorimetry
DtpA	dipeptide and tripeptide permease A, alternative name for YdgR
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EmrD	multidrug efflux pump from <i>E. coli</i>
FOS-12	n-dodecylphosphocholine, foscholine-12
g	earth's gravitational force
G3P	glycerol-3-phosphate
GFP	green fluorescent protein
GlpT	glycerol-3-phosphate transporter from <i>E. coli</i>
GPCR	G-protein coupled receptor
H^+	hydrogen, proton
<i>H. sapiens</i>	<i>Homo sapiens</i>
HCl	hydrochloric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
His-tag	histidine tag

HTP	high-throughput
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl- β -D-1-thiogalactopyranoside
KAc	potassium acetate
kbar	kilobar, unit of pressure
KCl	potassium chloride
kDa	kilo Dalton, unit of mass
L	liter
<i>L. lactis</i>	<i>Lactobacillus lactis</i>
LacY	lactose permease from <i>E. coli</i>
LB	Luria-Bertani medium
LED	light emitting diode
LeuT	leucine transporter, <i>Aquifex aeolicus</i>
L-O-F	loss of function mutant
mA	milliAmpere, unit of electric current
MCS	multiple cloning site
MFS	major facilitator superfamily
mg	milligram
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulfate
Mhp1	benzyl-hydantoin transporter from <i>M. liquefaciens</i>
ml	milliliter
<i>M. liquefaciens</i>	<i>Microbacterium liquefaciens</i>
MnCl ₂ *4 H ₂ O	manganese chloride tetrahydrate
MWCO	molecular weight cut-off
Na ⁺	sodium ion
Na ₂ O ₃ S ₂	sodium thiosulfate
NaCl	sodium chloride
NCS-1	nucleobase cation symport-1
ng	nanogram
Ni ²⁺	divalent nickel ion

Ni-beads	nickel ions immobilized on agarose or sepharose beads
Ni-NTA	nickel nitriloacetic acid, nickel ions immobilized on agarose or sepharose
nm	nanometer, unit of wavelength
NSS	neurotransmitter sodium symporter
N-terminus	amino terminus
OD ₆₀₀	optical density, absorbance at 600 nm
OPA	o-phthalaldehyde
OppA	oligopeptide permease from <i>S. typhimurium</i>
PEPTs	mammalian peptide transporters
PCR	polymerase chain reaction
P _i	inorganic phosphate
PIPES	piperazine-N,N'-bis-2-ethanesulfonic acid
POT	proton oligopeptide transporters
PTR	peptide transporter
PVDF	polyvinylidene difluoride
rpm	rounds per minute
<i>S. cerevisiae</i>	<i>Sacharomyces cerevisiae</i>
SDS	sodium dodecyl sulfate
SDS-PAA	SDS-polyacrylamide
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SLS	static light scattering
SOB	Super-Optimal broth
SOC	Super-Optimal catabolite repression
SSS	solute sodium symporter
<i>S. typhimurium</i>	<i>Salmonella typhimurium</i>
TAE	Tris/acetate/EDTA buffer
TB	Western blot transfer buffer
TBS	tris buffer saline
TBS-T	tris buffer saline/Tween
TCA	trichloroacetic acid
TDG	β-D-galactopyranosyl-1-thio-β-D-galactopyranoside

TEMED	N,N,N',N'-Tetramethylethylenediamine
TMD	transmembrane domain
tppB	tripeptide permease, <i>S. typhimurium</i>
V	Volts, unit of voltage
<i>V. parahaemolyticus</i>	<i>Vibrio parahaemolyticus</i>
v/v	volume to volume, (volume of solute (ml) / volume of solution (ml)) x 100)
vSGLT	sodium glucose/galactose symporter from <i>V. parahaemolyticus</i>
w/v	weight per volume, (mass of solute (g) / volume of solution (ml)) x 100)
WT	wildtype
YdgR	tppB from <i>Escherichia coli</i> , meaning of abbreviation not known

1. Introduction

A cell is surrounded by a semipermeable membrane, which separates it from the environment. Furthermore, in eukaryotic cells membranes also divide a cell into different compartments. Each compartment serves various different purposes. This compartmentalization has a number of advantages, for example, the enrichment of certain compounds such as enzymes or ions, or the partitioning of metabolic reactions. However, dividing a cell into different compartments demands the presence of a system that can shuttle compounds such as substrates, metabolites or catalysts from one compartment to another. Small hydrophobic molecules can penetrate the membrane but hydrophilic compounds must be transported. For this purpose, cells have evolved membrane channels and transport proteins. Both types are integral membrane proteins that allow for the passage of different molecules. The difference between these types of proteins is basically the mechanism of transport. Channels can either be constantly open or shuttle between an open and a closed state. The transport itself, however, is passive. The transported substrate diffuses across the membrane down its concentration gradient. In contrast to channel proteins, transporters translocate a substrate actively. This means that compounds can be transported against their concentration gradient at a certain energy expense. Transporters are divided into primary and secondary active transporters and a smaller third group, according to their mode of transport. Primary transporters such as P-type ATPases and ATP-binding cassette (ABC) transporters use energy generated by ATP hydrolysis to translocate their substrate. Secondary transporters, however, couple the translocation of their substrates to the movement of other molecules down their concentration gradient. This movement is energetically favourable and hence drives the active transport. A third class of transporters couple the transport to an energetically favourable chemical modification of the respective substrate (Law et al., 2008, Locher et al.2003). Transporters can either function as uniporters, symporters or antiporters. Uniporters transport one substrate using the energy stored in the substrate gradient. Symporters and antiporters translocate two or more substrates across the membrane at the same time; the latter transports two substrates in opposite directions. Both are energized by the electrochemical

gradient. The lactose permease LacY is an example of a symporter. A lactose molecule is imported with the concomitant transport of a proton. In contrast, GlpT, the glycerol-3-phosphate transporter, is an antiporter. Upon substrate import, inorganic phosphate is exported (Fig. 1A).

1.1. Alternating access model of transport

The largest group among secondary transporters is the major facilitator superfamily (MFS). This family has a vast variety of substrates, ranging from peptides, nucleotides and sugars to ions. Most of the MFS family members are predicted to have twelve transmembrane helices, and are thought to function as monomers. The N- and C-termini are located on the cytoplasmic side. There are a few exceptions, where family members have 14, 24 or 6 membrane spanning domains. The latter most likely functions as a homodimer (Wang et al., 2008). Most transporter structures are of MFS family members or members of closely related families such as the benzyl-hydantoin transporter Mhp1 (Weyand et al., 2008), which belongs to the nucleobase cation symport-1 family (NCS-1), or the sodium glucose/galactose transporter vSGLT (Faham et al., 2008), a family member of the solute sodium symporters (SSS) or the leucine transporter LeuT (Yamashita et al., 2005, Singh et al. 2008), a member of the neurotransmitter sodium symporters (NSS).

A general model describing the mode of action of secondary transporters is the alternating access model (Widdas, 1952, Jardetzky, 1966). The transporter undergoes conformational changes resulting in alternating access to the ligand binding site (Fig.1A). The protein is open to one side of the environment at a time, for example, either to the extracellular or to the intracellular space. The model is supported by all secondary transporter structures solved to date. However, transport proteins can not only occupy an inward-facing (Ci) and an outward facing (Co) conformation, but also an occluded state where the substrate binding site is not accessible from either side (Yamashita et al., 2005, Yin et al., 2006).

There are no structures of secondary transporters from mammals so far since high expression levels are extremely difficult to reach. It is thought that alternating access can be accomplished by two different mechanisms, referred to as the rocker-switch and the

alternating gate. In contrast to the rocker-switch model, the alternating gate or gated pore is a more channel-like protein that has a gate on each side, which open in an alternating mode. A transporter working with a rocker-switch mechanism moves both halves of the protein (Fig. 1B), and undergoes a more extensive conformational change than its counterpart (Karpowich et al., 2008).

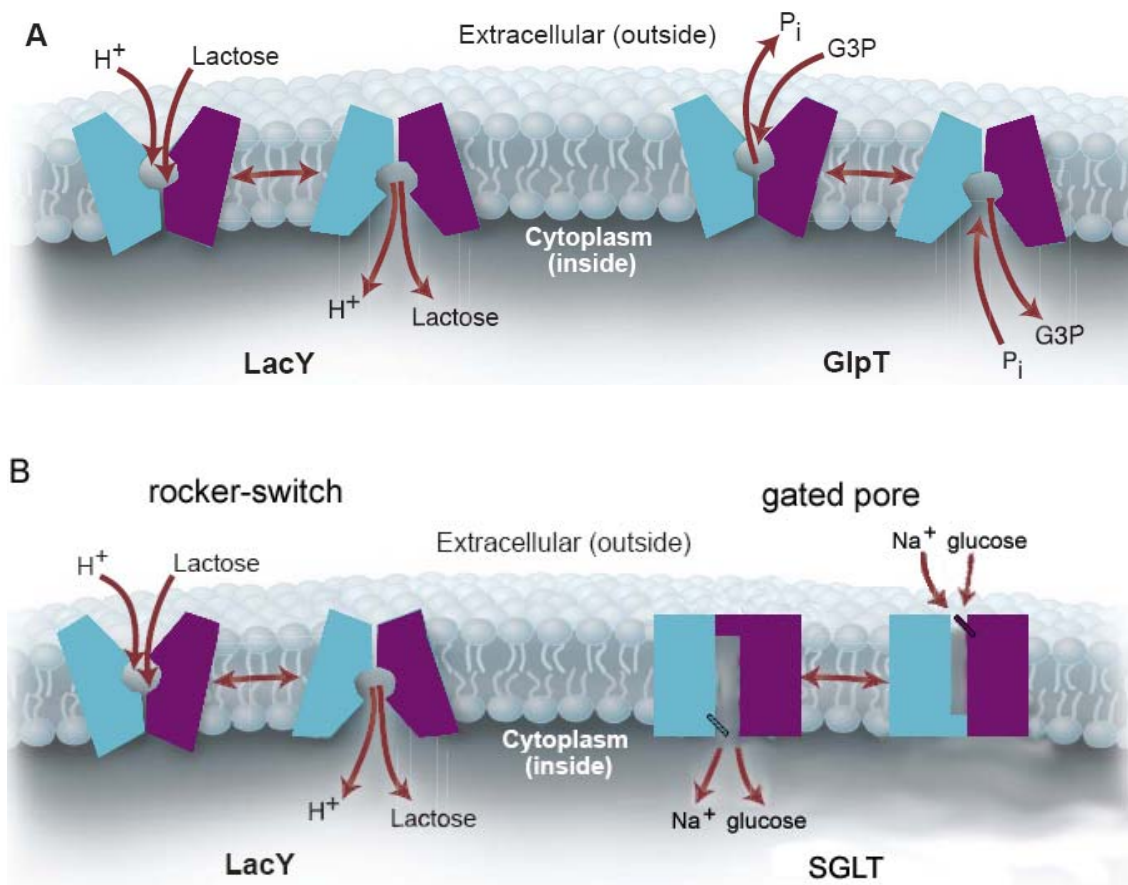


Figure 1. The alternating access model of transport.¹ (A) The lactose permease LacY is a symporter dependent on the proton gradient. The glycerol-3-phosphate transporter GlpT works in an antiport mechanism. Upon glycerol-3-phosphate uptake, inorganic phosphate (P_i) is exported. The transporters are accessible only from one side of the membrane at a time. They undergo a conformational change that is thought to translocate the substrate to the other side. (B) The alternating access model allows two mechanistic possibilities: the rocker-switch and the gated pore. LacY and GlpT (A) work in a rocker switch mode. The two halves of the protein undergo a conformational change, which makes the substrate binding site either accessible to the cytoplasmic or the extracellular side. In the gated pore or alternating gate, the quasi channel-like protein possesses a gate at each side of the pore. Through a small conformational change, these gates alternately open and close. According to Karpowich et al., vSGLT, the galactose transporter, is an example of an alternating gate.

¹ Figure adapted from Locher et al., 2003

Others disagree with this strict separation. The idea is rather that all MFS family members function in the same way as indicated by all structures solved so far. A small gate, i.e. a single amino acid or short part of a helix, serving as a primary gating element, excludes the substrate from the environment. In a second step that involves larger conformational rearrangement of transmembrane domains (TMDs), the substrate is translocated across the membrane, leading to inward- or outward facing cavities (Diallinas, 2008) (Fig. 2).

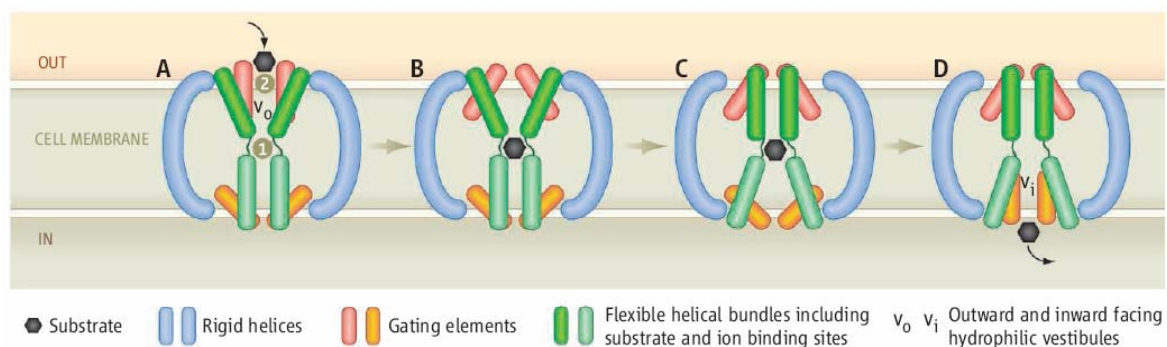


Figure 2. Conformational changes in MFS family members upon substrate uptake.² Upon substrate (black hexagon) binding, extracellular gating elements (red) exclude it from the environment (A, B). A larger conformational movement of helices (B to C, green and turquoise) switches the transporter from an outward- to an inward-facing conformation, where the substrate can be finally released (opening of the intracellular gate (yellow)).

Most secondary transporter structures have been solved in an inward-facing conformation, for example the structures of the glucose/galactose transporter from *Vibrio parahaemolyticus*, vSGLT (Faham et al., 2008) or the *Escherichia coli* glycerol-3-phosphate transporter GlpT (Huang et al., 2003) and lactose permease LacY (Abramson et al., 2003). One transporter crystallized not only in an outward-facing conformation but also in the substrate-bound occluded state is the benzyl-hydantoin transporter Mhp1 from *Microbacterium liquefaciens* (Weyand et al., 2008). Although the allocation of the diverse helices is different in the respective transporters, they all display a similar architecture. Using LacY as an example, they are comprised of two parallel repeats (Mhp1 and vSGLT, for example, contain inverted repeats instead) and intra- or extracellular cavities as shown in Figure 3A, through which the substrate can access the binding site. Furthermore, they possess kinked or broken helices, which are located at the central part of the transporter

² Figure taken from Diallinas, 2008

close to the substrate binding site. Because of these similarities, structures representing most steps in the transport cycle are now available.

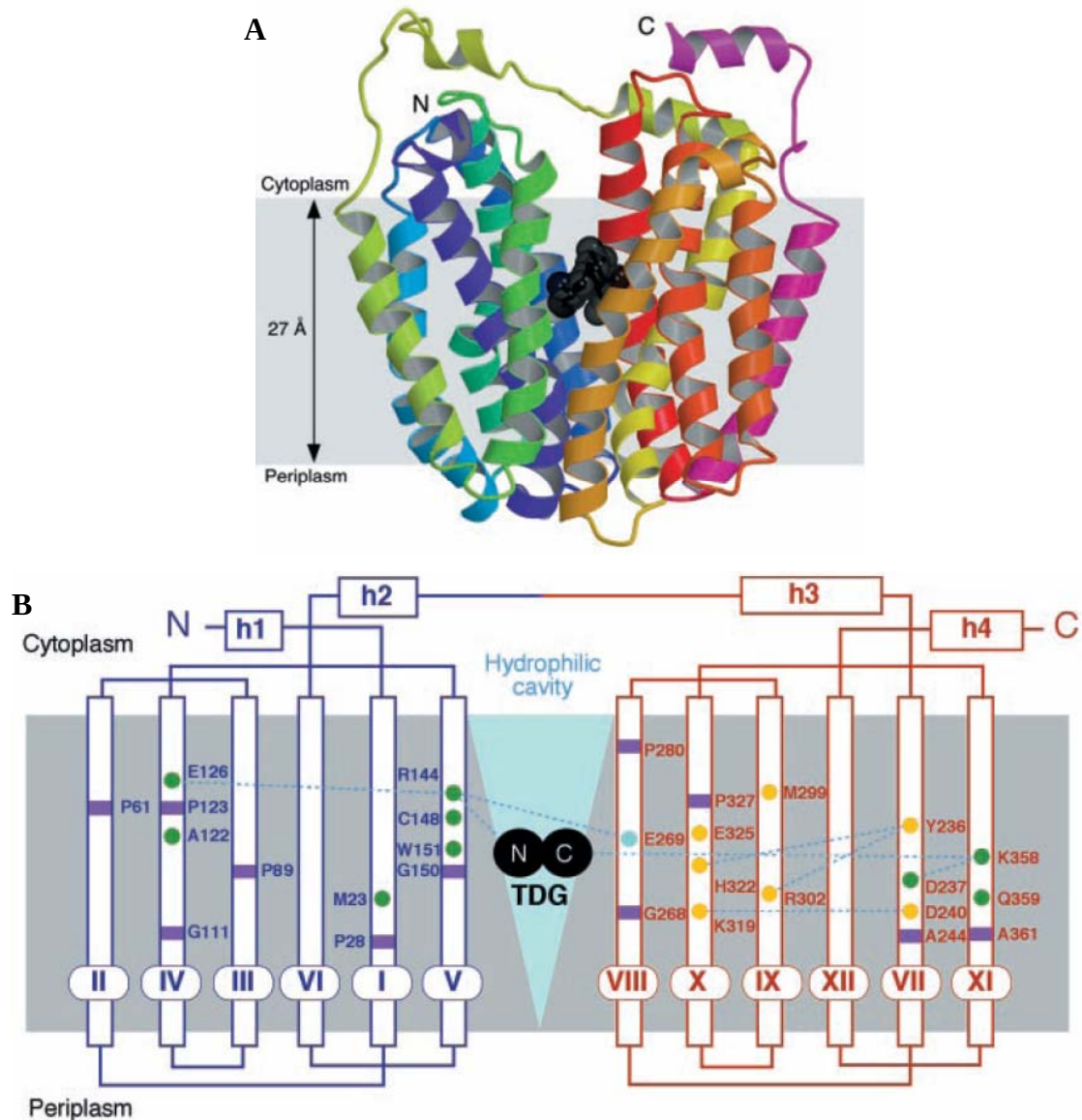


Figure 3. Overall structure of LacY.³ The figures are based on the C154G mutant structure with a bound substrate homolog, TDG (β -D-galactopyranosyl-1-thio- β -D-galactopyranoside). **(A)** Ribbon representation of LacY viewed parallel to the membrane. The 12 transmembrane helices are colored from the N-terminus in purple to the C-terminus in pink; TDG is represented by black spheres. **(B)** Secondary structure schematic of LacY. The N- and C-terminal domains of the transporter are colored blue and red, respectively. Residues at the kinks in the transmembrane helices are marked with purple rectangles; residues marked with green and yellow circles are involved in substrate binding and proton translocation, respectively; residue Glu269, represented by a light blue circle, is involved in both substrate binding and proton transfer. The hydrophilic cavity is designated by a light blue triangle, and TDG is shown as two black circles; h1 to h4 denote surface helices.

³ Figure plus legend taken from Abramson et al., 2003

1.2. The POT family of transport proteins

As already mentioned, cells need to take up nutrients from their environment in order to survive. Amino acids, for example, are taken up by a class of peptide transporters, the proton oligopeptide transporters (POT) (Paulsen et al. 1994), in the form of short peptides (Daniel et al., 2006). These transporters are conserved among almost all species, from humans to bacteria. Comparing family members throughout species reveals three strikingly conserved regions. The first of these so-called PTR motifs (Peptide Transporters) on transmembrane domain 1 is followed by a second motif comprising parts of TMD 2 and 3 plus their interjacent loop. The third motif, in helix 5, the FYING-PTR motif, is the most conserved among all species (Table 1).

Table 1. Alignment of conserved motifs of the SLC15 peptide transporter family.

	Motif 1	PTR 2-1 motif	FYING-PTR motif
	*EF*ERF*YYG*	G***AD***GK**TI***S**Y**G	*FS*FY*AIN*GSL*
<i>H.sapiens</i> PEPT	22NEFCERFSYYGM	69GALIADSWLGKFKTIVSLSIVYTTG	162FFSIFYLAINAGSLL
<i>C. elegans</i> PEP1	55NEFCERFSYYGM	102GSIVADGYIGKFWTIFSVSILYAIG	184FFSMFYFAINAGSLI
<i>S.cerevisiae</i> Ptr2	88VELSERFSYYGL	44GGYVADTFWGKYNTICCGTAIYIAG	143VFMIFYLAINVGSLS
<i>L. lactis</i> DtpT	24TEFWERFSYYGM	78GGWVADRLLGASRTIFLGGILITLG	152GFNIFYLAINMGSLI
<i>E. coli</i> YdgR	22IELWERFGYYGM	77GGWLGDKVLGTKRVIMLGAIVLAIG	151AFSIFYLAVNIGSFF

Humans possess four members of this family, two of which are well characterized, the peptide transporters PEPT1 (SLC15A1), and PEPT2 (SLC15A2). PEPT1, the low-affinity, high-capacity transporter, is mainly expressed in brush border membranes of the small intestines. PEPT2, which has a high affinity but a low capacity, is expressed in the lung, and both are expressed as a renal isoform (Weitz et al., 2007). Although Na⁺ driven transport processes are relatively more frequent in mammals, these peptide transporters use the proton gradient as a driving force, and are therefore also called “archaic” transporters (Daniel et al., 2006). PEPT1 and PEPT2 possess a large number of possible substrates, which include essentially 400 or 8000 possible dipeptides or tripeptides, respectively. Furthermore, they transport β -lactam antibiotics, which represent a broad class of penicillin derivatives, and also other therapeutic drugs such as angiotensin-converting enzyme

inhibitors (ACE inhibitors). More interesting, the substrate does not require a peptide bond for recognition by the mammalian PEPTs (Doring et al., 1998). There is no crystal structure available, but topological studies suggest 12 transmembrane domains. (Covitz et al., 1998). *Salmonella typhimurium* harbours three different oligopeptide transport systems, the oligopeptide permease OppA and the dipeptide permease DppA, which are ABC transporters. The third one is a tripeptide permease system, which members belong to the described POT family. The only characterized representative is called tppB (tripeptide permease B). The respective homologue identified in *Escherichia coli* is called YdgR (or sometimes also *E. coli* tppB or DtpA). YdgR was described as a dipeptide and tripeptide transporter, which could also transport various other substrates (Weitz et al., 2007). However, there is also no structural data available. It is not determined yet whether the *E. coli* protein also has twelve membrane spanning domains as its human homologue, or fourteen as suggested by prediction programs. The family contains three other members homologous to the *S. typhimurium* proteins. YhiP is a di- and tripeptide transporter as well (Harder et al., 2008), and YbgH and YjdL are two poorly characterized proteins. YdgR was identified as a target regulated by the EnvZ/OmpR two-component regulatory system (Goh et al., 2004). EnvZ is an integral membrane protein kinase, which phosphorylates its response regulator OmpR upon environmental stress. Similar to its family members, transport activity of YdgR was demonstrated to be proton-dependent (Weitz et al., 2007). Substrate specificity assays showed that the transporter prefers di- and tripeptides in their L-stereomeric form. Also, YdgR transports peptidomimetics such as β -lactam antibiotics and ACE inhibitors.

