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High pressure modulation of ToxR mediated signal transduction

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Vorwort

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Abbreviations

AAS	amino acid sequence
ACP	acyl carrier protein
ATP	adenosine-5'-triphosphate
bp	base pair
cat	chloramphenicol acetyltransferase (gene)
Da	dalton
DNA	desoxyribonucleic acid
dNTP	desoxynucleotid phosphate
DSM	DSMZ, Braunschweig, Germany
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
Fig.	Figure
g	gram
G	Gravity
GFP	Green fluorescent protein
GP	Generalized polarization
h	hour
HHP	High hydrostatic pressure
HP	High pressure
HPLC	high pressure liquid chromatography
L.	*Lactococcus*
lacZ	β-galactosidase (gene)
M	Mega (10^6), molar
m	Milli (10^{-3}), meter
min	minutes
NCBI	National center for Biotechnology Information
OD	optical density
p	pressure [Pa]
P.	*Photobacterium*
PCR	polymerase chain reaction
S.	*Saccharomyces*

SDS	sodium n-dodecylsulfate
s	second
Tab.	Table
TMS	Trans membrane segment
TMW	Technische Mikrobiologie Weihenstephan
TRIS	tris (hydroxymethyl) aminomethan
v/v	volume / volume
vol.	volume
w/v	mass / volume
ΔV	reaction volume
µ	Micro (10^{-6})

Contents

1 Introduction

1.1 The general effects of high hydrostatic pressure (HHP)

In physical or biochemical processes, all high hydrostatic pressure (HHP) effects accompany system volume changes. These changes are governed by the principle of Le Chatelier. The effect of pressure can be described by two fundamental relationships:

$$\left(\frac{\partial \ln K}{\partial p}\right)_T = -\frac{\Delta V}{RT} \qquad \left(\frac{\partial \ln k}{\partial p}\right)_T = -\frac{\Delta V^{\neq}}{RT}$$

Where K is the equilibrium constant, k is the rate constant, p is the pressure, T is the absolute temperature in Kelvin and R is the gas constant (ml atm K^{-1} mol^{-1}). ΔV is the difference between the final and initial volume in the entire system at equilibrium, including the solute and the surrounding solvent. $\Delta V^{\neq}$ is the apparent volume change of activation and represents the difference in volume between the reactants and the transition state. Therefore every reaction that is accompanied by a volume decrease can be enhanced by elevated pressure. And every reaction that is accompanied by a volume increase will be inhibited by elevated pressure (Gross & Jaenicke, 1994). Reaction volumes for biochemically important reactions by 25°C are shown in Table 1.1.

Table 1.1.: Reaction volumes associated with selected biochemically important reactions at 25°C (Gross & Jaenicke, 1994).

Reaction	Example	ΔV
		ml/mol
Protonation/ion-pair formation	$H^+ + OH^- \rightarrow H_2O$	+ 21.3
	Imidazole + $H^+ \rightarrow$ Imidazole • H^+	- 1.1
	TRIS + $H^+ \rightarrow$ TRIS • H^+	- 1.1
	$HPO_4^{-2} + H^+ \rightarrow H_2PO_4^-$	+ 24.0
	$CO_3^{-2} + 2H^+ \rightarrow HCO_3^- + H^+ \rightarrow H_2CO_3$	+ 25.5[a]
	Protein-COO^- + $H^+