It would be very important to understand the structural features and exact mechanisms of transport of this family, since a large number of drugs and antibiotics are transported by this family. Furthermore, certain drugs can be coupled to amino acids and thereby become a substrate for these transporters. The working effect of a drug is often diminished because of its low bioavailability, i.e. that it cannot be taken up into cells. A structure of a POT would be useful because a drug could then be designed in a way so that it is taken up by a certain transporter or in a certain tissue where it is needed.

1.3. X-ray crystallography

Determining the structure of a biological macromolecule is a very important step towards understanding its function and mode of operation. One method of doing so is structure determination by X-ray crystallography. Not only inorganic materials such as salts or metals can form crystals but also organic macromolecules. Highly purified protein forms crystals under certain conditions. These crystals are then subjected to X-rays, and the scattered electrons produce a three-dimensional electron density map, from which the positions of the respective atoms, amino acids and their side chains, can be determined. In contrast to other structure determination methods such as electron microscopy, for example, X-ray structures with a very high resolution can be obtained. Crystallization itself, however, is a challenging task. Protein contacts in the crystal are influenced by pH, ionic strength and the presence or absence of ions in the solution. In principle, the optimal crystal growing conditions have to be found empirically.

Crystallizing an integral membrane protein is even more demanding. Therefore, structural information is relatively sparse, although approximately 20 to 30 % of most genomes encode for membrane proteins. First, the respective protein has to be produced and purified in milligram amounts. This can often be very difficult because overexpression in the membrane tends to be toxic for the cells, or the expression leads to formation of aggregates or inclusion bodies. It is possible to solubilize these aggregates using high concentrations of chaotropes, such as guanidinium chloride or urea, but also SDS or alkaline pH. However, these solubilized proteins are often inactive. A second major problem arises when trying to crystallize a membrane protein. Integral membrane proteins are surrounded by lipids in the lipid bilayer, and are generally very hydrophobic. When a membrane protein is purified from its native environment, the hydrophobic surfaces tend to cluster to protect themselves from a hydrophilic environment, and the protein aggregates. To overcome this problem, these proteins have to be purified either in an organic solvent or solubilized in an aqueous solvent containing detergent. The latter is done more frequently because organic solvents often render a protein inactive. A detergent is an amphiphilic molecule consisting of a hydrophobic tail group and an amphiphilic head (Fig. 4B, left), which forms a micelle above a certain concentration, also called the critical micelle concentration (cmc). When a hydrophobic protein is present, detergent molecules adsorb

around the hydrophobic transmembrane helices, thereby replacing the lipid bilayer (Fig. 4B, right). There are hundreds of different detergents with various combinations of head and tail groups, and they differ greatly in their structures and solubilizing power. Three widely used examples are depicted in Figure 4A, sodium dodecyl sulfate (SDS), octoxynol-9 (Triton X-100) and β -D-octylglucoside (β -D-OG). SDS is a rather aggressive detergent, whereas β -D-OG is milder, and frequently used for crystallization.

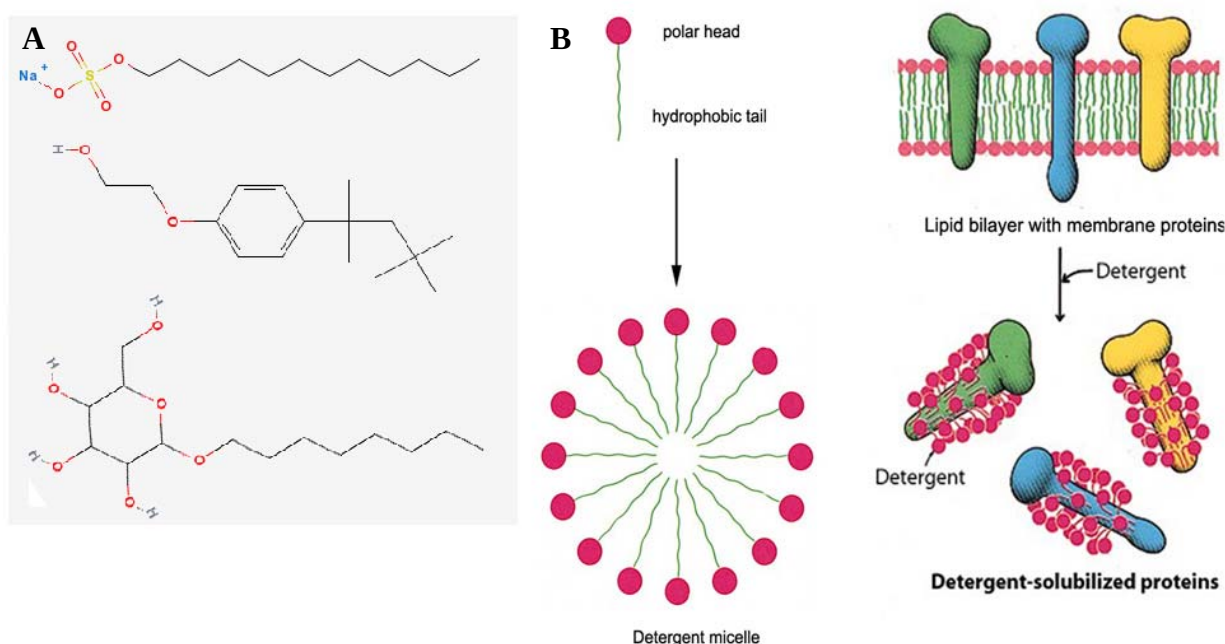


Figure 4. Detergents. (A)⁴ Structural representation of three common detergents. The uppermost compound is one of the most widely used detergents, sodium dodecyl sulfate (SDS). Triton X-100, also called octoxynol-9 (middle) and β -D-octylglucoside (undermost) are milder detergents. (B)⁵ Schematic representation of a detergent molecule with its polar head group (pink) and a hydrophobic tail (green) forming a micelle (left panel). Membrane proteins are embedded in the lipid bilayer. Upon addition of detergent, they are present in a solubilized form (right panel).

The lipid bilayer surrounding an integral membrane protein is usually quite complex in its composition, depending on the organism as well as the compartment. Replacing it by a detergent is therefore only a crude substitution, and can disturb intra-protein interactions or

⁴ Figure taken from <http://pubchem.ncbi.nlm.nih.gov/>

⁵ Figure adapted from Stryer, L. Biochemistry. W H Freeman

render a protein inactive. So far it is not possible to predict the optimal detergent for a respective protein, but rather it has to be determined empirically. However, having identified an optimal detergent for purification does not mean that it is also ideal for other purposes. Certain detergents solubilize a protein very well, but render it unstable. Others are relatively mild detergents, and stabilize the protein, but seem to disturb crystallization. More aggressive, shorter chain length detergents are better for crystallization, but can also damage protein integrity.

Until now (until January 2009), approximately 10 crystal structures of secondary transporters have been solved. They have provided many valuable insights into the mechanisms of transport. They all support the alternating access model of transport. As already mentioned, some of them work as a rocker-switch and some rather as an alternating gate. However, many questions still remain unanswered. Furthermore, in order to understand the exact mechanism of transport, modelling the protein based on one of the available structures is simply often not enough to yield satisfying results since it is only a model based on a different protein. Therefore, a method to enhance crystallization of membrane proteins would be gladly welcomed.

1.4. Protein thermostability

As already mentioned, protein crystallization is screened in various buffers, detergents and additives in order to yield ordered, highly diffracting crystals. Often, a vast number of proteins from different organisms are tested for high expression levels and enhanced stability. An observation made over the years, is that the more thermostable a protein is the more readily it crystallizes. Therefore, homologues from thermophiles are often good a choice.

A different approach is to make a protein more stable by mutagenesis. Engineering of proteins can, of course, have other purposes than enhancing thermostability, for example altering the specificity of an enzyme, boosting the enzymatic activity or enhancing overexpression (Martinez Molina et al., 2008). There are certain criteria that make a protein more thermostable. These features were found when comparing proteins from mesophilic and thermophilic organisms. In order to maintain protein integrity at higher

temperatures, thermophiles need to possess proteins with higher stability. These strategies include ionic interactions, hydrophobic interactions and π stacking, covalent bonds in disulfide bridges or post-translational modifications, such as lysyl-methylations. Also, a high ratio of charged to uncharged amino acids, especially on the protein surface, contributes to thermal stability, probably due to an increase in ionic interactions. Thermophiles often possess a higher CG content in their coding sequences, which favours the presence of certain amino acids. Other amino acids such as methionine or asparagine are less abundant because they are not stable at higher temperatures. (Trivedi et al., 2006). However, these strategies cannot be generalized. Thermophiles and hyperthermophiles use different strategies and combinations of these, rendering their proteins more thermostable. It is hence not yet possible to predict the thermostability of a protein, or add certain features to increase it. Also, it seems that many attributes accounting for thermostability in soluble proteins do not necessarily translate to membrane proteins. A general characteristic of integral membrane proteins is their modest stability, which can lead to rapid unfolding and inactivation. It was calculated that approximately 10 % of random mutations improved the stability of a membrane protein (Bowie, 2001; Zhou et al., 2000). This might not be true for all membrane proteins, but when comparing this to soluble proteins, a much lower percentage of mutations increased stability. Thus, it seems that possible stabilizing interactions in membrane proteins have not been selected for by evolution. Being optimized to its lipid environment, lower stability and therefore higher flexibility might be necessary for optimal protein function, especially in transporters, which have to undergo large conformational changes across the membrane.

Some of the known membrane protein crystal structures are of proteins from thermophiles. The Na^+/Cl^- dependent leucine transporter, LeuT, for example, was purified and crystallized from the thermophile *Aquifex aeolicus* (Yamashita et al., 2005, Singh et al. 2008). Recently, the structure of the $\beta 1$ -adrenergic receptor (Serrano-Vega et al., 2008) has been solved using mutagenesis as a tool for conformational thermostabilization. This G-protein coupled receptor (GPCR) was subjected to alanine-scanning mutagenesis. This method is simple but also labour-intensive. Every residue in the protein is individually mutated to an alanine. Six combined point mutations in the $\beta 1$ -adrenergic receptor (β -AR) enhanced the melting temperature by 21°C. The same approach was applied to another GPCR, the human adenosine A_{2a} receptor. There is no crystal structure of this mutant yet,

but different mutations increased the thermal stability by 17°C in the antagonist bound form and 9°C in the agonist bound form, respectively (Magnani et al., 2008).

Similarly, the structure of the lactose permease LacY was solved using a thermostable cysteine mutant for crystallization (Abramson et al., 2003). The reason why enhanced thermostability facilitates crystallization is not really known. A fact, however, is that transporters are quite dynamic and flexible proteins. This might be disadvantageous for crystal formation since a crystal is a highly ordered structure. It is thought that certain mutations arrest the transporter in one conformation of the transport cycle, thereby prohibiting movement and leading to a more rigid structure and decreased flexibility. Therefore, the protein might be more thermostable. Indeed, it was demonstrated that the LacY mutant is locked in an inward-facing conformation (Smirnova et al., 2003).

1.5. Outline of the diploma project

The main aim of this diploma project was to establish an easy and efficient screen to identify thermostable mutants of the *Escherichia coli* dipeptide transporter YdgR. This screen consists of two steps: one to first distinguish between active transporters and loss-of-function mutants, and a second step to measure their thermostability. To accomplish this, a library of transporter mutants had to be generated, and two assays had to be established and optimised. The first one is a growth assay, by which mutant proteins that retain transport activity are separated from the non-transporters. Second, a high throughput purification and thermostability assay had to be established. Furthermore, several loss-of-function mutants were biochemically characterized.

2. Materials and Methods

2.1. Materials

The chemical compounds listed here were purchased either from Fluka or Sigma, the enzymes and protein ladders from Fermentas if not stated otherwise.

2.1.1. General Media, Solutions, Reagents and Enzymes

2.1.1.1. Media

<u>LB-medium:</u>	Luria-Bertani medium 10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L dH ₂ O (deionized water), pH 7, autoclaved
<u>SOB:</u>	Super-Optimal broth ⁶ 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 mM KCl in 1 L dH ₂ O, autoclaved
<u>SOC:</u>	Super-Optimal catabolite repression SOB containing 2 % glucose
<u>SOC salt:</u>	SOC containing 25 mM magnesium salt (12.5 mM MgCl ₂ , 12.5 mM MgSO ₄)
<u>SOB CC:</u>	SOB for competent cells 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.5 mM KCl in 1 L dH ₂ O, autoclaved, 10 mM sterile MgCl ₂

2.1.1.2. Chemical compounds

<u>APS:</u>	ammonium peroxodisulfate 10 % (w/v) solution in dH ₂ O, sterile-filtered, stored at -20°C
-------------	---

⁶ According to Doug Hanahan - the author of "J. Mol. Biol. 166(4): 557-580", where SOB was first described.

<u>IPTG:</u>	isopropyl β -D-1-thiogalactopyranoside 0.5 M stock solution in dH ₂ O, sterile-filtered, stored at -20°C
<u>DTT:</u>	dithiothreitol 1 M stock solution in dH ₂ O, sterile-filtered, stored at -20°C
<u>EDTA:</u>	ethylenediaminetetraacetic acid 0.5 M stock, pH 8
<u>Ala-Ala:</u>	H-Ala-Ala-OH (alanyl-alanine) 1 M stock solution in dH ₂ O, prepared freshly or stored at -20°C
<u>β-Ala-Lys-AMCA:</u>	N-7-amino-4-methylcoumarin-3-acetic acid labeled alanyl-lysine 5 mM stock in DMSO, stored at -80°C

2.1.1.3. Detergents

<u>SDS:</u>	sodium-dodecyl sulfate 10 % stock in dH ₂ O
<u>DDM:</u>	n-dodecyl- β -D-maltoside, Anatrace cmc (H ₂ O) ~ 0.17 mM (0.0087 %), micelle size: 72 kDa, prepared freshly as 10 % stock
<u>FOS-12</u>	n-dodecylphosphocholine (foscholine-12), Anatrace cmc (H ₂ O) ~ 1.5 mM (0.047 %), prepared freshly as 10 % stock

2.1.1.4. Enzymes

<u>Lysozyme:</u>	50 mg/ml solution in dH ₂ O, stored at -20°C
------------------	---

2.1.1.5. Antibiotics

<u>Ampicillin:</u>	100 mg/ml stock solution in dH ₂ O, sterile-filtered, stored at -20°C, used as a 1:1000 dilution
<u>Carbenicillin:</u>	50 mg/ml stock solution in dH ₂ O or 50% glycerol/dH ₂ O, sterile-filtered, stored at -20°C, used as a 1:1000 dilution
<u>Kanamycin:</u>	50 mg/ml stock solution in dH ₂ O, sterile-filtered, stored at -20°C, used as a 1:1000 dilution
<u>Alafosfalin:</u>	L-alanyl-L-1-aminoethylphosphonic acid 1 M stock in dH ₂ O

2.1.1.6. Buffers

<u>5x SDS LB:</u>	5x SDS loading buffer 62.5 mM Tris pH 8, 10 % (v/v) glycerol, 2 % (w/v) SDS, in dH ₂ O, Bromophenol Blue (tip of a spatula for approximately 40 ml), stored at room temperature, 50 µl of β-mercaptoethanol added freshly to 950 µl of solution
<u>1x SDS RB:</u>	1x SDS running buffer: 25 mM Tris/HCl pH 8.3, 200 mM glycine, 0.1 % (w/v) SDS, prepared as 5x stock, stored at 4°C
<u>10x TAE buffer:</u>	Tris/acetate/EDTA 0.4 M Tris/HCl pH 8, 10 mM EDTA-Na ₂ -salt, 0.2 M acetic acid
<u>6x DNA LD:</u>	6x DNA loading dye 0.25 % (w/v) Bromophenol Blue, 50 % (w/v) sucrose in dH ₂ O, stored at 4°C
<u>1x TB:</u>	Tris buffer, Western blot transfer buffer 25 mM Tris/HCl pH 8, 192 mM glycine, 10 % methanol; prepared as 10x stock without methanol, stored at 4°C
<u>1x TBS:</u>	tris buffered saline 10 mM Tris/HCl pH 8, 150 mM NaCl, prepared as 10x stock, stored at 4°C
<u>1x TBS-T:</u>	TBS Tween 0.1 % Tween-20 added to 1x TBS, stored at 4°C
<u>Inoue-buffer:</u>	10 mM HEPES pH 6.7, 250 mM KCl, 15 mM CaCl ₂ *2 H ₂ O, 55 mM MnCl ₂ *4 H ₂ O
<u>Krebs-buffer:</u>	modified: 25 mM Tris pH 7.5, 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl ₂ , 0.8 mM MgSO ₄ , 0.09 % glucose, in dH ₂ O, sterile-filtered, stored at 4°C

2.1.2. Bacterial strains

Bacterial strains used in these experiments are given in Table 2.

Table 2. Bacterial strains with respective genotypes.

Bacterial strain	Genotype	Description
DH5 α	<i>E. coli</i> F- ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 deoR, recA1 endA1 hsdR17(rk- mk+ phoA supE44 λ - thi-1 gyrA96 relA1	DH5 α cells are widely used for simple and efficient DNA transformation and preparation.
BL21(DE3) Stratagene	<i>E. coli</i> B F- dcm ompT hsdS(rB- mB-) gal λ (DE3)	The BL21 strain carries a chromosomal copy of the T7 promoter. It provides high-level protein expression, but has the potential risk of leaky expression.
BL21 Gold(DE3) pLysS Stratagene	<i>E. coli</i> B F- ompT hsdS(rB- mB-) dcm+ Tetr gal λ (DE3) endA Hte [pLysS Camr]	These cells provide a tighter control due to the expression of T7 lysozyme (T7 RNA polymerase inhibitor)
C43(DE3)	Strain derived from C41 (DE3), which in turn is derived from BL21(DE3)	This strain carries an undefined mutation that allows for the overexpression of otherwise toxic proteins i.e. integral membrane proteins (Walker et al., 1996).
JW1626	<i>E. coli</i> K12 BW25113 dYdgR, rrnB3 #lacZ4787 hsdR514 # (araBAD)567 # (rhaBAD)568 rph-1	YdgR knock-out strain, Keio-collection (single-gene deletions of 3985 non-essential <i>E. coli</i> K12 genes) (Baba et al., 2005).
XL10-Gold Stratagene	<i>E. coli</i> TetrD(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1	The cells provide enhanced transformation efficiency and are therefore extremely useful when

	gyrA96 relA1 lac Hte [F' generating plasmid DNA libraries proAB lacIqZDM15 (larger, more complex libraries). Tn10 (Tetr) Amy Camr
Fusion-Blue Clontech	<i>E. coli</i> K-12 endA1, hsdR17 High transformation efficiency with (rK12-,mK12+), supE44, thi-1, an additional blue-white screening recA1, gyrA96, relA1, lac capability (when using a plasmid F'[proA+B+, that provides α -complementation). lacIqZ Δ M15::Tn10(tetR)]

2.1.3. Plasmids

A pCS19 vector (derived from pQE (Ehrmann et al. 1999), Fig. 5), a pET vector (Novagen) and a pJET vector (GeneJET Cloning Kit, Fermentas) were used for all cloning and expression experiments conducted here.

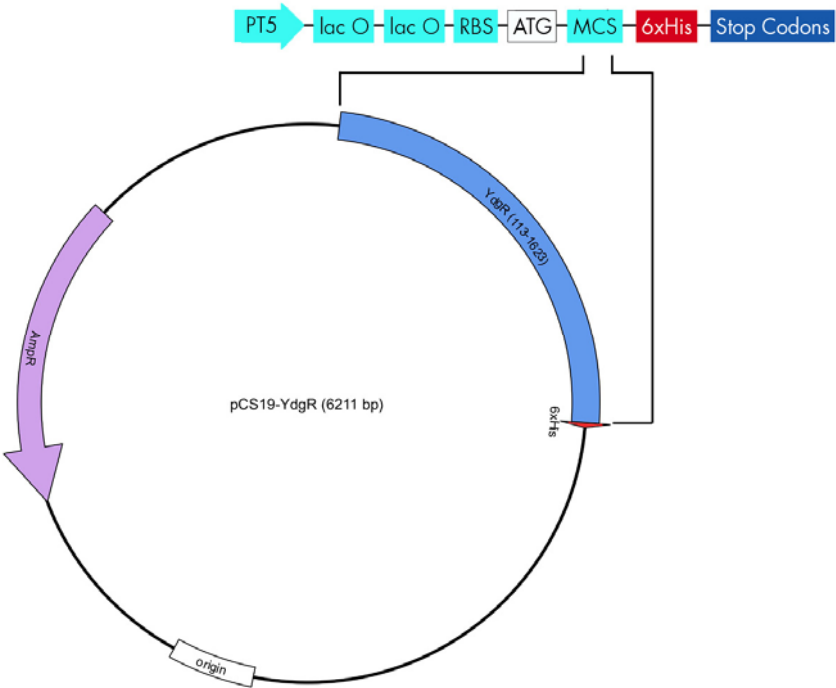


Figure 5. Schematic representation of the pCS19 vector. The vector carries an ampicillin resistance gene, and has a YdgR gene with a C-terminal His-tag inserted in its multiple cloning site (MCS).

2.2. Methods

2.2.1. Molecular cloning techniques

2.2.1.1. Agarose gel electrophoresis

Agarose gel electrophoresis separates DNA molecules according to their size. PCR or restriction fragments as well as purified plasmids were analyzed on 1 % or 2 % agarose gels depending on the size of the fragment. Agarose was dissolved in 1x TAE buffer by heating it in the microwave. SYBR Safe DNA gel stain (Invitrogen) was added to visualize separated DNA bands.

2.2.1.2. DNA gel extraction and PCR purification

DNA was purified using either the QIAquick gel extraction or PCR purification kit (Qiagen), according to the provided protocol using an Eppendorf microcentrifuge. The purified DNA was eluted in either 30 or 50 µl of dH₂O.

2.2.1.3. Restriction digest and ligation

DNA restriction digests were performed overnight in a 50 µl reaction mixture containing 1x buffer (according to enzyme requirements or Fermentas double digest function: <http://www.fermentas.com/doubledigest/index.html>), 0.1 U/µl enzyme, alkaline phosphatase (only when cutting vector) and 2-5 µg DNA. In order to verify a vector insert by restriction, fast digest enzymes were used according to protocol. Vector and insert were ligated for two hours with T4 ligase in ligation buffer in a 5x molar excess of the insert. The ligation mixture was directly transformed into competent *E. coli*.

2.2.1.4. Generation of competent *E. coli* cells

BL21(DE3) and XL-10 gold competent *E. coli* cells were made according to the Inoue-protocol⁷. Purchased competent cells from a -80°C stock were thawed, shaken in SOC for 30 minutes and plated on an LB plate. A single colony was picked, inoculated into 25 ml SOB (see “SOB for competent cells” for recipe) in a 100 ml flask and grown for seven hours at 37°C at 250 rpm. 10, 4 and 2 ml were inoculated in 250 ml SOB in a 1 L flask and

⁷ from Sambrook J., Molecular Cloning, 3rd Edition (2001), Cold Spring Harbour Laboratory Press

were incubated at 18 °C overnight at 190 rpm. The cells were grown to an OD₆₀₀ of 0.6 and incubated in an ice-water bath for 10 minutes. The cultures were pelleted at 2500 g for 10 minutes at 4°C. The pellet was dried, washed in 80 ml of ice-cold Inoue-buffer, and resuspended in 20 ml of Inoue buffer. 1.5 ml of DMSO were added and the suspension was aliquoted and immediately frozen in liquid nitrogen and stored at -80°C.

2.2.1.5. Transformation of competent *E. coli*

Fifty nanograms of plasmid DNA were transformed into 50-100 µl of *E. coli* competent cells. The cells were incubated with the DNA for 15 minutes on ice, then heat-shocked for 1 minute at 37°C, and cooled on ice for a further two minutes. 500 µl SOC medium were added and the cells were incubated with shaking at 37°C. The transformed bacteria were plated onto LB plates containing the respective antibiotics. The transformation for In-Fusion as well as library cloning were performed in Fusion-Blue or XL-10 Gold competent cells delivered with the respective kits according to provided transformation protocols.

2.2.1.6. Random mutagenesis

Random mutagenesis of a gene can be a useful tool to establish a library of clones with improved or altered features. Such a desired characteristic is for example loss or gain-of-function, increased thermostability or enhanced production of a protein. In principle, the infidelity of a DNA polymerase is exploited to introduce mutations randomly. The mutation rate depends primarily on the error rate of the polymerase, but can further be influenced by varying the concentration ratios of components in the PCR reaction.

Here, I introduced random mutations into distinct regions of the YdgR gene by using the GeneMorph II Random Mutagenesis Kit (Stratagene). First, the gene fragment of interest was amplified using the provided, error-prone polymerase (Mutazyme II). The purified target fragment then served as a so-called “megaprimer” in a second PCR reaction, in which the whole target plasmid was amplified. The parental DNA plasmids were digested with the DpnI restriction enzyme, which targets methylated and hemi-methylated DNA, leaving the new plasmids containing the desired mutations intact (Fig. 6).

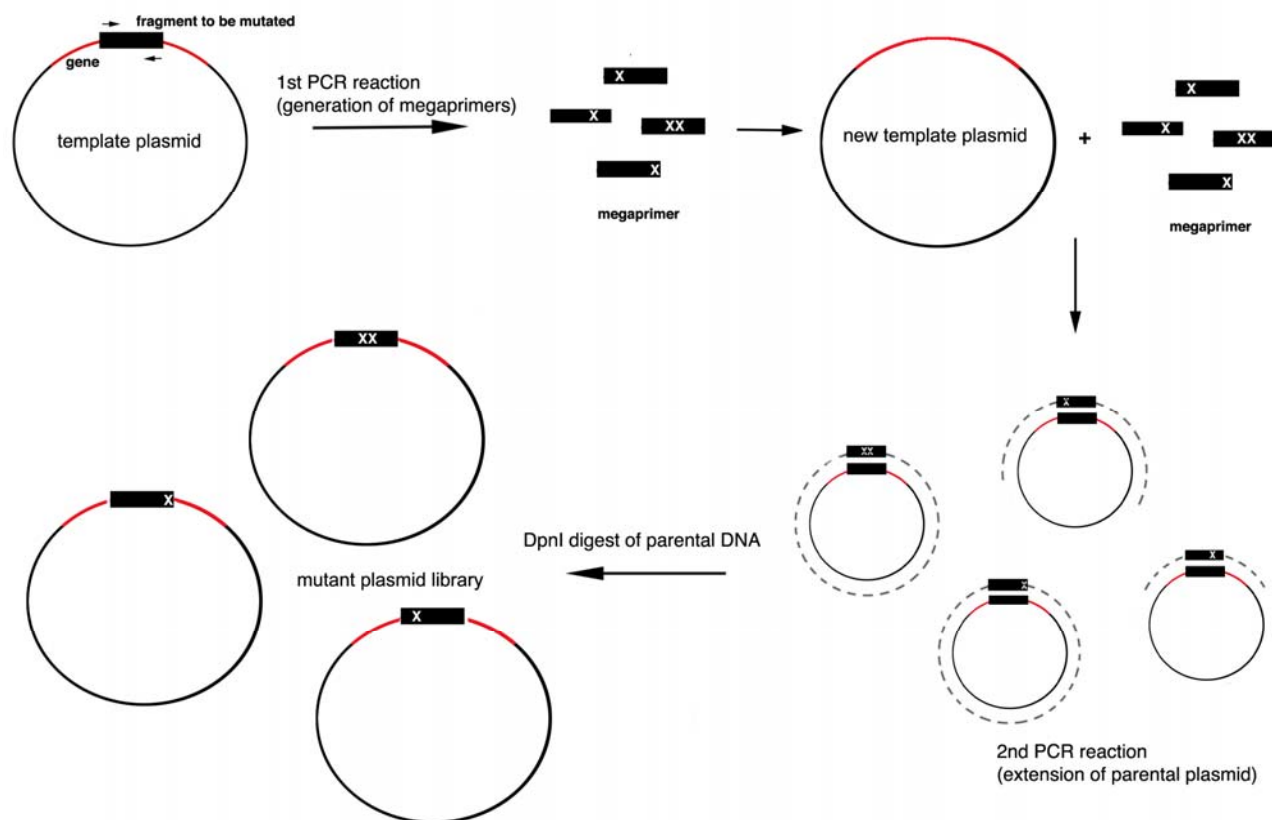


Figure 6. Principle of GeneMorph (Stratagene) random mutagenesis. A targeted gene region is randomly mutated exploiting the infidelity of a Taq polymerase mixture (Mutazyme II, Stratagene). The mutagenized fragment is used as a megaprimer to amplify a template plasmid. After this PCR reaction the parental plasmid is digested by a DpnI digest, leaving a mutated plasmid library.

I introduced random mutations into 4 different regions of the YdgR gene, i.e. helix 6 and helix 7, Helix 8 and 9, and Helix 11 and all of their adjacent loops (Fig. 7). Particular regions were left out, for example Helix 1 and Helix 5, which contain rather conserved regions and thus mutations would probably lead to more severe phenotypes, such as misfolding or mislocalisation. Furthermore, the c-terminal helices are less likely to participate in the core fold as it could be seen in the structures of the leucine transporter LeuT (Yamashita et al., 2005), benzyl-hydantoin transporter Mhp1 (Weyand et al., 2008) or the sodium/galactose symporter vSGLT (Faham et al., 2008), therefore mutations in this region might have a relatively limited effect on thermostability. Furthermore, it has been shown that thermostable mutations appeared frequently at the hinge regions, i.e. in the loop residues close to the membrane surface (Zhou et al., 2000; Bowie, 2001).

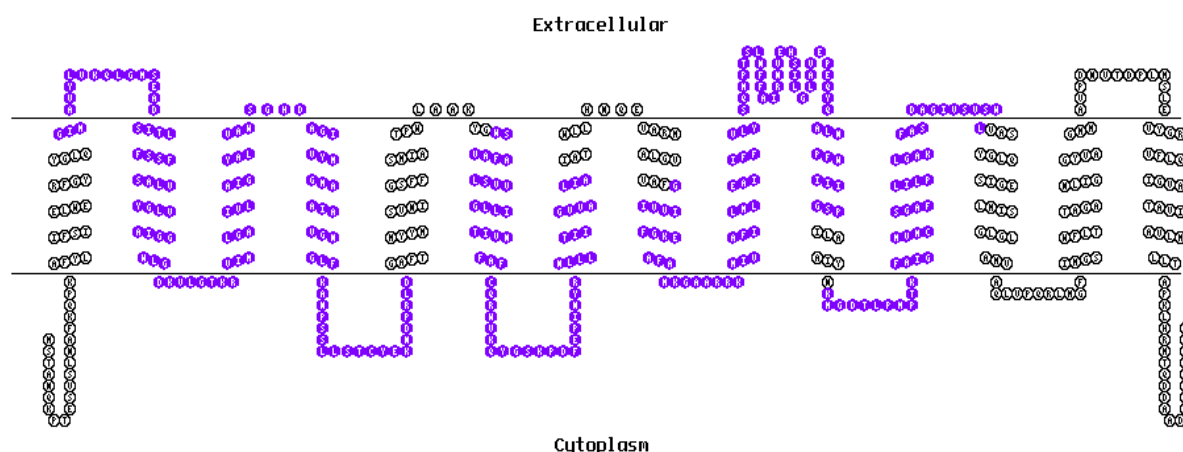


Figure 7. Targeted regions of YdgR.⁸ The fragments targeted in the random mutagenesis reactions are highlighted in purple.

The mutagenic PCR reaction was performed in 50 μ l of 1x Mutazyme reaction buffer containing 250 ng of the respective primer mixture (Tab. 3), 800 μ M dNTPs, 2.5 U/ μ l Mutazyme II DNA polymerase and varying amount of template plasmid pET-YdgR-GFP. The reaction cycle is shown in Table 4. As already mentioned, the mutation frequencies greatly depend on the components in the PCR reaction and on the fragment length. Therefore, the concentration of template DNA was varied in order to achieve a sufficient mutation density. Before the elongation of the megaprimer, these frequencies were determined by blunting the amplified, mutated fragments and cloning them into the pJET vector using the GeneJET Cloning Kit (Fermentas). Ten different clones for each DNA template concentration were sequenced. In the second PCR reaction, the elongation of the plasmid was performed according to protocol with the reaction cycle shown in Table 4 (with gel-purified fragments).

⁸ Figure generated with www.sacs.ucsf.edu/TOPO2-run/wtopo2.pl

Table 3. Primer pairs used in the first PCR reaction.

Primer	Sequence	Used annealing temperature
Loop1/4_Fw	5' ATGGCTGTTTACCTGGTTAAAC 3'	50
Loop1/4_Rev	5' CAGACGCGGGTCGTTTTTCTCATA 3'	
Loop6/7_Fw	5' TGGAGTGTTGCGTTTTCGTTGAGC 3'	53
Loop6/7_Rev	5' GCGATCAGTGCCACAACACCAATAA 3'	
Helix8_Fw	5' TATCGCCACCTGGCTGCTG 3'	50
Helix8_Rev	5' ATCATTTTACGACGCGCAGCACC 3'	
Helix 9/loop_Fw	5' CTTCGCGATGAAAGGTGCTGC 3'	50
Helix 9/loop_Rev	5' GACTACCGATGATGATCCAGAAC 3'	
Helix 11_Fw	5' AAGATGGGCGATACCCTGCC 3'	49
Helix 11_Rev	5' CAGCCAGCTTACAGACACGAT 3'	

Table 4. Mutagenic PCR reaction cycles

Fragment mutation (PCR 1)			Fragment elongation (PCR 2)	
Step	Temperature	Time	Temperature	Time
Initial denaturation	95°C	2 minutes	95°C	1 minute
Denaturation	95°C	30 seconds	95°C	50 seconds
Annealing	50-55°C	30 seconds	60°C	50 seconds
Extension	72°C	30 seconds	68°C	12.5 minutes
Final extension	72°C	10 minutes	-	-

The values for the mutation frequencies are not very accurate since only 10 clones were analyzed for each determination. However, they should ensure that there are not too many wildtype genes left in the screen.

Library Loop 1/4 Although only 5 or 10 ng were used in the mutagenesis reaction, the libraries had mutation frequencies of 20 and 30 %, respectively. The mutagenesis has to be repeated with even lower concentrations

Library Loop 6/7 Using 25 ng of pET-YdgR-GFP, 60% carried a single mutation, 30 % had two point mutations or insertions, respectively, and 10 % had four point mutations.

Library Helix 8/9 Originally, the libraries for helix 8 and helix 9 should have been established separately. However, the achieved mutation frequencies for this part of the protein were always too low. Therefore, these two fragments were combined using Helix 8 forward and the Helix 9/loop reverse primers for the PCR. A template concentration of 10 ng yielded 43 % of the clones with one point mutation, 29 % with two, 14 % with three and 14 % with no mutation.

Library Helix 11 Two nanograms were used for the first PCR. They showed a mutation frequency of 100 % (1 point mutation)

2.2.1.7. GFP fusion cloning

A GFP-tag (green fluorescent protein) was added to the C-terminus of YdgR and the mutant Y156A in the pCS19 vector using two different approaches, namely cloning with restriction enzymes and In-Fusion Cloning (Clontech). The GFP gene was amplified from a pET-GFP vector using the primers given in Table 5.

Table 5. Primer pairs for cloning of a C-terminal GFP-tag.

Primer name	Function	Sequence
GFPTag_fw	Standard cloning	5'GAAGATCTGAAAACCTGTACTTCC AGGG 3'
GFPTag_rev		5'TCGGATCCTCAGTGGTGGTGGTGG TG 3'
INF_GFPTag1_fw	In-Fusion cloning	5'GCCGTAGCGAGATCTGAAAACCTG TACTT CCAGGG 3'
INF_GFPTag2_fw	In-Fusion, additional GGs linker	5'AGCCGTAGCGAGATCTGGAGGTTC CGAAAACCTGTACTTCCAGGG 3'
INF_GFPTag3_fw	In-Fusion, control for GFP expression	5'GGAGGATCCAGATCTATGGAAAAC CTGTA CTTCCAGGG 3'
INF_GFPTag_rev	Reverse primer for all three reactions	5'TCAGCTAATTAAGCTACTAGTTCA GTGGTG GTGGTGGTG 3'

The standard PCR reaction (Tab. 6) was performed in 50 µl 1x HF Buffer and 0.02 U/µl Phusion polymerase (Finnzymes) containing 800 µM dNTPs, 200 nM primers and 25 ng DNA template (pET-YdgR-GFP). The fragment was gel purified and cut with the restriction enzymes BglII and BamHI. The vector pCS19-YdgR was treated with BglII and alkaline phosphatase. Digested vector plus the fragment in a 5 x molar excess were ligated, transformed into DH5α and plated onto LB-ampicillin plates. Clones were checked for an insert via a restriction analysis and sequenced. The following mutant proteins were also cloned as a GFP construct in pCS19: M154K, L190V, A184T, N196K and F197I. The fragments were cut out of the pCS19 vector containing the respective mutant gene, gel purified, digested with EcoRI and BglII, and ligated into pCS19-GFP, from which the wildtype YdgR had been excised with the same restriction enzymes.

Table 6. Reaction cycle of GFP cloning.

Step		Temperature	Time
Initial denaturation		98°C	30 seconds
Denaturation	25x	98°C	15 seconds
Annealing		63°C	30 seconds
Extension		72°C	30 seconds
Final extension		72°C	5 minutes

Whereas standard cloning procedures require restriction sites, the In-Fusion cloning system gets by without. The primers are designed normally, but with a 15 base pair extension, which is homologous to the linearized vector ends. The amplified fragment anneals and recombines into the vector (Fig. 8).

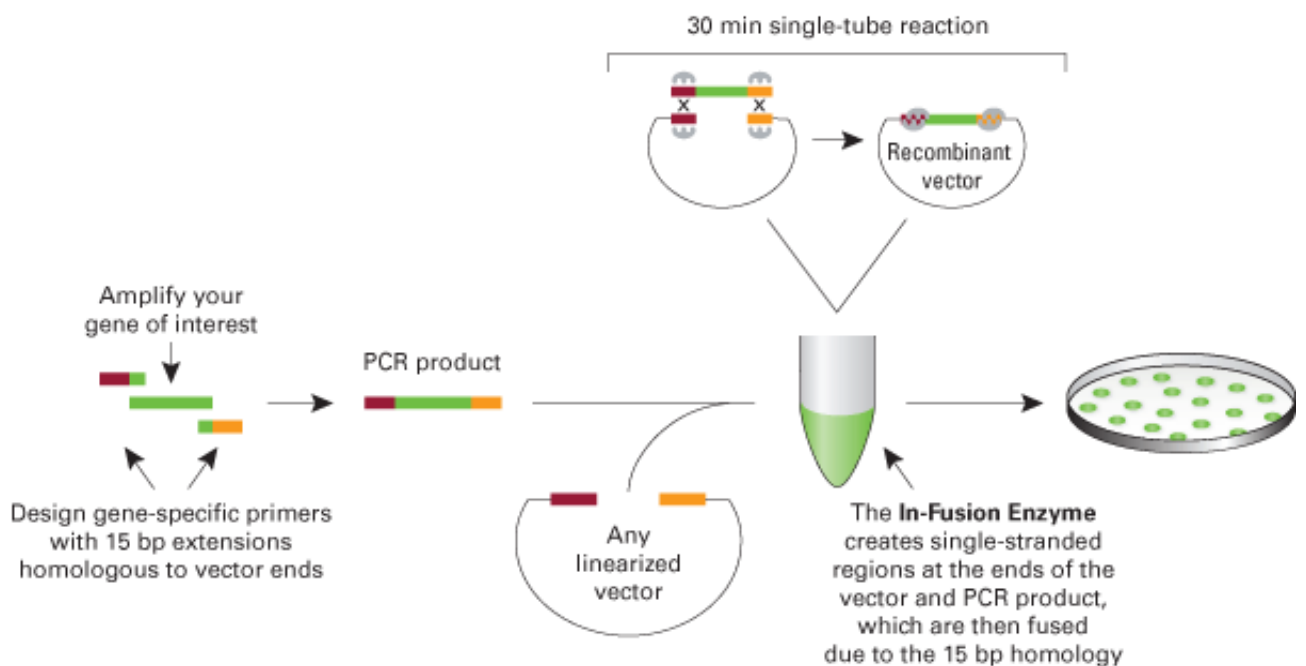


Figure 8. The In-Fusion cloning protocol.⁹ A gene of interest is amplified using primers with a 15 bp extension homologous to the linearized vector ends. The PCR product can then anneal and insert into the vector by homologous recombination.

⁹ Figure taken from Clontech http://www.clontech.com/products/detail.asp?product_id=206261&tabno=2

2.2.2. In vivo Assays

2.2.2.1. Growth Assay

A growth assay was established to distinguish between active and loss-of-function mutants of YdgR. The mutant libraries were transformed into *E. coli* BL21(DE3) and inoculated into 1 ml LB/carbenicillin overnight cultures. In the assay, 2 μ l of these cells were grown in 96 well plates in 200 μ l of LB medium containing the antibiotic carbenicillin. Additionally, the antibiotic alafosfalin, which inhibits cell wall synthesis and is specifically taken up by YdgR, was added to an end concentration of 200 μ g/ml. Protein expression was induced with 0.01 mM IPTG at OD₆₀₀ 0.3 (determined by using Synergy™ HT Multi-Mode Microplate Reader, Biotek; corresponds to an OD of 0.6 in a normal spectrophotometer, Ultrospec 6300 pro, Amersham). OD₆₀₀ was measured after inoculation, before induction, one hour after induction, and seven hours after induction.

2.2.2.2. Transport assay

Comparing the uptake level of substrate by mutant proteins to wildtype also required an in vivo transport assay. Using this assay, it was possible to determine whether the cells expressing YdgR or its mutant forms could still transport the dipeptide alanyl-lysine labeled with the fluorescent dye AMCA (N-7-amino-4-methylcoumarin-3-acetic acid). For this purpose BL21(DE3) cells were transformed with the respective pCS19 constructs. Two milliliter overnight cultures with LB containing 2 % glucose and carbenicillin were set up. 250 μ l were pelleted, resuspended in fresh LB/glucose/carbenicillin and transferred to 25 ml of fresh LB/glucose/carbenicillin in a 100 ml baffled flask. The cells were grown for approximately 2.5 hours until they reached an OD₆₀₀ of 0.6, when they were induced with 0.1 mM IPTG. After one hour of induction, the cells were placed on ice and 3.25×10^9 cells (as estimated from OD₆₀₀) were resuspended in 1 ml modified Krebs-buffer. 40 μ l of cells were incubated with 10 μ l of 500 μ M β -Ala-Lys-AMCA (diluted from stock in Krebs-buffer) and 50 μ l of Krebs-buffer (negative control: 60 μ l Krebs-buffer) for 15 minutes in a MultiScreen filter plate (Millipore) at 37°C with shaking. A competitor was also tested (50 μ l of 20 mM alanyl-alanine dissolved in Krebs-buffer instead of buffer only). The supernatant was filtered through the plates using a Millipore Multiscreen_{HTS} vacuum manifold. The cells were then washed with three times 200 μ l of Krebs-buffer.

Substrate fluorescence was measured with a Synergy plate reader (excitation wavelength: 360 nm, emission: 460 nm, Tungsten lamp, sensitivity 50). Serial dilutions of the cells from each construct were plated to confirm the actual cell concentration. The protein levels were compared by Western blotting.

2.2.2.3. GFP-fluorescence assay

As for the growth assay, BL21(DE3) cells were transformed with YdgR-GFP or the respective mutants. Colonies were inoculated into 1 ml overnight cultures. Cells grown in 96 well plates for 2.5 hours were induced with 0.01 mM IPTG and YdgR-GFP fluorescence was determined after another 4 hours. Absorbance at 600 nm was also measured to confirm similar cell density of the samples.

2.2.3. Protein expression

2.2.3.1. Small-scale high-throughput purification (HTP)

2.2.3.2.1. Expression

The mutant libraries and the respective controls (wildtype, Y156A mutant, empty pCS19 vector) were transformed into BL21(DE3) (see growth assay). 30 µl of pre-culture were inoculated into 2 ml LB/carbenicillin in 24 well plates, and grown to OD 0.6 (37°C, 200 rpm). After induction with 0.1 mM IPTG, the temperature was reduced to 18°C and the cell were incubated with shaking overnight. The bacteria were harvested directly in the plates (4000 g, 10 minutes) and frozen at -20°C (also when processed the same day because freezing enhanced the lysis).

2.2.3.2.2. Purification

The cells were thawed, resuspended in 350 µl lysis buffer (20 mM Tris, pH 8, 150 mM NaCl, 10 % glycerol, 15 mM imidazole, 1 % DDM, 5 mM Ala-Ala, Complete protease inhibitors (Roche), 1 mg/ml lysozyme, 10 U/µl Benzonase (Sigma) and 2 mM MgCl₂), and incubated on a shaker at 4°C for two hours. 40 µl of the same buffer with a higher salt concentration was added to increase the end concentration to 300 mM salt (Ni-NTA

binding optimum). Unlysed cells and cell debris were pelleted for 10 minutes at 4000 g. In the meantime, 96 well Ni-NTA plates with 50 µl beads (His Multitrap HP, GE Healthcare) were equilibrated with wash buffer (20 mM Tris, pH 8, 300 mM NaCl, 30 mM imidazole, 10 % glycerol, 0.04 % DDM and 5 mM Ala-Ala). The supernatant was added to the beads, and the plates were incubated with shaking overnight at 4°C. The samples were then filtered through the plates using the Millipore vacuum manifold and washed with 1 ml wash buffer. The protein was eluted in 50 µl elution buffer (20 mM Tris, pH 8, 300 mM NaCl, 10 % glycerol, 150 mM imidazole, 0.02 % DDM and 5 mM Ala-Ala) and centrifuged at 500 g for 2 minutes (to avoid bubble formation in the solution during vacuum suction).

2.2.3.2. Large-scale purification

2.2.3.2.1. Expression

YdgR was recombinantly expressed in C43(DE3) cells. This *E. coli* strain carries a mutation that leads to a larger membrane surface area and thus to an increase in protein yield (Walker et al., 1996). Therefore, the respective constructs were freshly transformed into this strain. 30 ml LB/glucose/carbenicillin medium in a 100 ml baffled flask was inoculated with one colony and grown overnight at 30 °C (200 rpm). These overnight cultures were pelleted and resuspended in 10 ml fresh medium. 3 ml of this cell suspension was transferred to 2 L LB/glucose/carbenicillin medium in 5 L baffled flasks (pre-rinsed with sterile dH₂O). The cells were grown at 37°C at 190 rpm until they reached an OD₆₀₀ of 0.6. After induction with 0.1 mM IPTG (final concentration), the temperature was shifted to 18°C and the cells were grown overnight at 130 rpm to reduce the foaming. Cells were harvested by centrifugation at 15 000 g for 15 minutes, resuspended in 50 ml resuspension buffer per liter cell culture (50 mM Tris pH 8, 300 mM NaCl, 10% glycerol and 2 mM EDTA) and frozen at -80°C.

2.2.3.2.2. Membrane preparation

A very important step towards the purification of membrane proteins is the isolation of membranes. First, it already serves as a purification step since cytosolic proteins are removed, and second, the starting material for the actual purification is more concentrated. The thawed cells were filtered through a 47 mm disc filter (Millipore) to remove lumps. After addition of Complete protease inhibitors (Roche), the cells were lysed using a French press (1.7 kbar). The lysate was centrifuged at 26 940 g for 45 minutes to remove the unlysed cells and cell debris. The supernatant was ultracentrifuged at 100 000 g for one hour. The pelleted membranes were homogenized using a glass homogenizer in homogenizer buffer (10 mM Tris pH 8, 20 mM NaCl, 20% glycerol). The protein concentration was determined by the Lowry method, and the membranes were diluted to 6 mg/ml and frozen in liquid nitrogen and stored at -80°C.

2.2.3.2.3. Purification

YdgR was purified using a two-step protocol, consisting of metal affinity chromatography and size-exclusion chromatography. The gene is expressed with a 6x-His tag on the C-Terminus, and can therefore bind to nickel ions (Ni^{2+}) immobilized onto a stationary phase such as agarose or sepharose. Before the chromatography steps can be performed, however, the YdgR protein has to be solubilized from the membrane.

2.2.3.2.3.1. Solubilization

First, the membranes are thawed and diluted 1:1 with a dilution buffer resulting in an end-concentration of 20 mM Tris, pH 8, 300 mM salt, 10 % glycerol, 1 % DDM and 5 mM of Ala-Ala (and also 0.5 mM DTT if the protein was not used for assays where DTT would disturb the reaction). After an hour of rotating at 4°C, the samples are centrifuged at 100 000 g to spin down the non-solubilized membranes.

2.2.3.2.3.2. Immobilized metal affinity chromatography (IMAC)

The supernatant from ultracentrifugation was diluted 1:1 with a buffer containing 30 mM imidazole (end-concentrations: 15 mM imidazole, 0.5 % DDM). One milliliter of Ni-beads

(Qiagen, equilibrated in 20 mM Tris, pH 8, 300 mM NaCl, 10 % glycerol, 5 mM of Ala-Ala and 0.04 % DDM) per 10 ml of 6 mg/ml membranes was added and the samples were incubated with rotating overnight at 4°C. The beads were poured into a Biorad polyprep chromatography column and washed with ten times the column volume of washing buffer (20 mM Tris, pH 8, 300 mM NaCl, 10 % glycerol, 30 mM imidazole, 0.04 % DDM and 5 mM of Ala-Ala). The proteins were eluted in the same buffer containing 150 mM imidazole and 0.02 % DDM in five times two ml fractions (ten times the column volume with 10 minutes incubation time between the first and the second fraction to maximize protein yield). The fractions were analyzed on a Coomassie stained SDS-gel (Fig. 9). The YdgR protein band runs at approximately 39 kDa. YdgR has an expected molecular weight of 54 kDa assessed from its length (500 amino acids) However, it runs lower due to the abnormal migration behavior sometimes observed with membrane proteins (Weitz et al., 2007). There was very little protein in the wash steps. The majority of protein eluted in the first two elution fractions, which were pooled and concentrated in a Vivaspin 6 (Sartorius) with a 50 kDa molecular weight cut-off (MWCO) to 500 µl. The micelle including the protein should have a molecular mass of approximately 150 kDa, therefore, a MWCO of 50 kDa should allow for concentration of the protein.

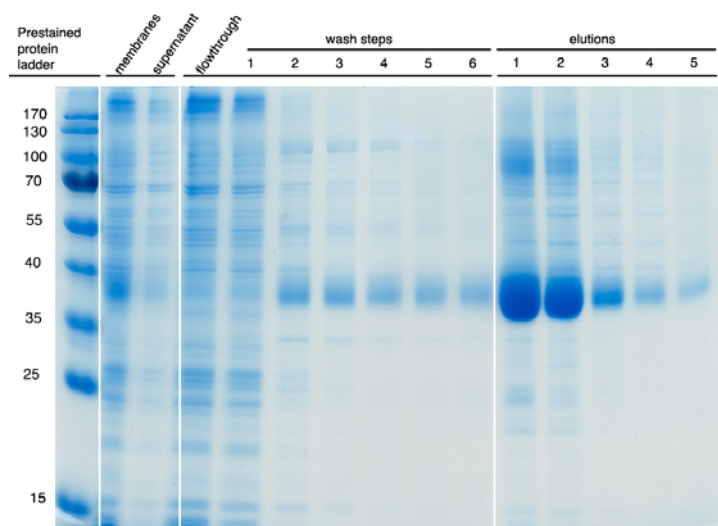


Figure 9. Coomassie stained SDS-gel of immobilized metal affinity chromatography (IMAC). YdgR containing a C-terminal His-tag was purified over a nickel-agarose column. The fractions were analyzed on a Coomassie stained SDS-PAGE. The protein runs at approximately 39 kDa, and eluted in the first four milliliters of the elution fractions.

2.2.3.2.3.2. Size exclusion chromatography

Size exclusion chromatography or gel filtration is a chromatographic method that separates compounds according to their size (the so-called hydrodynamic volume, which not only includes the molecular weight, but also the shape of the molecule). The column is composed of a highly porous polymer material of a certain diameter. Smaller molecules are retained in these pores whereas larger particles can diffuse through much faster, and are therefore eluted first. This principle can not only be exploited to separate differently sized proteins, but also to exchange or remove buffer components.

Pooled and concentrated fractions from the Ni-NTA eluate were loaded onto a Sephadex 200 column (GE Healthcare), equilibrated in 20 mM Tris, 150 mM NaCl, 10 % glycerol and 0.04 % DDM and chromatographed with a flow of 0.5 ml/minute. YdgR eluted at a column volume of approximately 13 ml (peak maximum), which corresponds to 150 kDa (data Peggy Stolt-Bergner). Fractions were analyzed on a SDS-gel, pooled and concentrated if necessary. One example of a YdgR gel filtration chromatogram is given in Figure 10A . Fractions C1 to C13 can be seen in lane 4 to 15 on the Coomassie stained gel (Fig. 10B). Compared to the pool after the Ni-IMAC (lane 2), the protein is relatively pure. The Vivaspin flowthrough sample did not contain any protein, which means that the MWCO of the concentration column is small enough to retain the protein. Fractions C8 to C13 were pooled and frozen.

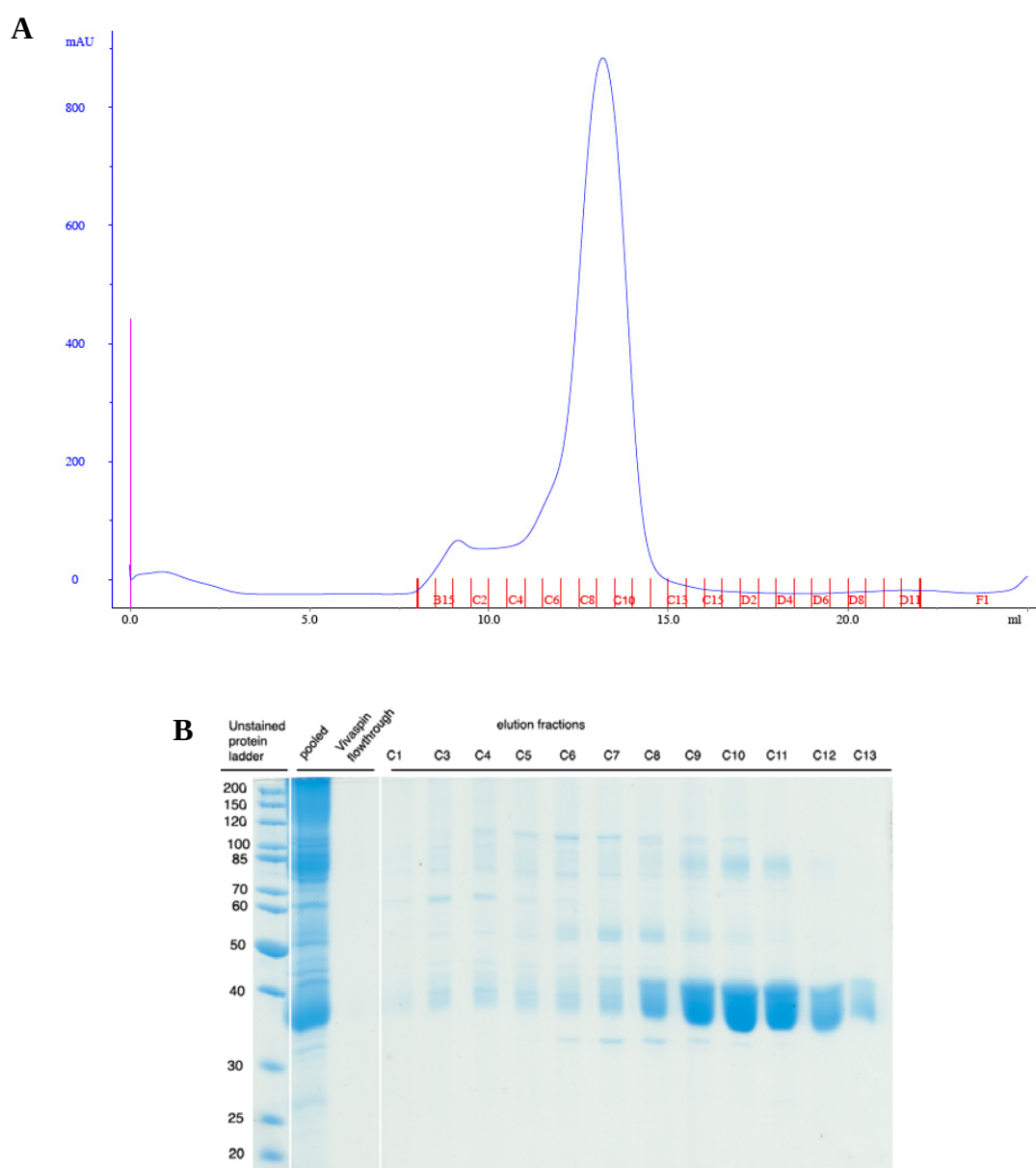


Figure 10. Gel filtration of IMAC purified YdgR. (A) Gel filtration chromatogram and **(B)** Coomassie stained SDS-PAGE of the eluate. YdgR eluted at approximately 13 ml, which corresponds to its molecular weight plus micelle (150 kDa).

2.2.4. Protein Analysis

2.2.4.1. SDS-polyacrylamid gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis is a widely used technique to separate proteins according to their molecular weight. The proteins are denatured in a buffer containing

SDS, which renders the protein negatively charged. As already mentioned, SDS (Fig 4A) is a detergent, an amphiphilic molecule with a negatively charged polar head (sulfate group) and a hydrophobic tail that adsorbs to the protein.

5x SDS loading buffer was added the protein samples, which were then loaded onto a 12 % SDS polyacrylamide gel. The gel is divided into a separating gel (lower part, 12 % bis/acrylamide, 375 mM Tris pH 8.8, 0.1 % SDS, 0.1 % ammonium persulfate (APS), 0.04 % N,N,N',N'-Tetramethylethylenediamine (TEMED)) and a stacking gel (upper part, loading pockets, 5 % bis/acrylamide, 125 mM Tris pH 6.8, 0.1 % SDS, 0.1 % APS, 0.1 % TEMED). The proteins were electrophoresed for one hour at 160 Volts.

2.2.4.2. Coomassie Staining

Coomassie dyes are commonly used to stain polyacrylamide gels by adsorption to certain amino acids in a protein. Here, a PageBlue protein staining solution from Fermentas was used, and gels were stained according to the provided fast protocol.

2.2.4.3. Silver staining

The advantage of silver over Coomassie staining is that its detection limit is about 100 times lower. In principle, silver ions bind to the protein, where they are reduced to metallic silver, and thus produce a band on the gel.

The PAA gel was fixed in 60 ml 50 % (v/v) acetone, 1.25 % (w/v) trichloroacetic acid (TCA) and 0.015 % (w/w) formaldehyde. After washing with dH₂O for 3x 5 seconds, 5 minutes and another 3x 5 seconds, the gel was incubated another 5 minutes with 60 ml 50 % (v/v) acetone. It was soaked in 60 ml 0.017 % (w/v) sodium thiosulfate (Na₂O₃S₂ in dH₂O), washed with water (3x 5 seconds) followed by an eight minute incubation in a solution of 0.27 % (w/v) silver nitrate (AgNO₃), 0.37 % (w/w) formaldehyde. After an additional wash step (dH₂O, 5x 5 seconds), the stain was developed in 60 ml 195 mM sodium carbonate, 0.015 % (w/w) formaldehyde and 0.004 % (w/v) Na₂O₃S₂ in dH₂O. The reaction was stopped in 1 % (v/v) glacial acetic acid.

2.2.4.4. Western blotting

A SDS-PAGE was run with a pre-stained protein ladder and transferred to a PVDF membrane (Millipore Immobilon-P PVDF, 0.45 μm). The membrane was first activated with methanol (5 minutes), washed in dH_2O (1 minute) and transfer buffer (5 minutes). The assembled gel sandwich was run in 1x transfer buffer at 400 milliampere (mA) for 1.5 hours at 4°C (Hoefer TE 22 Mighty Small Transphor Electrophoresis unit, GE Healthcare). The membrane was blocked in 5 % milk in TBS-T, incubated on a shaker with a Qiagen penta-His antibody (diluted in 5 % milk/TBS-T) at 4°C overnight. The blot was rinsed 3 times, washed 5x 7 minutes with TBS-T and incubated with a 1:20 diluted solution of the ECL Western blot detection kit (GE Healthcare).

2.2.4.5. Determination of protein concentration

There are several methods to determine protein concentration in solution. The following protein assays were used to quantitate proteins.

2.2.4.5.1. Absorbance 260/280 nm

Measuring absorbance at 260/280 nm, and calculating the concentration is quite simple, but not very sensitive (depending primarily on the absorbance of the amino acids tryptophan and tyrosine). 260/280 nm measurements were always performed using a Nanodrop spectrophotometer (Thermo Scientific) unless stated differently.

2.2.4.5.2. Lowry

In this colorimetric assay, a complex between the peptide bonds and divalent copper ions (Cu^{2+}) is formed in an alkaline solution (Biuret-reaction). Then, Cu^{2+} is reduced to monovalent copper (Cu^+), which in turn reduces the yellow Folin-reagent. The solution turns blue, and absorption can be measured at 750 nm. Here, the Lowry assay was performed using a BioRad Dc protein assay kit and a BSA standard curve.

2.2.4.5.3. Fluoraldehyde assay (Pierce)

The reagent o-phthalaldehyde (OPA) reacts with the amino terminus of peptide chains to form a compound that is excited at 330-390 nm and emits light at 436 - 475 nm and can

therefore be used to determine protein concentrations. However, it also reacts with amino acids containing a primary amino group or Tris, so the buffer conditions have to be chosen carefully, and a standard curve should be preferentially done with the same peptide/protein. The assay has a quite low detection limit and is compatible with detergent and imidazole, two prerequisites for measuring concentrations of the high throughput purified protein used in these experiments. Protein concentrations from 0.05 mg/ml to 0.3 mg/ml yielded a linear curve, and were therefore used to generate a standard curve. The samples from the HTP purification were measured undiluted (excitation: 360 nm, emission: 460 nm).

2.2.4.5.4. 660 nm assay (Pierce)

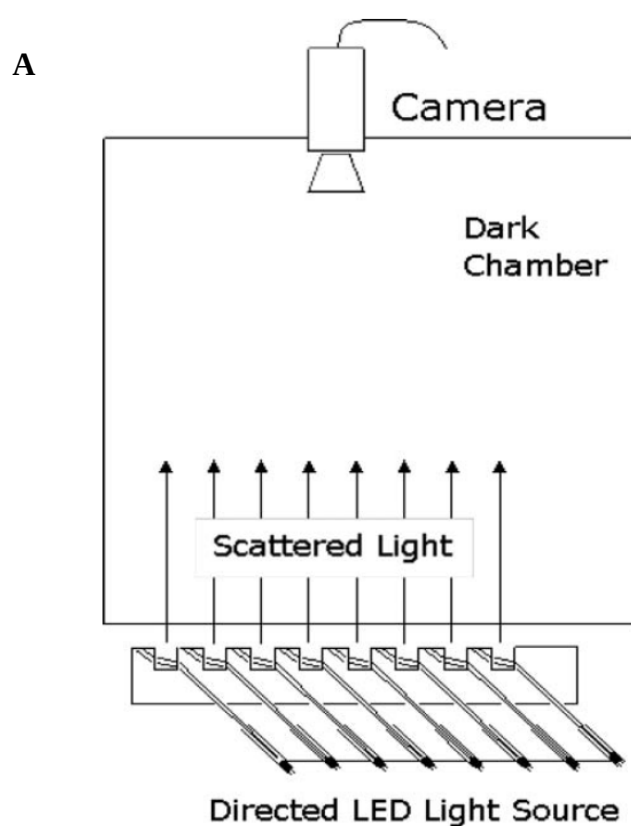
The reagent contains a complex that interacts with basic amino acids such as arginine, lysine and histidine, but also with phenylalanine, tyrosine and tryptophan, and absorbs at a wavelength of 660 nm. The assay was used in a microplate format according to the provided protocol.

2.2.4.6. Stargazer Screen (Harbinger biotech)

The stability of a protein can be measured by its melting temperature T_m , i.e. the temperature at which the protein unfolds or denatures. The T_m of a protein can be determined by several methods, for example differential scanning calorimetry (DSC). However, a major drawback of these methods is that only one sample can be measured at a time. As the protein unfolds, it forms particles of increasing size in solution, which can be measured by static light scattering. Exactly this principle is adopted by the Stargazer assay. The machine itself consists of a heating block with an underlying light source (LEDs) and an overhead camera (Fig. 11A). The samples are measured in 384 well plates, which allows one to assay many different conditions, for example buffers, ligands or mutant proteins, at the same time. The heating block heats the sample, and an image (Fig. 11B) is captured every 0.5 °C. The increase in static light (see circle, Fig. 11C) scattering is determined via a provided program (Harbinger Biotech, Intensities). The increase in light intensity is integrated into a melting curve (Bioactive program), from which the aggregation temperature (T_{agg}) can be calculated. The T_{agg} is not the same as the T_m , but

both values should lie in a very similar range. The Stargazer cannot be used to determine exact melting temperatures, but to compare aggregation temperatures in different conditions to each other.

Protein samples for Stargazer assays were pipetted into Nunc 384 well optical bottom black plates in 50 μ l aliquots, to a final concentration of 0.1 to 0.2 mg/ml (duplicates). 50 μ l of mineral oil were overlayed, and the plates were centrifuged at 3000 g for 5 minutes. Well A24 always contained only buffer and oil (temperature measure well).



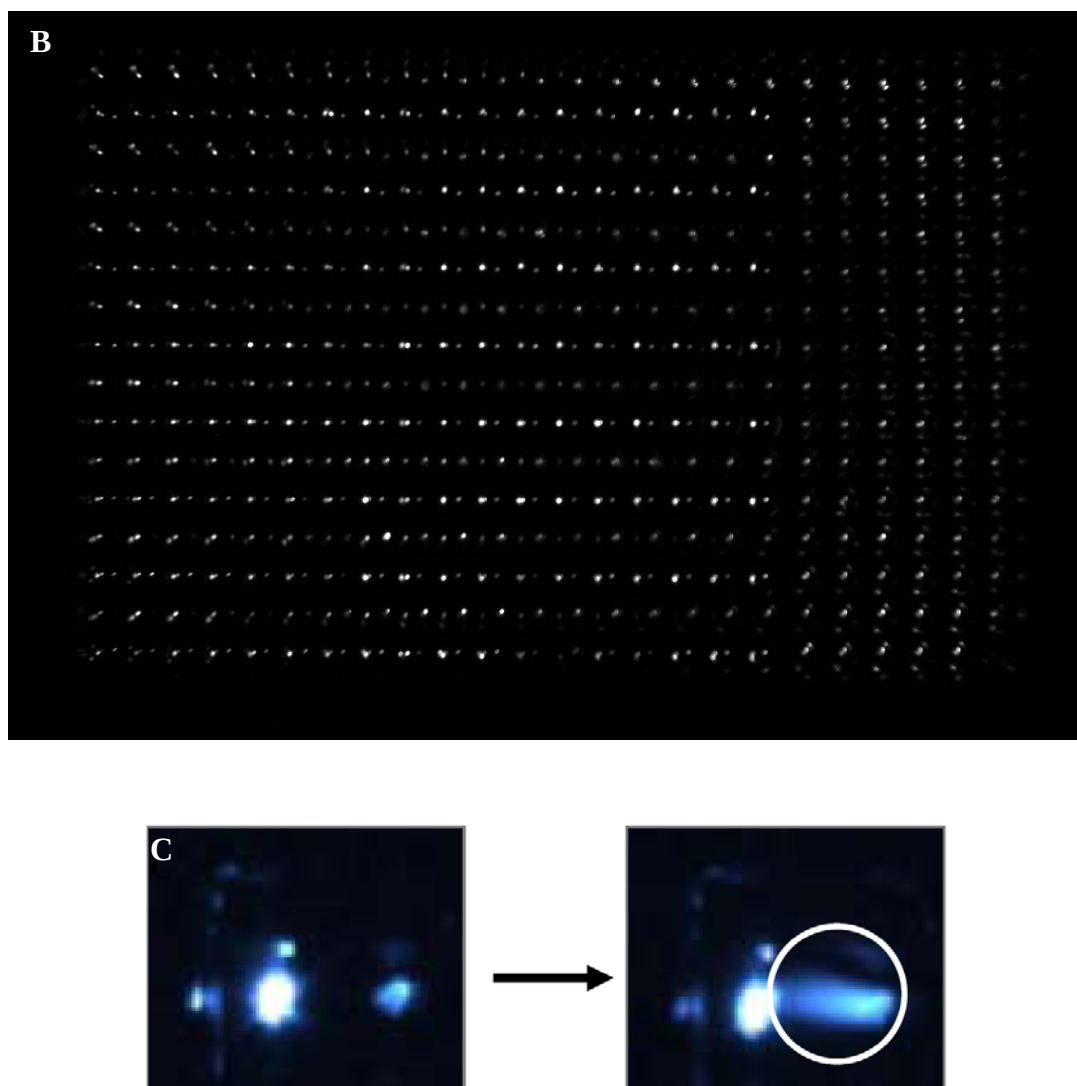


Figure 11. The Stargazer. (A)¹⁰ Schematic representation of the Stargazer setup. A heat block with an underlying LED source heats up to protein samples, while an overhead camera takes a picture every 0.5 °C. (B) Picture of a 384 well plate taken by the camera. (C)¹¹ Enlarged image of one well at two different temperatures. The scattered light lies between the reflected and the incident light. The increase in scattered light can easily be seen in the left panel (see circle)

¹⁰ Figure taken from Senisterra et al., 2006

¹¹ From StargazerTM-384, High through-put protein stability analyzer product brief, Harbinger Biotech

3. Results

The important thing in science is not so much to obtain new facts
as to discover new ways of thinking about them.

Sir William Bragg (1890-1971)

3.1. Optimization of the assays

3.1.1. Optimization of the growth assay

This simple and fast growth assay relies on the ability of YdgR to take up various substrates, including an antibiotic called alafosfalin (Hahn, 1981). This toxin is a phosphonopeptide (Fig. 12) and inhibits cell wall synthesis, and is a specific substrate for YdgR. YhiP, one of the family members, also transports alafosfalin, but to a lower extent than YdgR (Harder et al., 2008). Therefore, cells can only survive in the presence of this antibiotic when they express a loss-of-function YdgR mutant, but not when expressing wildtype protein (or a functional mutant).

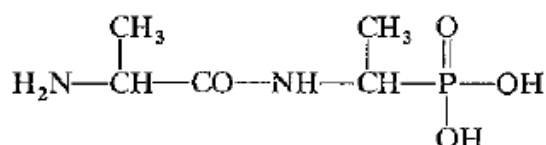


Figure 12. The phosphonopeptide alafosfalin.¹² YdgR takes up the peptidomimetic antibiotic alafosfalin, which inhibits cell wall synthesis.

First, the growth assay had to be optimised. Cells expressing the mutant proteins were grown in a 96 well plate format in the presence of alafosfalin, and their OD₆₀₀ was measured at different time points. Several different conditions for the assay were investigated. First, three different cell types were chosen, namely BL21(DE3), which is generally a good strain for expressing high levels of proteins, C43(DE3), a strain that carries an unknown mutation making it more suitable for membrane protein expression, and the YdgR knock out strain JW1626, which has the advantage of expressing recombinant YdgR-His without any native background. Furthermore, the cells were induced with either 0.01 or 0.1 mM IPTG, and were grown with either 200 or 500 µg/ml alafosfalin. Induction with higher concentrations of IPTG seemed to decrease cell growth, most probably because elevated levels of expressed YdgR were toxic for the cells. Also, a

¹² Figure taken from Hahn, 1981

high concentration of alafosfalin was detrimental to cell growth, even when recombinant YdgR was not expressed. Probably natively expressed YdgR, or its family members in the case of the delta strain, was enough to take up the noxious compound. After several experiments, it was clear that the BL21(DE3) cells grown in 200 µg/ml antibiotic and induced with 0.01 mM IPTG yielded the most reproducible and reliable results. In Figure 13B, several wildtype and mutant cultures are shown. The wildtype cells started to die one hour after induction, whereas the L-O-F mutant, a Y156A mutation in the FYING-PTR motif, expressing cells continue to grow. The uninduced cells treated with alafosfalin grew much slower than the control cells without the toxin (Figure 13A).

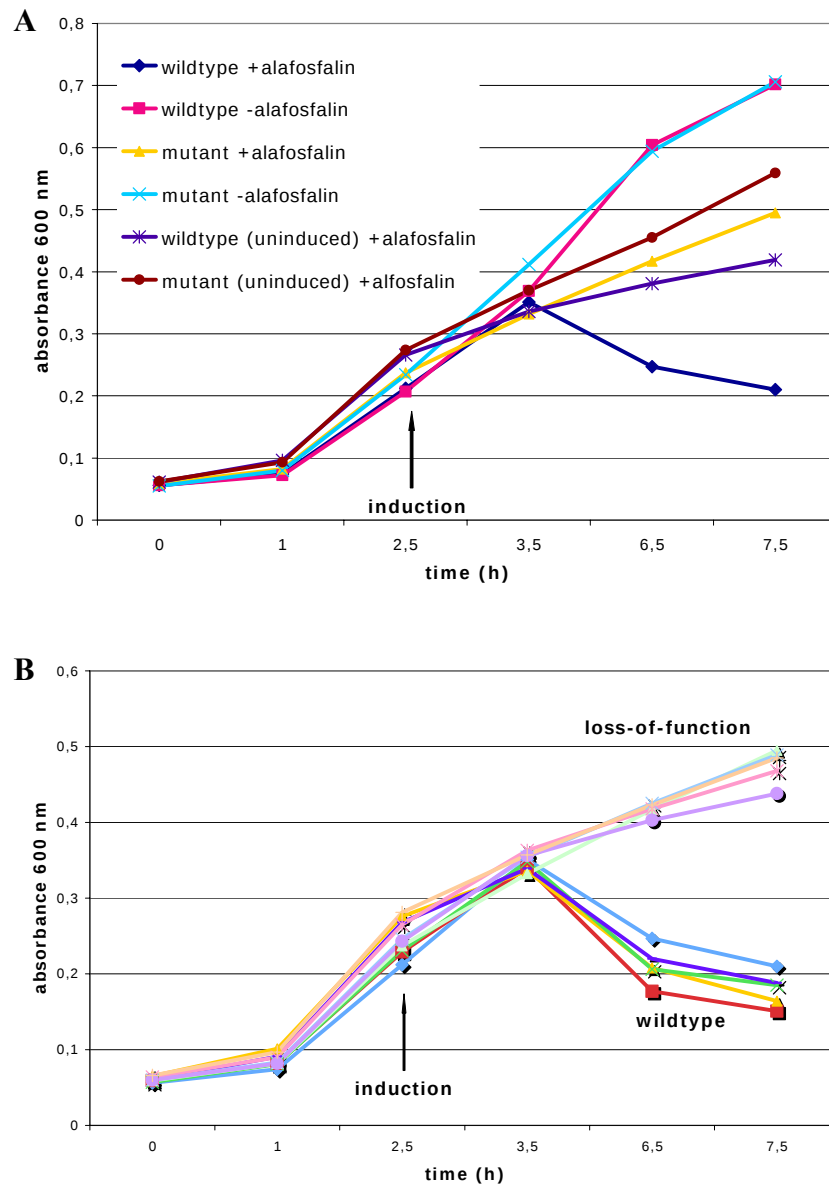


Figure 13. Growth assay with alafosfalin. (A) Only loss-of-function mutants can survive in the presence of the antibiotic alafosfalin (yellow) since they do not take up the toxin. Cells expressing wildtype protein (black) start to die approximately one hour after induction. Control cells without alafosfalin grow much better (wildtype: pink, L-O-F mutant: turquoise) than the uninduced control with the antibiotic (wildtype: purple, L-O-F- mutant: brown). (B) Control experiment with several wildtype (bright colors) and the loss-of-function mutant Y156A (pastel-colored) cultures. BL21(DE3) cells induced with 0.01 mM IPTG and grown in the presence of 200 μ g/ml alafosfalin yielded the most reliable results.

3.1.2. Optimization of the purification

3.2.1.1. Growth and expression

Concomitantly with the growth assay in the screen, the same mutant proteins should be expressed in small cultures for the high-throughput purification. Therefore, growth and protein production had to be optimised.

The pre-cultures already used in the growth assay were inoculated in 1 ml LB/carbenicillin in 96 well plates. After several trial purifications, it was decided that these cultures simply yielded too little protein. Therefore, the volume was increased to 2 ml, and the cells were grown in 4x 24 well plates instead where better aeration and therefore better cell growth and expression resulted than in the smaller wells. It was also tested, which of the three cell types, BL21(DE3), C43(DE3) and JW1626, expressed the most protein. YdgR containing a c-terminal GFP-tag and the Y156A mutant were expressed in these cell lines and analysed on a Western Blot (Fig. 14). BL21(DE3) exhibited the highest protein levels compared to the other two (see arrow).

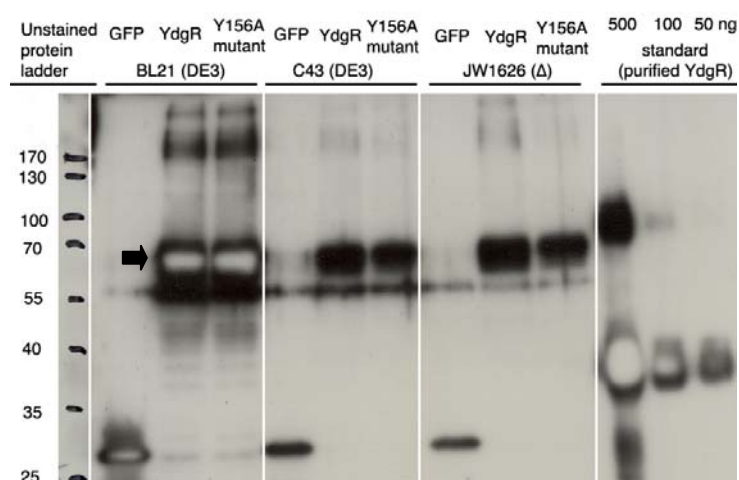


Figure 14. Expression of YdgR-GFP in different cell lines. Western blot of BL21(DE3), C43(DE3) and JW1626 expressing a YdgR-GFP construct. The *Escherichia coli* BL21(DE3) strain was found to express the highest levels of YdgR-GFP (arrow) when compared to C43(DE3) and knock-out strain JW1626. As a positive control, purified YdgR (without GFP-tag) was added. The empty vector expressed only GFP (band at 26 kDa).

3.2.1.2. Lysis

Initially, the lysis buffer contained 20 mM Tris, 300 mM salt, 15 mM imidazole. Furthermore, 1 % DDM (Fig. 15A) to solubilize the membrane, 5 mM substrate (A-A) to stabilize the YdgR transporter, 1 mg/ml lysozyme to enhance bacterial lysis, 10 U/ml Benzonase to digest nucleic acids, Complete protease inhibitor to protect YdgR from degradation and the reductive agent DTT were added. The buffer was added to the bacteria pellet, and lysis was carried out with shaking at 4°C for one hour. Since the lysis was not very efficient, I tried to optimise it by changing the lysis method. The original approach was compared to sonication, lysis with a different detergent (foscholine-12, FOS-12, Figure 15B) and the commercially available lysis buffer BugBuster (Novagen). A silver stained SDS-Page of the high throughput purified proteins is shown in Figure 15C. The membranes solubilized with the detergent FOS-12 yielded the highest level of protein, whereas DDM and sonication displayed fairly equal but lower concentrations. BugBuster, however, seemed to be the least suitable method since there are no protein bands visible on the silver stain.

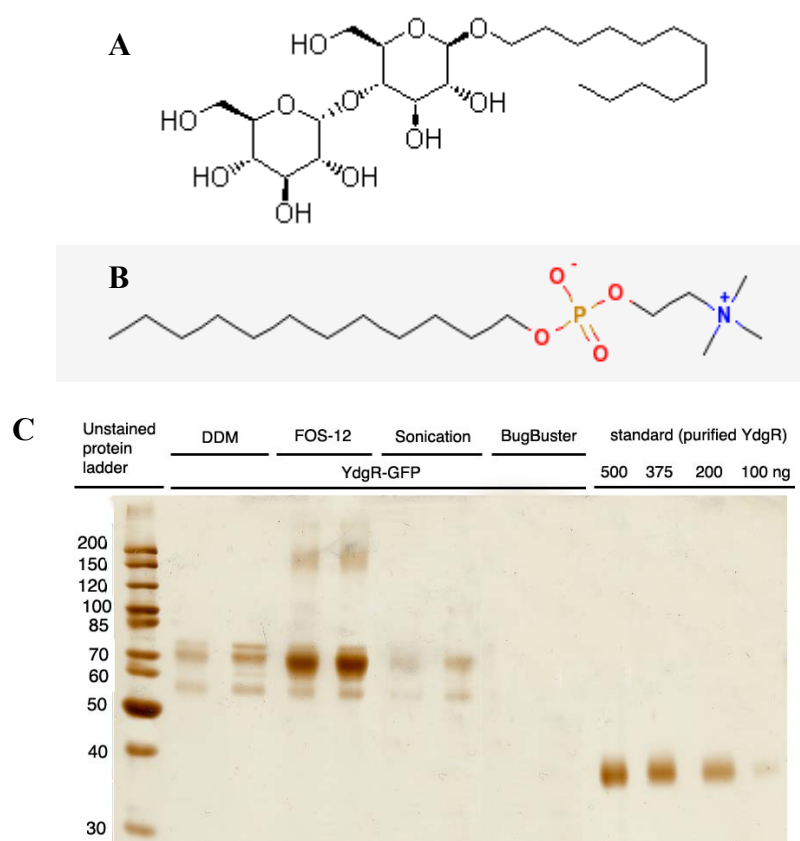


Figure 15. Optimization of lysis. Structure representation of the detergents **(A)**¹³ dodecylmaltoside and **(B)**¹⁴ foscholin-12. **(C)** Solubilization of YdgR from BL21 (DE3) by 4 different methods: The detergent foscholin-12 yielded the highest protein levels, whereas solubilization with DDM and sonication gave remarkably lower protein levels. Using the commercially available BugBuster (Novagen) did not work for YdgR solubilization.

FOS-12 is a fairly aggressive detergent, effective at solubilization, but often also detrimental for protein stability. Therefore, a purification of YdgR and a subsequent analysis on an analytical gel filtration column should allow for determination of the protein quality when solubilized with different detergents. The protein was solubilized out of prepared membranes with DDM or FOS-12 or a mixture of both. It was then purified over a Ni-NTA column and run on a gel filtration column (Superdex 200 PC 3.2/30, Äktaexplorer Ettan, GE Healthcare). The detergent concentrations for lysis, wash and elution buffers and their respective ratios are given in Table 7.

¹³ Figure taken from <http://www.chemblink.com/products/69227-93-6.htm>

¹⁴ Figure taken from <http://pubchem.ncbi.nlm.nih.gov/>

Table 7. FOS-12/DDM detergent ratios and concentrations.

	Lysis Buffer	Washing Buffer	Elution Buffer	FOS:DDM
	FOS-12/DDM	FOS-12/DDM	FOS-12/DDM	ratio
1	1 % FOS-12	0.2 % FOS-12	0.1 % FOS-12	
2	1 % FOS-12	0.2 % FOS-12, detergent exchange to 0.04 % DDM	0.02 % DDM	
3	1 % DDM	0.04 % DDM	0.02 % DDM	
4	1 % / 0.04 %	0.2 % / 0.01 %	0.2 % / 0.01 %	4:1
5	0.8 % / 0.7 %	0.2 % / 0.02 %	0.1 % / 0.01 %	2:1
6	0.5 % / 0.1 %	0.2 % / 0.04 %	0.1 % / 0.02 %	1:1
7	0.5 % / 0.2 %	0.2 % / 0.1 %	0.1 % / 0.04 %	1:2

Instead of a single peak, which is present in DDM purified protein, FOS-12 detergent indeed had a negative effect on protein stability. Before the peak corresponding to monomeric protein, there was a second peak, which most probably represents aggregated protein (Fig. 16). This peak could be diminished when FOS-12 was gradually decreased and DDM was increased. When the FOS-12 was replaced with DDM in a detergent exchange step during washing, the second peak disappeared, but the size still corresponds to aggregated protein. The conjecture that FOS-12 destabilized the protein is further enhanced by the fact that protein purified in FOS-12 or protein samples where FOS-12 has been added after purification, showed no melting curves in Stargazer assays, but rather behaved like aggregated protein. FOS-12 is therefore not suitable for high-throughput purification. The detergent exchange during purification is not an alternative either because the protein yield was much too low.

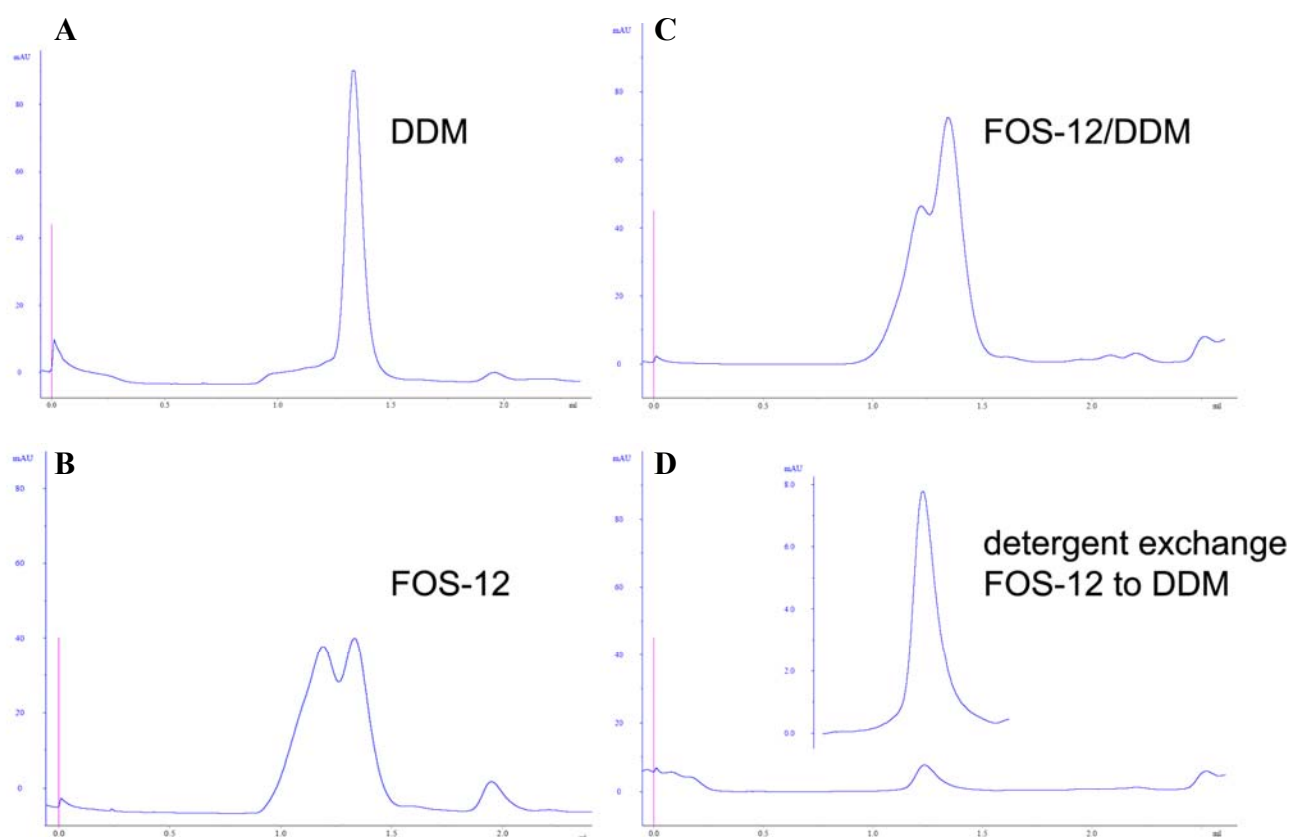


Figure 16. Gel filtration chromatograms of YdgR purified in FOS-12/DDM. (A) Protein purified in only DDM does not show any aggregates, only one peak corresponding to monomeric protein. (B) YdgR purified in FOS-12 gave two peaks, the first of which probably corresponds to aggregated protein. (C) The higher the DDM concentration, the lower this aggregate peak becomes. (D) A detergent exchange gives only an aggregate peak, and greatly decreases protein concentration (upper peak in panel D 10 times enlarged).

Since none of the tested lysis methods worked, and DDM seemed to be an excellent detergent for the purification of YdgR, I tried to enhance lysis in DDM containing buffer instead. It was observed that the lysed samples were very viscous, which might be detrimental for binding to the nickel-beads. Increasing the volume of the lysis buffer did not enhance the concentration of purified protein. By decreasing the salt concentration to 150 mM and adding 2 mM magnesium chloride to the buffer to improve Benzonase function, viscosity decreased considerably. Furthermore, the lysis time was expanded to two hours, and a freeze-thawing cycle ($-20^{\circ}\text{C} \rightarrow \text{room temperature}$) was added to improve lysis.

3.2.1.3. Purification

Initially, HTP purification trials were performed with Qiagen Ni-beads with a washing buffer volume of 500 μ l. This yielded a considerably pure protein, so about 500 mutants were screened in these conditions. However, it was not considered that switching from initial trials to the high throughput screen i.e. from 1 ml to 2 ml cultures and from Qiagen Ni-NTA in BioRad columns to GE Healthcare pre-pipetted Ni-plates, would lead to much less pure protein purifications (Fig. 17A). Increasing the washing buffer helped greatly (Fig. 17B), but it also reduced YdgR concentration, so the Stargazer assay could not be run in duplicates anymore. So far, the lysate had been incubated with the Ni-beads overnight to increase the protein yield. However, determination of protein concentration from samples incubated overnight or for ten minutes showed that actually a shorter incubation led to higher protein levels. A summary of the optimized purification protocol is given in Table 8.

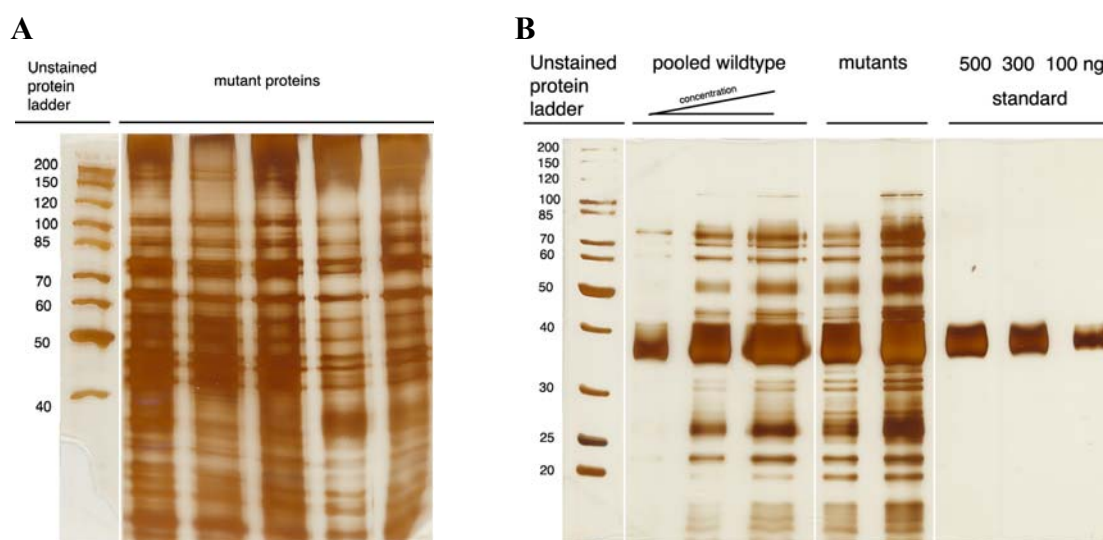


Figure 17. High-throughput purification of YdgR. The protein levels of HTP purified protein from (A) Qiagen Ni-NTA and (B) GE-Healthcare pre-pipetted plates were compared in a silver staining of a SDS-PAGE.

Table 8. Optimization of the HTP expression and purification.

	Initial protocol	Optimised protocol
Expression	1 ml culture	2 ml culture
	96 well plate	4x 24 well plates
HTP purification	100 µl lysis buffer	350 µl lysis buffer
	1 hour lysis	2 hours lysis
	Detergent: DDM	DDM
	300 mM NaCl in lysis buffer	150 mM NaCl
	No MgCl ₂	2 mM MgCl ₂
		Freeze-thaw cycle
	500 µl wash buffer	1 ml wash buffer
	Overnight binding to Ni-NTA	10 minutes binding

3.1.3. Establishment and optimization of the Stargazer thermostability assay

The Stargazer assay was a new methodology established in this laboratory. First, purified YdgR was assayed for its thermostability in different buffers in different concentrations. These experiments were all performed in small, black 384 well optical bottom plates, each well accommodating only 10 µl of protein sample (0.1 – 0.2 mg/ml). The advantage of the smaller plates compared to the bigger ones, which need 50 µl of protein solution, is obviously that much less sample protein is needed. However, during the initial experiments it turned out that the error in the smaller plates was much higher than in the larger volume plates.

YdgR had different stabilities in different buffer conditions. Furthermore, some buffers seemed to completely denature the protein, because no interpretable aggregation curve could be obtained. An example for three different buffers is shown in Figure 18. 50 mM MES with 150 mM NaCl, pH 6 gave a reasonable aggregation curve and an aggregation temperature of 61.2 °C (dark blue). The stability could be further increased by 4 °C in a 50 mM pyridine buffer with 150 mM NaCl, pH 5.5 (green). However, in 50 mM PIPES with 500 mM KAc, pH 7 (light blue) the curve is not sigmoidal and therefore could not be interpreted. Linear regression gave a thermal stability of 140°C, which is of course

relatively unlikely. Also, the concentration dependence of the assay was tested. In the small 10 μ l plates, the average aggregation temperature for both 0.1 and 0.2 mg/ml samples was about 64°C. Switching from the 10 μ l to the 50 μ l plates rendered the assay much more reproducible, and it was observed that there actually was a concentration dependency.

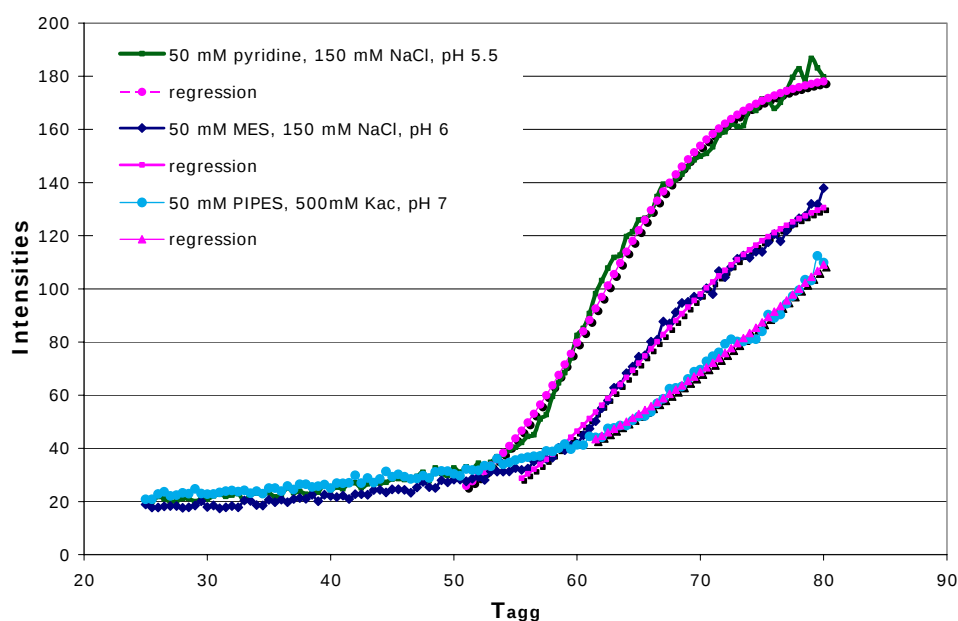


Figure 18. Aggregation temperature curves from a Stargazer experiment. Three different buffers are compared. When switching from 50 mM MES and 150 mM NaCl, pH 6 (dark blue) to 50 mM pyridine and 150 mM NaCl, pH 5.5 (green), the thermal stability is increased by 4°C. However, certain buffers such as 50 mM PIPES with 500 mM KAc, pH 7 (light blue) resulted in non-interpretable data.

3.2. Result of the screen

Summarizing the screen, the mutant library and wildtype, empty vector and the Y156A loss-of-function mutant as controls were transformed into BL21(DE3), a growth assay distinguished between functional and loss-of function mutants, the same mutant clones were grown as 2 ml cultures, from which the protein was high-throughput purified in Tris buffer and DDM, and the thermostability was determined in a Stargazer thermostability assay (Fig. 19).

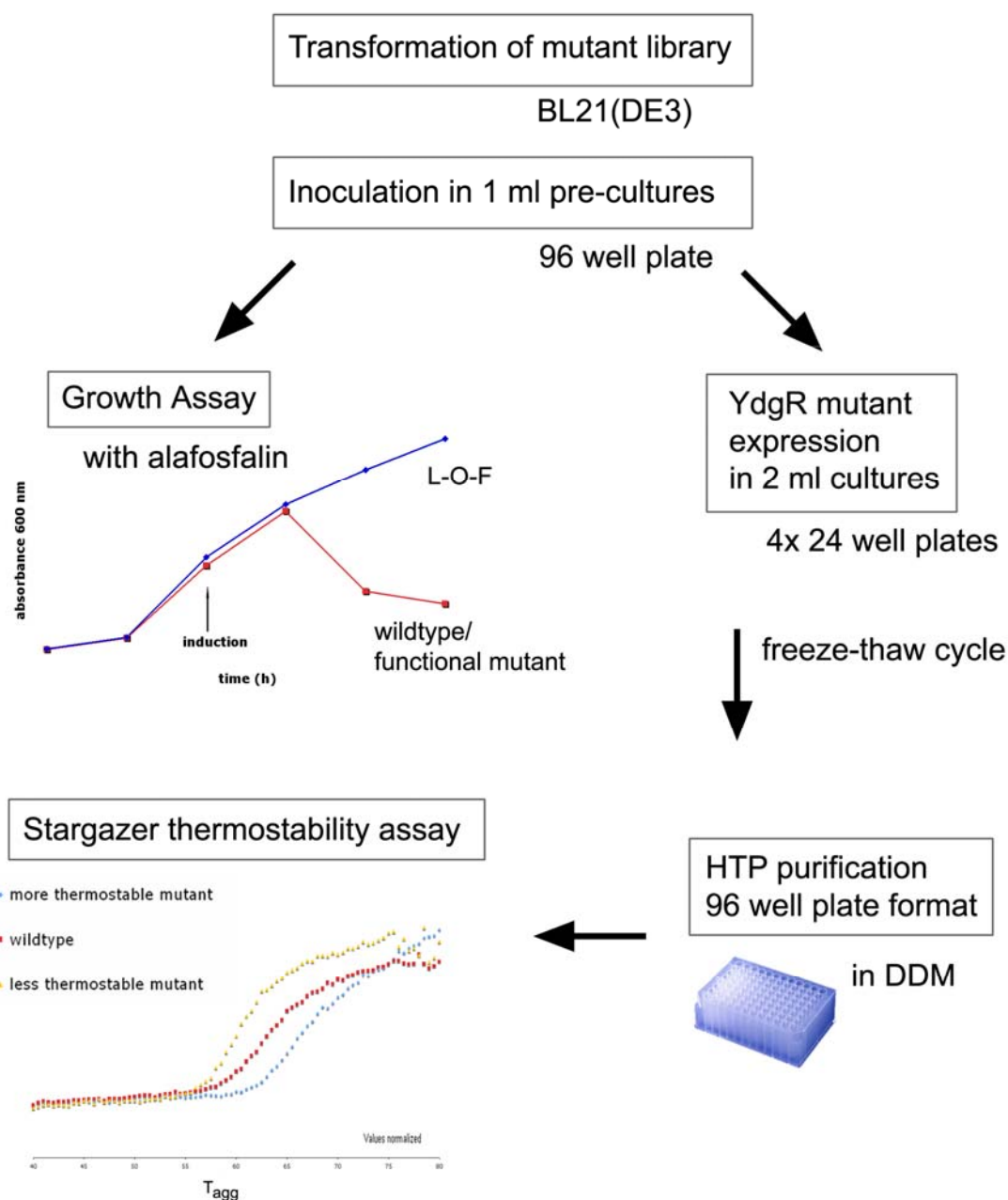


Figure 19. Scheme of the complete thermostability screening protocol. The mutant library is transformed into *E. coli* BL21(DE3), 1 ml overnight pre-cultures are used for both the growth assay (left) and the expression of the mutant clones in 2 ml cultures. The YdgR mutant proteins are high-throughput purified and analysed in a Stargazer thermostability assay.

I screened 720 mutants from the loop 6/7 library targeting transmembrane domains 6 and 7 and the interjacent loop domain. 630 of those mutants also underwent the Stargazer thermostability assay. Furthermore, 350 mutant clones in transmembrane helices 8 and 9

plus their intermediary loops were tested, 260 of which were assayed for their thermostability. The screen was performed in the above mentioned conditions (see Results 3.1.). The mutants from library loop 6/7 were purified with 500 µl wash buffer and hence were much dirtier than the protein purified later on.

3.2.1. Mutation frequencies

3.2.1.1. Library loop 6/7

The 720 mutants from this library resulted in 13 % loss-of-function mutants. Many of these were due to insertions or deletions usually resulting in a frame shift, and hence in a truncated version of the protein. The Stratagene mutagenesis kit promised an insertion frequency of 0.7 % and a deletion frequency of 4.8 %. The actual frequencies found were approximately 2 % single base insertions and 0.97 % single base deletions. However, many insertions were simply a duplication of parts of the mutated fragment indicating that the second PCR, in which the whole vector was amplified using the mutagenized fragment as a primer, did not work properly, possibly due to secondary structures in the fragment or base pairing of two fragments.

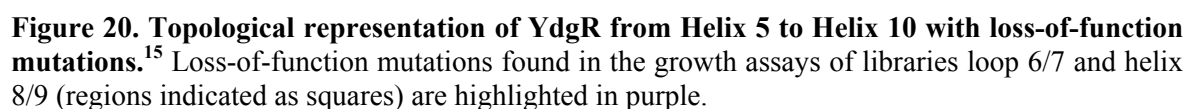
3.2.1.2. Library helix 8/9

Of the 350 mutant clones tested from this library, about 22 % were loss-of-function mutants. Sequencing analysis revealed that the second PCR step completing the plasmid worked much better for this library. Only two out of 76 loss-of-function mutants had a longer fragment inserted (i.e. 2.6 % of the L-O-F mutants, 0.6 % of the total library). The single base insertion and deletion frequencies amounted to 1.3 % and 15.8 %, respectively (corresponding to 0.3 % and 3.4 % of the total library). Generally, the quality of this library was better since also the mutation frequencies were relatively high: 26.3 % of the L-O-F mutants carried a single point mutation, 22.4 % had two and 3.9 % three point mutations (insertion and deletion clones as well as plasmids without interpretable sequencing data cover the remaining percent). The overall mutation frequency is not known since none of the functional mutants were sequenced.

3.2.2. Growth assay

In the first 180 clones screened of library loop 6/7, about 12 % were loss-of-function mutants. Only 18 % had point mutations, whereas over 59 % contained a long insert. In order to reduce DNA preparation and sequencing efforts, I established a colony PCR protocol to determine the length of the mutated fragment to exclude mutants with a long insertion. Taking together the results from all 720 mutants screened for transmembrane helix 6 and 7, 13 % of those were loss-function mutants, approximately half of which (53.2 %) had a long insertion (7 % of the total clones screened). Although the overall mutation frequency had been determined beforehand by sequencing 10 clones (6: single point mutation, 3: two point mutations or insertions, 1: four point mutations) in order to guarantee a reasonably high frequency, it turned out that the actual mutation frequencies were much lower, meaning that there were still many wildtype clones left in the screen. Sequencing of the loss-of-function mutants yielded only 8 single point mutations: M154K, A184T, L190V, N196K and F197I. The L190V mutant was found 3 times and the F197I two times. The remaining sequenced clones either had single base deletions or insertions, which led to a frameshift, or codons that were transformed into stop codons.

The following loss-of-function mutations were found in the growth assay of the screen of library helix 8/9: L249R, V252E, A264P, K274I, A285V, F289L, F289S, M295K (2 times), T297A, N300D, N300Y, F301I, A303G, R305C, N306I, Q320L, L324V (2 times) and P326Q. They are shown together with the loss-of-function mutations from library loop 6/7 in Figure 20 (purple, targeted regions: squares). Furthermore, four truncated versions of the protein resulted in a total loss-of-function: E263stop, K268stop, Q320stop and P326stop.



The determination of thermostability from library helix 6/7 yielded 45 candidates that had a higher aggregation temperature than the wildtype. Since the wildtype protein gave different temperatures from run to run, it was always high-throughput purified as an internal control, as well as the Y156A mutant, which was usually 2 to 3 °C lower than the wildtype. Also, the empty vector was expressed as a negative control, and never yielded any melting curves. Only 9 of these 45 sequenced thermostable mutants carried a mutation indicating that the screen detected a high number of false positives (see below). Table 9 lists the mutations that were found and their increase in aggregation temperature compared to the wildtype.

¹⁵ Figure generated with www.sacs.ucsf.edu/TOPO2-run/wtopo2.pl

threonine and an asparagine at position 220 mutated to tyrosine also appeared. The most notable mutation, however, was a 192 base pair or 64 amino acid insert, here referred to as Ins64. This mutation showed only a 0.3 °C increase in aggregation temperature, but remarkably, the protein could still transport the antibiotic alafosfalin in the growth assay.

Table 9. Increase of aggregation temperatures in different functional mutants.

Mutant	aggregation temperature Δ wildtype in °C	comment
L190V	+ 1.4/ + 3	found twice in thermostability screen, L-O-F
T193A	+ 7.85	
T193I	+ 0.7	
I194N	+ 0.7	
K210T	+ 1.9	
N217K	+ 3.1	
N217S	+ 0.9	
N220Y	+ 1.4	
Ins 64	+ 0.3	192 bp/64 amino acid insert

To confirm that these mutants were indeed more thermostable, all nine mutant proteins were purified in a large-scale protein purification (see 2.2.3.2.). The proteins were then analysed in a Stargazer assay to determine the stability of the highly pure protein. Unfortunately, none of the candidate proteins were more thermostable, except for the insertion mutant Ins64 (Fig. 21). Mutants T193I, T194A and N220Y were within a 0.7 °C range compared to the wildtype. K210T and N217K were 1.2 and 1.4 °C lower, I194N and N217S 2.4 and 2.6 °C. The L190V mutant, which showed up two times in the thermostability screen, was even 4.5 °C decreased. The only mutant that was surprisingly more thermostable was the Ins64 mutant, which was 2°C higher than the wildtype.

Unexpectedly, the aggregation temperatures were very concentration dependent, as can be seen in Figure 21, where the temperature difference between 0.1 mg/ml samples and 0.2 mg/ml samples is shown. The aggregation temperature of the wildtype in its lower concentration is 9°C higher than the sample with the higher concentration. This feature had

not been observed in previous Stargazer experiments (see Results 3.1.3.). However, this explained the false positive thermostable mutants detected in the Stargazer assay since the concentrations in the high-throughput purified protein samples differed to a very large degree. Library helix 8/9 was not analysed for thermostability since it was already clear that the Stargazer assay is tremendously concentration dependent.

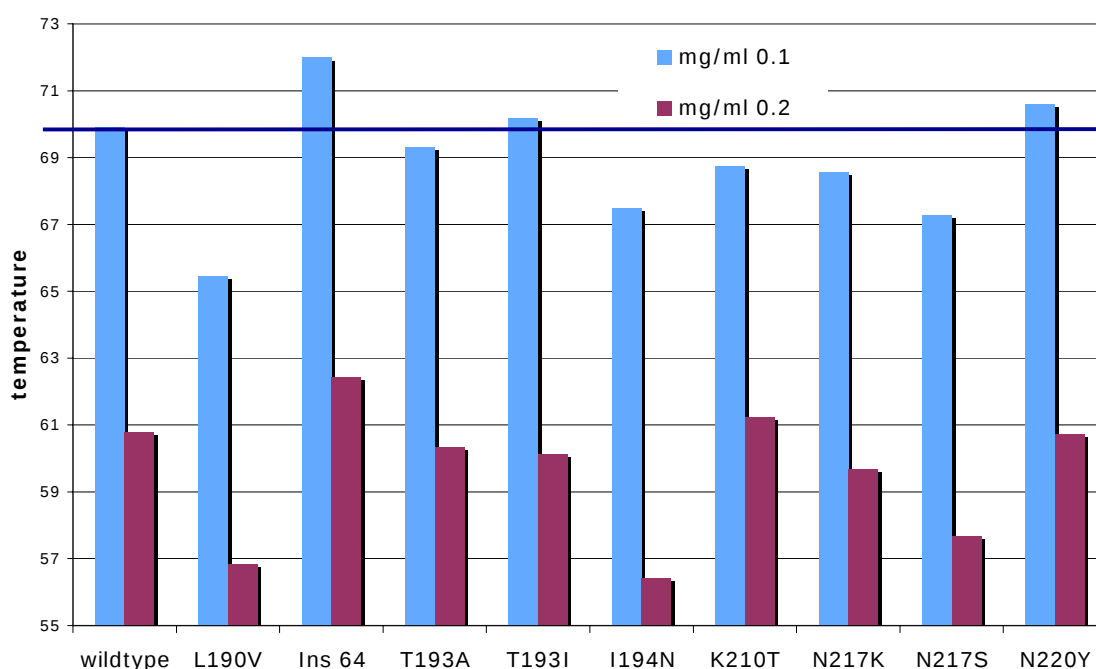


Figure 21. Aggregation temperatures of purified thermostable candidates. The diagram compares the aggregation temperature of different mutants in two different protein concentrations (blue: 0.1 mg/ml; purple: 0.2 mg/ml). None of the candidates is actually more thermostable than the wildtype except the insertion mutant Ins64.

3.3. Optimization of the screen

Since the aggregation temperature was found to be concentration dependent, this caused an immense problem for the thermostability screen because the concentrations in the high throughput purified protein samples varied greatly. Also, another major problem arose. The highly pure protein already has a quite high aggregation temperature. Since the Stargazer measures protein aggregation from 25°C to 80°C, proteins with aggregation temperatures

above 70°C do not give interpretable results. Additionally, the high throughput purified protein was further stabilized by the impurities abundant in the purification. This poses an additional problem for accuracy when wanting to observe minor temperature changes in the range of 2 to 3°C. In order to solve this problem, two strategies were developed. First, the concentration from the HTP purifications had to be determined much more accurately. However, since it would be too laborious an effort to bring 96 different samples to exactly the same concentration, a “standard curve” plotting temperature against concentration should be established so that the temperatures and concentrations obtained from the screen could be normalized to this standard curve. Second, the aggregation temperature of YdgR should be reduced, in order to obtain lower and therefore more accurate values of the Stargazer assayed HTP proteins.

3.3.1. Concentration dependence of the Stargazer assay

The concentration dependence had not been tested in the larger volume plates before the purified candidates with increased thermostability were assayed (see Results 3.1.3.). Thereafter, the aggregation temperature of purified wildtype protein in increasing concentrations from 0.05 mg/ml to 0.6 mg/ml was determined. As can be seen in Figure 22, the aggregation temperature decreased with increasing protein concentrations. The curve is relatively linear from a concentration between 0.1 and 0.25 mg/ml and flattens out at higher concentrations. The data point at 0.05 mg/ml was not interpretable since the aggregation curve was distorted.

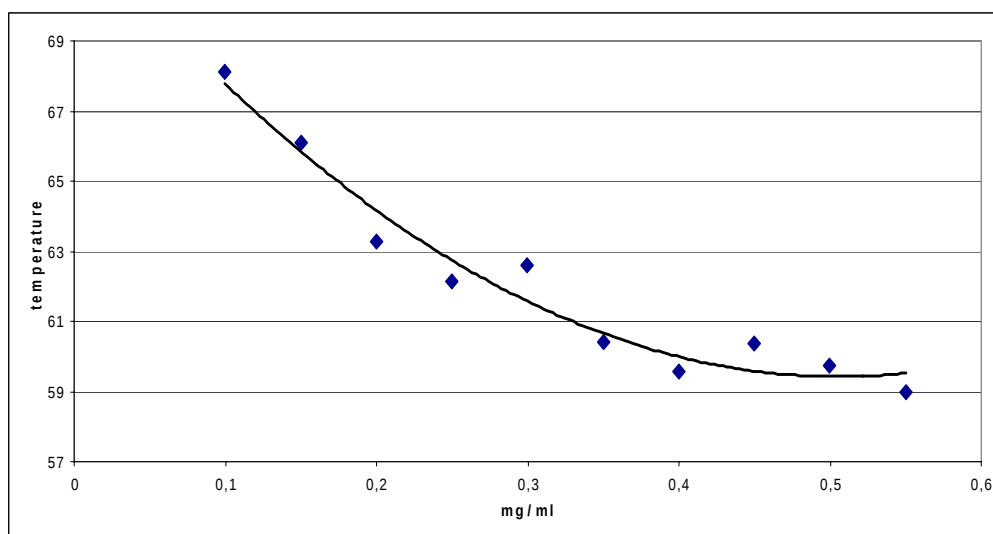


Figure 22. Concentration dependency of aggregation temperatures. The determined aggregation temperatures depend on the protein concentration of the Stargazer sample. The curve shows that the higher the concentration is, the lower the temperature. In a concentration range from 0.1 mg/ml to 0.25 mg/ml the decrease seems to be linear.

3.3.2. Determination of protein concentration

As mentioned previously, the protein concentration in the HTP purifications had only been approximated to lie between 0.1 mg/ml and 0.2 mg/ml. This was done by measuring the absorbance at 280 nm of about 10 to 15 sample concentrations with the Nanodrop spectrophotometer, and analysing the whole 96 well plate in a Synergy plate reader (also at 280 nm), calculating a standard curve, and calculating the other values from this curve. However, the values were very inaccurate when comparing the concentrations measured with the Nanodrop to calculated ones, but it was considered to be enough to give rise to interpretable Stargazer melting curves. Although measuring the absorbance at 260/280 nm is normally a very accurate method, this assay was not sensitive enough to obtain accurate concentrations in small volume samples. Many proteins assays are not compatible with certain buffer components, especially detergents and reducing agents. Three other assays were tested: the Lowry assay in microplates, which was not compatible with the high imidazole concentrations, and the Pierce assay 660 nm and the Pierce fluoraldehyde assay, both of which react with amino acids with measurable light absorbance. The latter turned out to be very accurate in the low concentration range of the HTP purifications. One

drawback, however, was that the reagent also reacted with the free amino groups of the Tris buffer. However, since HEPES worked for the purification as well, and more important, reduced the thermal stability (see below, 3.4.3.), it was decided that the purification be carried out in HEPES buffer instead of Tris.

3.3.3. Decreasing the thermostability of the wildtype protein

As already discussed, the thermostability of the HTP purified proteins should be decreased to yield more reliable aggregation temperatures. 90 mutants of library helix 8/9 were purified without glycerol to yield lower aggregation temperatures. In a Stargazer screen with protein purified under the original conditions, 10 % glycerol in the buffer enhances the stability by approximately 5.4 °C at a concentration of 0.1 mg/ml and 5.8 °C at 0.2 mg/ml (Fig. 23). Also, DTT was beneficial for YdgR stability, and resulted in an increase of 5.1 °C or 3.7 °C, respectively (see bars for thermostability without glycerol, with and without DTT). Addition of 200 mM substrate alanyl-alanine increased protein stability greatly (+11.3 and 12.4 °C, respectively). Notably, the temperature difference between the 0.1 and 0.2 mg/ml samples is not as pronounced as for the other conditions when substrate is present.

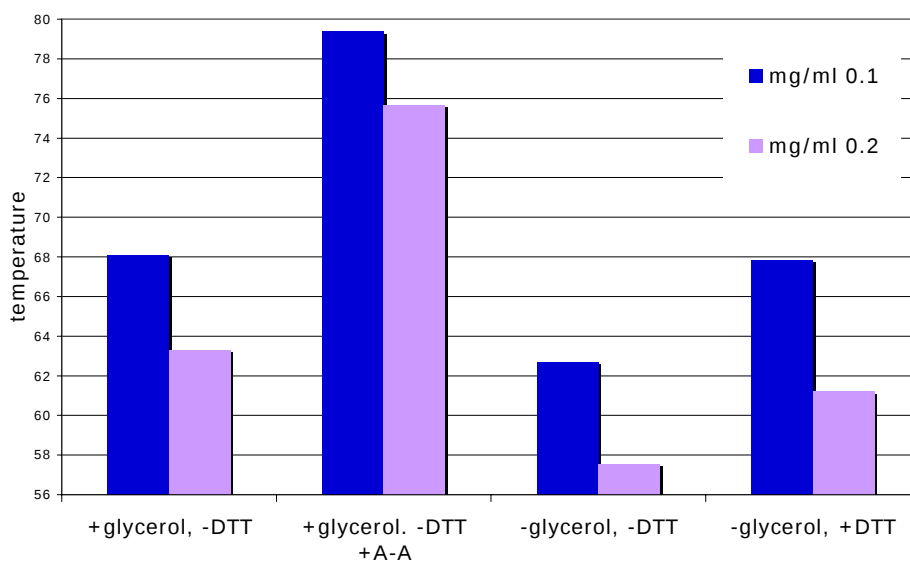


Figure 23. Stabilization and destabilization of YdgR. Upon addition of certain buffer components, the protein is stabilized. Alanyl-alanine, glycerol and DTT increase the thermal stability.

Purification without glycerol (no glycerol in the wash and elution buffers) reduced the temperature, but also resulted in low quality aggregation curves, and was therefore not optimal for the Stargazer assay. DTT had already been excluded from the HTP purification quite early in the optimization process. The buffer was then changed from Tris to HEPES, since lower aggregation temperatures had been observed in this buffer (experiment from Bettina Spitzenberger).

3.3.4. HTP standard curve

To be able to calculate enhancements in thermostability in the HTP assay, the “standard curve” was repeated with HTP purified YdgR in HEPES. The purification was performed using the standard GE Healthcare pre-pipetted Ni-plates as well as self-pipetted Qiagen Ni-NTA. YdgR concentrations were determined by measuring at 260/280 nm in the Nanodrop, and protein samples from 0.05 to 0.55 mg/ml were analysed in a Stargazer assay. As in the curve from the pure protein (Fig. 22), the decrease in temperature is relatively linear at first, and flattens out at higher concentrations (Fig. 24). An expansion of the linear part of the curve can be seen in the lower part of Figure 24 with the respective trend-lines and equations. The protein purified from the self-pipetted plate seemed to have slightly higher thermostability, and also exhibits slightly higher purity than the protein purified over the commercially available Ni-NTA 96 well plates (data not shown).

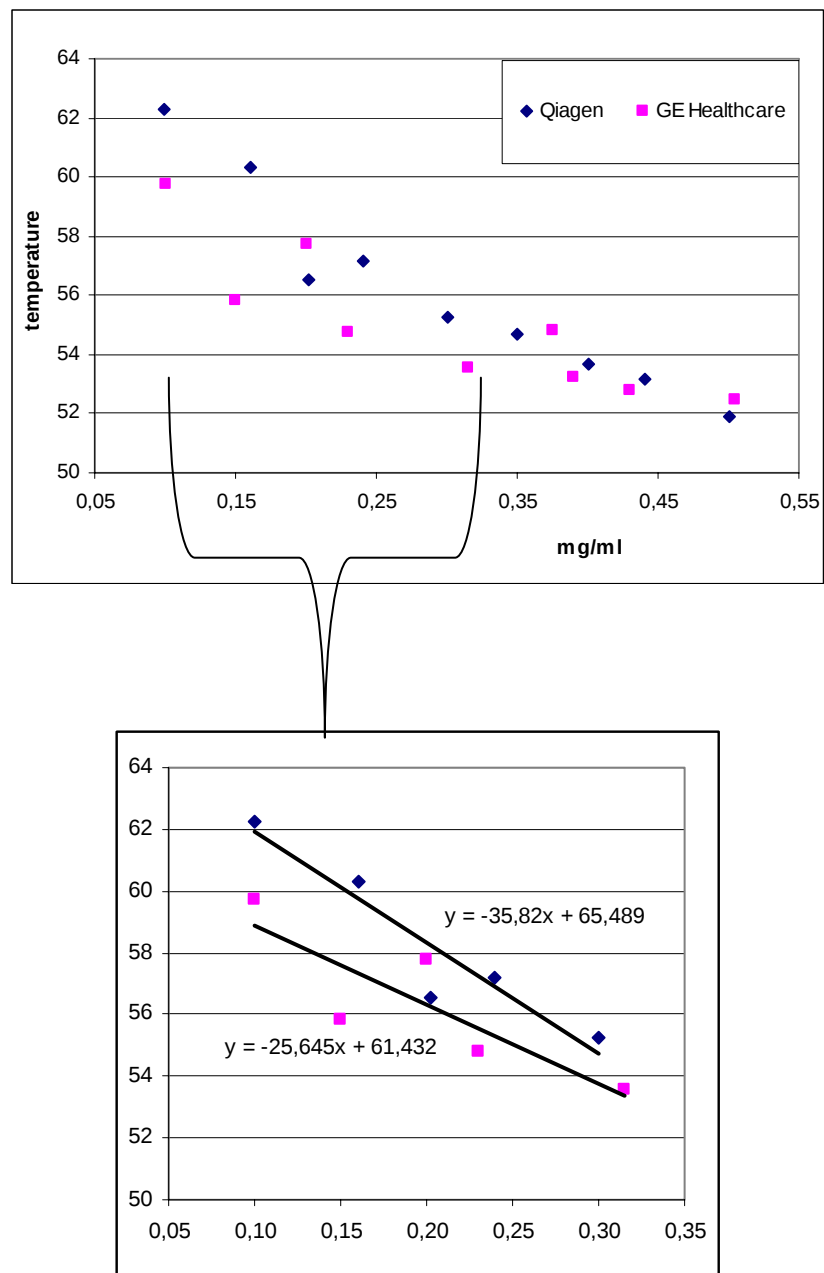


Figure 24. High-throughput purification standard curve. Since the aggregation temperature is very much concentration dependent, thermal stability of HTP purified protein was determined, and plotted against its concentration. From this standard curve, which is linear in the initial range (see arrow, enlargement, see also Fig. 22), obtained values from future HTP purifications can be back-calculated.

3.4. Characterization of loss-of-function mutants

In the screens of libraries helix 6/7 and helix 8/9 twenty-three loss-of-function mutants with single amino acid substitutions were found. Reason for a loss of function could be a misfolded protein, incorrect localisation or a protein locked in a single conformation that has therefore lost its transport ability. However, the growth assay with alafosfalin does not rule out that the mutant proteins may still transport other substrates. In order to characterize these mutants further, I performed the experiments described below.

3.4.1. Localisation to the plasma membrane

3.4.1.1. Small-scale membrane preparation.

First, proper localisation to the plasma membrane was verified by expressing the wildtype and mutant proteins in small scale cultures (see Materials and Methods, 2.2.3.1.), preparing membranes and analyzing them on a Western blot. With the help of Thomas Pollak, I could confirm that most of the mutant proteins with a single amino acid change were localized to the plasma membrane. In Figure 25A, cells expressing YdgR wildtype (+ control), the M154K and the A184T loss-of-function mutants before and after induction as well as purified membranes, and the supernatant yielded in the ultracentrifugation step are shown. There is sparse leaky expression of YdgR before induction, but it is greatly enhanced after induction with 0.1 mM IPTG and overnight expression at 18°C. Also, the negative control, i.e. cells harbouring the empty vector, displayed a weak background band. The protein signal (5 µg of protein) in the membranes is much stronger compared to the cells and the supernatant. However, because this Western is overexposed, one cannot compare the relative protein amount. However, it can be easily seen that there is little protein remaining in the cytosolic fraction (supernatant). The generation of additional inclusion bodies, however, cannot be excluded. Figure 25B shows some examples of 5 µg of membranes analyzed on a Western Blot with a 1:20 dilution of ECL. All proteins except the A264P, R305C, and the L324V mutants were abundant in the membranes. Also, the Q320L mutant exhibited only a very weak signal when the blot was exposed longer. Whether these three proteins were not expressed properly or whether they remained in the cytoplasm as inclusion bodies remains to be tested.

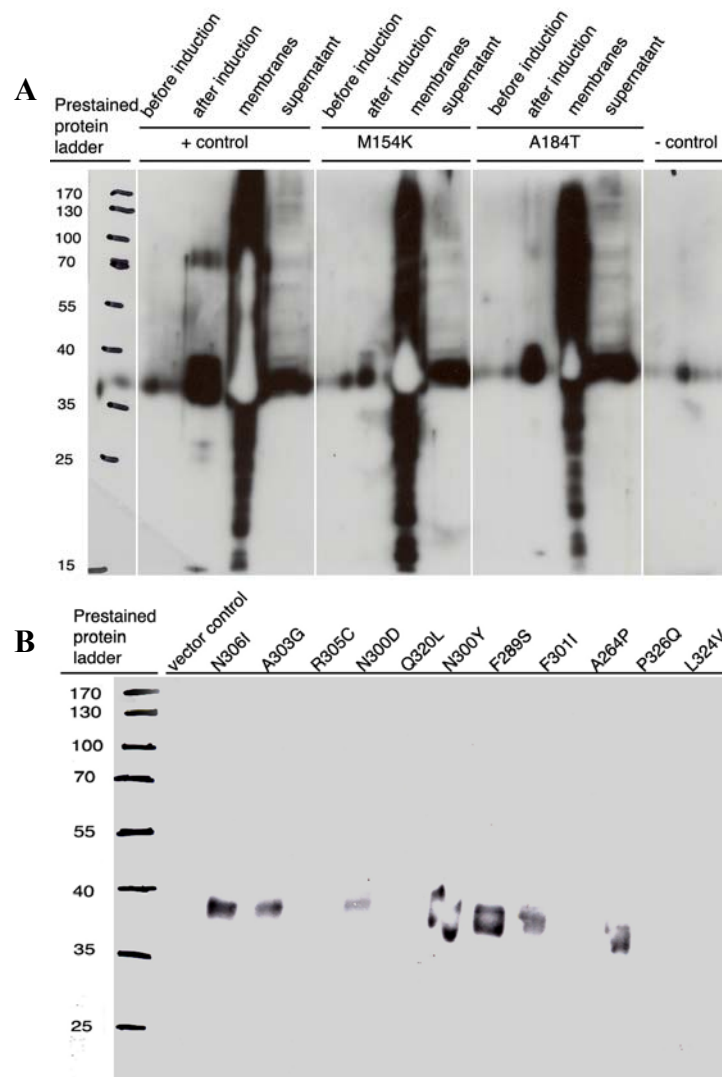


Figure 25. Membrane expression of L-O-F mutants. The membrane expression of YdgR mutant proteins was confirmed on a Western blot. All mutants except four (A264P, R305C, L324V, Q320L) were positively expressed in the plasma membrane.

3.4.1.2. GFP fluorescence

One other possibility to verify proper membrane localisation is to label the recombinantly expressed protein with a GFP-tag. When a protein tagged with GFP is overexpressed in its correctly folded form, or in this case in the native membrane inserted state, the GFP is able to fold correctly and fluoresce. However, when the protein is accumulating in inclusion bodies and in an unfolded/aggregated state, GFP is not properly folded and hence does not fluoresce (Drew et al., 2001). The wildtype and 6 loss-of-function mutants (M154K, Y156A, A184T, L190V, N196K, F197I) were cloned with a C-terminal GFP-tag (see

Materials and Methods). All of the tested mutants showed GFP fluorescence (Fig. 26) and are therefore most probably inserted properly in the plasma membrane. Compared to the uninduced cells, the fluorescence signal approximately doubled except for the expressed GFP positive control. The soluble GFP is naturally expressed in the cytoplasm and at much higher levels than when attached to a membrane protein. Cells expressing the positive control therefore showed an elevated level of fluorescence.

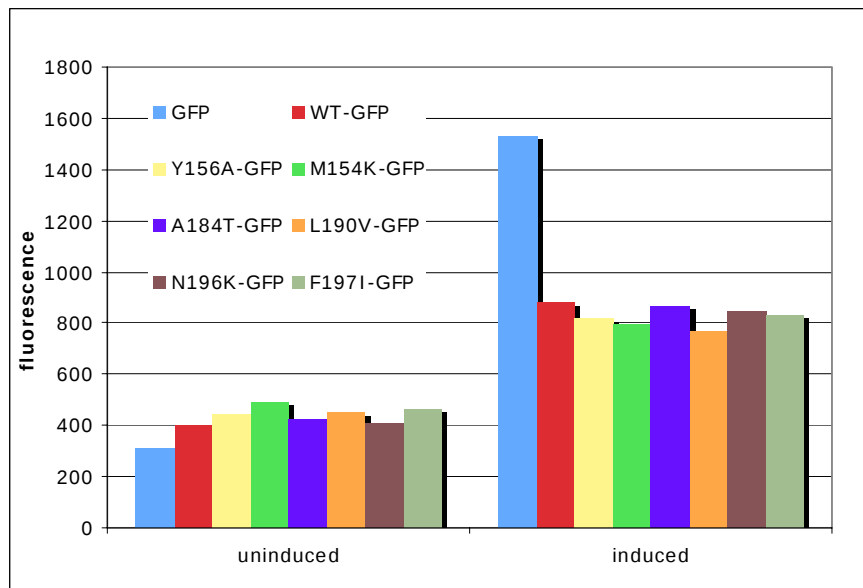


Figure 26. GFP-fluorescence of membrane expressed L-O-F mutants. GFP fusion protein variants were tested for GFP fluorescence. All of the so far tested mutants expressed GFP, and are therefore most likely properly folded in the membrane. These results also mirror the small-scale purifications (see Fig. 25)

3.4.2. Transport assay

As already mentioned, YdgR transports a vast number of substrates, not only di- and tripeptides, but also drugs and peptidomimetics such as the antibiotic alafosfalin used in the growth assay. Although the transporter is not very specific concerning its substrates, it is unlikely that it binds and transports all of its substrates with the same affinity or rate, respectively. The loss-of-function mutants discovered in the growth assay were identified as proteins that had lost the ability to transport alafosfalin. However, it would be possible that they are still able to transport other substrates. In order to investigate this, I performed an in-vivo transport assay using AMCA-labeled alanyl-lysine. The label AMCA, N-7-

amino-4-methylcoumarin-3-acetic acid, fluoresces upon excitation at 360 nm and the uptake of the dipeptide into the cells can be measured.

The wildtype YdgR and the loss-of-function mutants Y156A, L190V, and N196K were tested in this transport assay. Additionally, the insertion mutant Ins64, which was able to transport alafosfalin, was also examined. Compared to the cells transformed with empty vector, the cells expressing wildtype YdgR transport about twice as much substrate. The transport activity of the control is most probably due to the natively expressed YdgR and to residual traces of dye that have not been washed away. The number of cells should be the same for every construct tested. However, it must also be taken into account that not every mutant is expressed at the same level, and that some of the cells measured by absorbance at 600 nm might be dead. For this reason, a dilution series of the applied cells was carried out to determine the number of live cells by counting the number of colony forming units. The number of live cells was relatively even among the mutants, while the wildtype and vector samples contained four to five times more live cells (empty vector: 7×10^8 cells, wildtype: 5.8×10^8 cells, Y156A: 2.5×10^8 cells, L190V: 1.4×10^8 cells, N196K: 1.6×10^8 cells, Ins64: 1.3×10^8 cells). A Western blot comparing protein levels was also carried out as a control (Fig. 27B). The quality of the Western blot is relatively poor because the signal was much too strong, and the blot could only be photocopied after incubation with the ECL reaction kit instead of developing it. However, it can be easily seen that there is no protein in the vector control and that the wildtype, Y156A and L190V protein levels are relatively even. The N196K mutant exhibits a slightly weaker band, and the Ins64 mutant displays less than half the protein concentration when compared to the wildtype. When comparing these results to the results of the uptake assay (Fig. 27A), it can be concluded that the mutants Y156A and N196K actually are loss-of-function mutants, whereas the L190V could still transport the AMCA labeled dipeptide. The insertion mutant Ins64 also showed very low uptake levels, but may still be a functional transporter since only half as much protein is expressed when compared to the wildtype. The assay definitely requires optimization since the errors are too broad with the frequent occurrence of outliers, and there seems to be residual fluorescence that is not due to the uptake by YdgR, but rather to AMCA-labeled substrate retained on the filter-plates used in this assay.

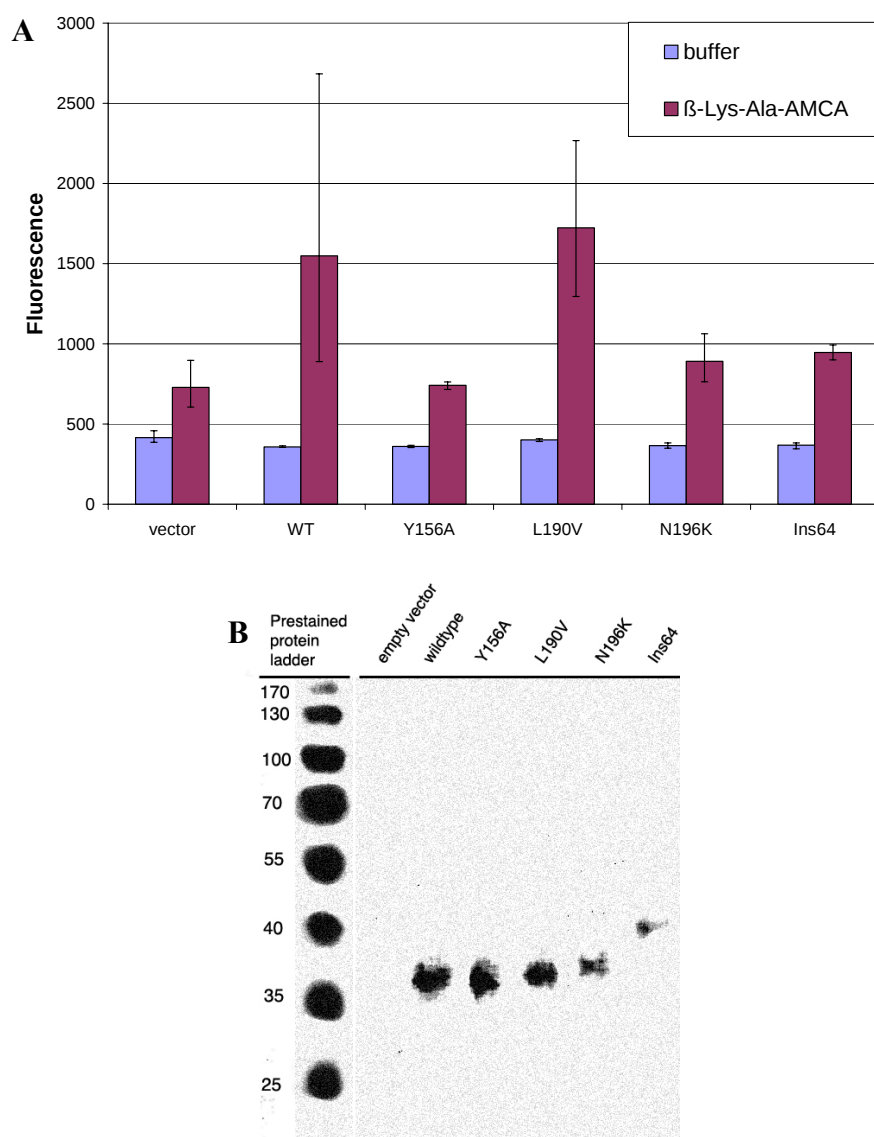


Figure 27. In-vivo transport assay. AMCA-labeled alanyl-lysine is a substrate for YdgR. (A) The diagram shows the transport functions of wildtype and several mutants i.e. the buffer control (blue) and the uptake of AMCA-Ala-Lys (purple). The L-O-F mutants Y156A and N196K did indeed lose their ability to transport. L190V was defective for the uptake of alafosfalin, but could still transport the dipeptide. Ins64 could still transport AMCA-Ala-Lys, what can only be seen when **(B)** comparing protein expression levels of the different constructs.

3.4.3. Thermostability of loss-of-function mutants.

Since the loss-of-function mutants have lost their ability to transport, it would be possible that some of these mutant proteins are trapped in a certain conformation. A mutant protein that is less flexible or cannot transition from one state to another, i.e. from the open to the

closed state, for example, might be also more thermostable than the wildtype protein. One of the loss-of-function mutants, L190V, was defective in alafosfalin, but not in β -Ala-Lys-AMCA uptake. Its thermostability has already been determined with the other thermostable candidates (see Results 3.2.3.), and it was approximately 3°C lower than the wildtype. Four other loss-of-function mutants (M154K, A184T, N196K, F197I), the wildtype protein and Ins64 as a positive control were purified over Ni-NTA (without gel filtration) and thermostability was measured in a Stargazer experiment. All the different mutants were purified at the same time, and thermostability was measured with freshly purified protein (Fig. 28). As in previous experiments, the aggregation temperature of the Y156A mutant was decreased. The positive control Ins64, which was already identified as a thermostable variant, showed an increase of 9.5°C (0.1 mg/ml) and 13.4°C (0.2 mg/ml), respectively. Remarkably, three mutants, namely M154K (+3.5°C), N196K (+3.9°C) and F197I (+4.3°C) exhibited enhanced thermostability, the latter two also at 0.2 mg/ml (N196K: +2.1°C, F197I: +0.4°C). Also, the temperature of the A184T mutant was slightly increased (+1.3 and +0.95, respectively).

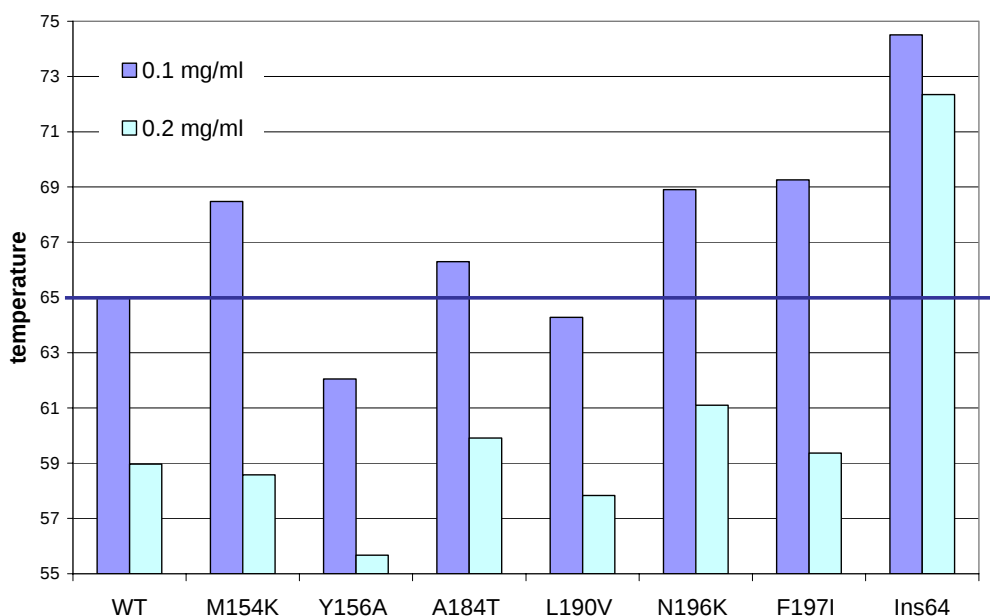


Figure 28. Aggregation temperatures of purified L-O-F mutants. The diagram compares the aggregation temperature of several purified loss-of-function mutants in two different protein concentrations. Indeed, three of the mutants, M154K, N196K and F197I exhibit an elevated thermal stability when compared to the wildtype. Ins64 served as a positive control.

The aggregation temperatures of all mutants, except for the insertion Ins64, increased enormously upon addition of substrate, either 200 mM alanyl-alanine or 100 mM alafosfalin (Fig. 29). This stabilizing effect is most probably due to binding of the substrate to the transporter. Ins64 could transport both alafosfalin and alanyl-lysine-AMCA, but it seemed that the addition of substrate does not stabilize this mutant protein. In this case, substrate binding may be very weak.

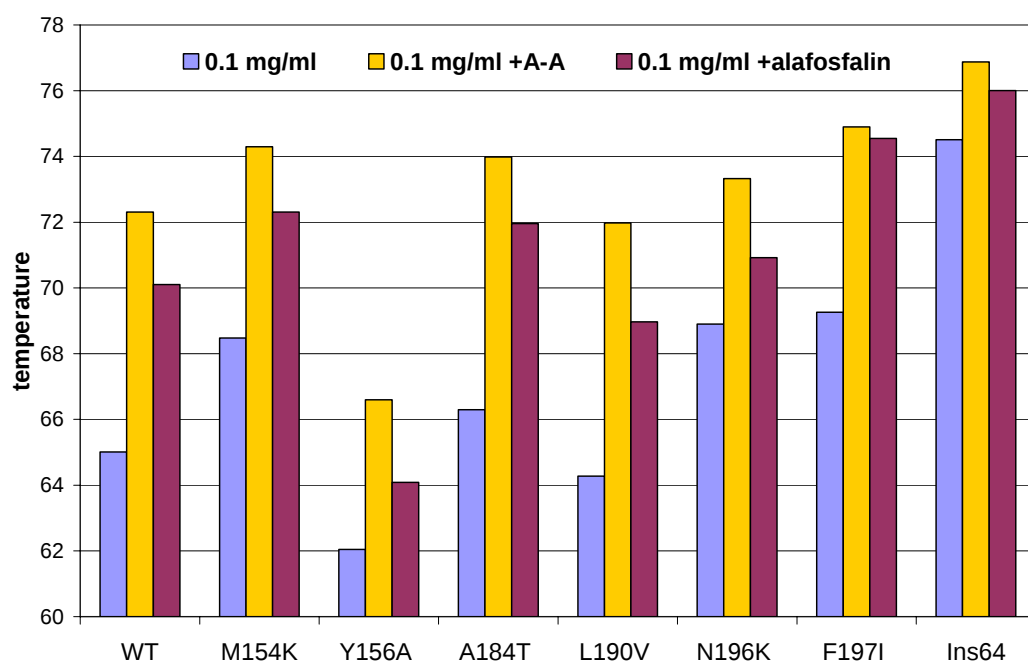


Figure 29. Stabilizing effects of substrate addition. Upon addition of 200 mM Ala-Ala or 100 mM alafosfalin, wildtype YdgR is remarkably stabilized. This is also true for most of the mutants except for the insertion mutant Ins64.

4. Discussion

In this diploma project, a simple and efficient screen to identify thermostable mutants of the *Escherichia coli* dipeptide transporter YdgR was developed and optimized. In summary, the screen consists of two steps. First, a growth assay distinguishes between active transporters and loss-of function mutants. In a second step, these proteins are first high-throughput purified and then subjected to a thermostability assay.

The initial screen yielded eight mutant candidates that were suspected to be more thermostable than the wildtype. However, after purifying them to high purity, and assaying them with the Stargazer thermostability assay, they proved to be false positives. Only one of the candidate proteins actually possessed a higher thermal stability than the wildtype. This one exception was a protein with a 192 bp insertion (64 amino acids) that coded for the predicted helices 6 and 7. Therefore, it is possible that this mutant protein contains two additional transmembrane helices. It is not clear why a larger protein with even more hydrophobic regions should be more thermostable. On the other hand, these helices might be stabilizing each other, resulting in a less flexible and therefore more stable structure. However, the insertion mutant is not useful for structure determination by X-ray crystallography because the insertion probably alters the overall protein architecture. However, it was later used as a positive control. The high number of false positives, and probably also false negatives, was due to the tremendous concentration dependency of the Stargazer thermostability assay. Furthermore, the high aggregation temperatures of the HTP purified proteins impeded interpretable results. In order to solve these problems, two strategies were developed. First, a standard curve plotting the aggregation temperatures against the protein concentration was established, in order to be able to normalize the aggregation temperatures obtained in the screen to this standard curve. Second, the overall aggregation temperature was reduced by altering buffer components, and also the purity of the HTP purified protein was increased. These strategies should now greatly reduce the numbers of false positives and negatives, and lead hopefully to the discovery of thermostable active transporters.

A second major part of this diploma thesis was to identify and characterize YdgR loss-of-function mutants. The screen yielded 23 loss-of-function mutant proteins with a single mutation that lost the ability to transport the YdgR specific antibiotic alafosfalin. All of these mutants except three were determined to be properly expressed in the membrane, several also in a GFP fluorescence assay to verify proper membrane insertion. The GFP-fluorescence assay might be a rather quick alternative to small-scale membrane preparation and Western blotting. However, the assay remains to be optimized and tested with negative controls that do not insert into the plasma membrane. Furthermore, an in-vivo transport assay should provide insights into the specificity and transport abilities of the various mutant proteins. However, the transport assay used here has to be optimized in order to obtain reliable and reproducible results.

As already mentioned in the introduction, two membrane proteins, namely the β -adrenergic receptor 1 (Serrano-Vega et al., 2008) and the lactose permease LacY (Abramson et al., 2003) have been crystallized as protein variants with enhanced thermal stability. The β -AR was subjected to alanine scanning and a combination of six point mutations lead to an increase in thermostability of 21°C. LacY contained only a single point mutation that rendered the protein more thermostable, a cysteine to glycine substitution. Both methods bear immense disadvantages compared to the method applied in this project. First, the generation of mutants for the β -AR was very labour intensive since every amino acid had to be mutated to an alanine using site-directed mutagenesis. In my project, a random mutant library was generated. This guarantees a mutation of every amino acid targeted in the random mutagenesis if the library is large enough to statistically cover all of them. Also, the library does not only contain mutations to alanine but to all other essential amino acids. Serrano-Vega et al. showed that mutations to other amino acids than alanine rendered the proteins usually not more thermostable than the alanine itself. Nevertheless, this might not be true for other proteins. LacY contains eight cysteines, each of which was mutated to create a LacY mutant devoid of cysteines. The increase in thermostability of the C154G mutation was rather a coincidental side-effect. Therefore, it would be impossible to predict such an effect of a cysteine mutation in a different protein.

An enormous advantage of the introduced screening method is also that the assay determining the thermal stability is not dependent on any enzymatic activity. This makes

the method broadly applicable to other proteins. Determining the thermostability of an enzyme is often rather easy since a certain enzymatic activity can be measured. Transporters, however, often lack an enzymatic activity. In the case of the β -AR, the increase in thermostability was determined by a radioligand binding assay. The protein was purified over Ni-IMAC, and 20 – 100 μ g of protein were used in the binding assay. Non-bound radioligand had to be removed via a gel filtration step. Again, this is a very time-consuming and laborious assay. Similarly, the thermostability of the LacY mutant was determined measuring substrate binding to wildtype and mutant protein after heating. Also here, relatively high concentrations (120 -140 μ M) of highly pure protein (90-95 %) were needed to perform these assays. The determination of the thermal stability in a Stargazer assay is therefore highly advantageous. First, only 5 -10 μ g of each HTP purified protein is necessary to perform the Stargazer assay. Second, the assay is easy and fast, and no radioactive substrate is needed.

As also mentioned in the introduction, transporter proteins are rather flexible. The cysteine LacY mutant was shown to be locked in a certain conformation (Smirnova et al., 2003) since it can still bind to substrate but cannot translocate it across the membrane. Here, six YdgR loss-of-function mutants were assayed for their thermostability. Two of them, N196K and F197I, indeed exhibited a higher aggregation temperature than the wildtype protein. The mutations evidently lie directly beside each other, strongly indicating that they actually render YdgR more thermostable. Also, another mutant, M154K, showed a slight increase in aggregation temperature, but only at 0.1 mg/ml Stargazer sample concentration. The first two are good potential candidates, which should now be further characterized and eventually used for crystallization trials. Also, these mutations should be combined in order to test for further enhancement of the aggregation temperature. These residues could also be mutated to alanines, since Serrano-Vega et al. found mutations to alanine to be the most stabilizing. Most important, 17 loss-of-function mutants remain to be tested for their thermostability. Furthermore, the screening of YdgR should be continued in order to find more thermostable candidates and interesting loss-of-function mutants.

In summary, this screening method provides a number of advantages. It neither relies on enzymatic activity nor on the presence of certain amino acid residues in the protein. The assays are rapid and easy, also when compared to the other two described methods. Furthermore, the functionality of the transporter is determined in the first step, the growth

assay, and identified loss-of-function mutants give insights into the significance of particular residues or amino acid stretches. More important, this screen should be broadly applicable to other proteins, and could be a very useful method to increase the number of solved membrane protein structures.

5. References

- Abramson J, Smirnova I, Kasho V, Verner G, Kaback HR, Iwata S. Structure and Mechanism of the Lactose Permease of *Escherichia coli*. *Science* 301, 610-14 (2003)
- Allen JG, Atherton FR, Hall MJ, Hassall CH, Holmes SW, Lambert RW, Nisbet LJ and Ringrose PS. Phosphono-peptides, a new class of synthetic antibacterial agents. *Nature* 272, 56 – 58 (1978)
- Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular Systems Biology*. (2006)
- Bowie JU. Stabilizing membrane proteins. *Current Opinion in Structural Biology* 11, 397-402 (2001)
- Covitz K-MY, Amidon GL and Sadee W. Membrane Topology of the Human Dipeptide Transporter, hPEPT1, Determined by Epitope Insertions. *Biochemistry* 37, 15214-15221 (1998)
- Daniel H, Spanier B, Kottra G and Weitz D. From Bacteria to Man: Archaic Proton-Dependent Peptide Transporters at Work. *Physiology* 21, 93-102 (2006)
- Diallinas G. An Almost-Complete Movie. *Science* 322, 1644-45 (2008)
- Doring F, Will J, Amasheh S, Clauss W, Ahlbrecht H and Daniel H. Minimal Molecular Determinants of Substrates for Recognition by the Intestinal Peptide Transporter. *Journal of Biological Chemistry* 273, 23211–18 (1998)

- Drew E, von Heijne G, Nordlund P and de Gier J-W. Green fluorescent protein as an indicator to monitor membrane protein overexpression in *Escherichia coli*. *FEBS Letters* 507, 220-224 (2001)
- Faham S, Watanabe A, Besserer GM, Cascio D, Specht A, Hirayama BA, Wright EM, Abramson J. The crystal structure of a sodium galactose transporter reveals mechanistic insights into Na⁺/sugar symport. *Science* 321, 810-4 (2008)
- Goh E-B, Siino DF and Igo MM. The *Escherichia coli* tppB Gene Represents a New Class of OmpR-Regulated Genes. *Journal of Bacteriology* 186, 4019-24 (2004)
- Hahn FE. Alafosfalin, a New Synthetic Antibacterial Compound. *Naturwissenschaften* 68, 90 (1981)
- Harder D, Stolz J, Casagrande F, Obrdlik P, Weitz D, Fotiadis D and Daniel H. DtpB (YhiP) and DtpA (TppB, YdgR) are prototypical proton-dependent peptide transporters of *Escherichia coli*. *FEBS* 275, 3290-98 (2008)
- Huang Y, Lemieux MJ, Song J, Auer M, Wang DN. Structure and Mechanism of the Glycerol-3-Phosphate Transporter from *Escherichia coli*. *Science* 301, 616-20 (2003)
- Jardetzky O. Simple Allosteric Model for Membrane Pumps. *Nature* 211, 969-70 (1966)
- Karpowich NK and Wang D-N. Symmetric Transporters for Asymmetric Transport. *Science* 321, 781-82 (2008)
- Law CJ, Maloney PC, Wang DN. Ins and Outs of Major Facilitator Superfamily Antiporters. *Annual Review in Microbiology* 62, 289-305 (2008)
- Locher KP, Bass RB and Rees DC. Breaching the Barrier. *Science* 301, 603-4 (2003)

- Magnani F, Shibata Y, Serrano-Vega MJ, and Tate CG. Co-evolving stability and conformational homogeneity of the human adenosine A2a receptor. PNAS 105, 10744–49 (2008)
- Martinez Molina D, Cornvik T, Eshagi S, Haeggström JZ, Nordlund P and Sabet MI. Engineering membrane protein overproduction in Escherichia coli. Protein Science 17, 673-80 (2008)
- Miroux, B and Walker JE. Over-production of proteins in Escherichia coli: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. J Mol Biol 260, 289-98 (1996)
- Paulsen IT and Skurray RA. The POT family of transport proteins. Trends in Biochemical Sciences 19, 404 (1994)
- Senisterra GA, Markin E, Yamazaki K, Hui R, Vedadi M and Awrey D. Screening for Ligands Using a Generic and High-Throughput Light-Scattering-Based Assay. Journal of Biomolecular Screening 11(8); (2006)
- Serrano-Vega MJ, Magnani F, Shibata Y and Tate CG. Conformational thermostabilization of the β 1-adrenergic receptor in a detergent-resistant form. PNAS 105, 877-82 (2008)
- Singh SK., Piscitelli CL, Yamashita A. and Gouaux E. A Competitive Inhibitor Traps LeuT in an Open-to-Out Conformation. Science 322, 1655-61 (2008)
- Smirnova IN and Kaback HR. A Mutation in the Lactose Permease of Escherichia coli That Decreases Conformational Flexibility and Increases Protein Stability. Biochemistry 42, 3025-31 (2003)
- Spiess, C, Beil, A, and Ehrmann, M. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell 97, 339-47 (1999)

Trivedi S, Gehlot HS and Rao SR. Protein thermostability in Archaea and Eubacteria. *Genetics and Molecular Research* 5, 816-27 (2006)

Weitz D, Harder D, Casagrande F, Fotiadis D, Obrdlik P, Kelety B and Daniel H. Functional and Structural Characterization of a Prokaryotic Peptide Transporter with Features Similar to Mammalian PEPT1. *Journal of Biological Chemistry* 282, 2832-39 (2007)

Weyand S, Shimamura T, Yajima S, Suzuki S, Mirza O, Krusong K, Carpenter EP, Rutherford NG, Hadden JM, O'Reilly J, Ma P, Saidijam M, Patching SG, Hope RJ, Norbertczak HT, Roach PC, Iwata S, Henderson PJ, Cameron AD. Structure and molecular mechanism of a nucleobase-cation-symport-1 family transporter. *Science* 322, 709-13 (2008)

Widdas WF. Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. *Journal of Physiology*, 23-39 (1952)

Yamashita A, Singh SK, Kawate T, Jin Y, Gouaux E. Crystal structure of a bacterial homologue of Na⁺/Cl⁻-dependent neurotransmitter transporters. *Nature* 437, 215-23 (2005)

Yin Y, He X, Szewczyk P, Nguyen T, Chang G. Structure of the Multidrug Transporter EmrD from *Escherichia coli*. *Science* 312, 741-44 (2006)

Zhou Y and Bowie JU. Building a thermostable protein. *The Journal of Biological Chemistry* 275, 6975-79 (2000)

Inoue-protocol

Sambrook Joseph & Russel David W., Molecular Cloning, A Laboratory Manual, 3rd Edition 2001, Cold Spring Harbour Laboratory Press

Detergent scheme

Stryer Lubert, Berg, Jeremy M. & Tymoczko, John L.. Biochemistry. W H Freeman
from http://photoscience.la.asu.edu/photosyn/faculty/Allen/protein-structures/membrane-proteins/lipids_detergents.html

DDM

<http://www.chemblink.com/products/69227-93-6.htm>

FOS-12, β -OG, Triton X-100, SDS

<http://pubchem.ncbi.nlm.nih.gov/>

Figure In-fusion Cloning

http://www.clontech.com/products/detail.asp?product_id=206261&tabno=2

YdgR topology image

www.sacs.ucsf.edu/TOPO2-run/wtopo2.pl

Curriculum vitae

Elisabeth Malle

Personal data

Date of birth	September 29 th , 1983
Place of birth	St. Veit a.d. Glan, Austria
Citizenship	Austrian

Education

1990 – 1994	Primary school, Ludmannsdorf, Austria
1994 – 2002	High school, BG Mössinger, Klagenfurt, Austria
since 2002	Undergraduate studies in Molecular Biology at the University of Vienna, Austria
2006	ERASMUS semester at the University of Uppsala, Sweden
2007 – 2008	Diploma thesis at the Structural Biology Department, Group Stolt-Bergner, Institute for Molecular Pathology (I.M.P.), Vienna, Austria

Internships and teaching

2008	Teaching experiences (tutor) in university practicals: Biochemistry (Fritz Pittner), Yeast and plant genetics (Markus Teige) and Molecular Biology, in-vitro techniques (Cécile Brocard), University of Vienna
------	--

- Sep 2007 University of Vienna, Department of Biochemistry, Vienna, Austria
Optimization of meat-freshness biosensors on the basis of gold-nanoparticles
Group Fritz Pittner, Supervisor: Nadira Ibrisomovic
- Okt 2006- Dec 2006 University of Vienna, Institute of Microbiology and Genetics, Vienna, Austria
Expression of Mrs2p in E. coli and Iron transport into mitochondria by Mrs12/Rim2 protein
Group Rudolf Schweyen, Supervisor: Dr. Elisabeth Froschauer
- Mar 2006 – Jun 2006 Biomedical Center Uppsala, Institute for Biochemistry and Organic Chemistry, Uppsala, Sweden
Tripeptidyl-peptidase II: Characterization of mutants defective in substrate binding site
Group Birgitta Tomkinson, Supervisor: Birgitta Tomkinson
- Jul 2005 – Aug 2005 Medical University Innsbruck, Division Cell Biology, Innsbruck, Austria
Proteomics of MAP Kinase Signaling
Group Lukas Huber, Supervisor: Dr. Sandra Morandell
- Sep 2005 – Okt 2005 General Hospital Munich Schwabing, Clinical Chemistry, Munich, Germany:
Mitochondrial Deletion and Depletion in Myopathy
Group Rita Horvath, Supervisor: Dr. Rita Horvath

Publications

Reichenbach J, Schubert R, Horvath R, Petersen J, Fütterer N, Malle E, Stumpf A, Gebhardt BR, Koehl U, Schraven B, Zielen S. Fatal neonatal-onset mitochondrial respiratory chain disease with T cell immunodeficiency. *Pediatr Res.* 60(3):321-6 (2006)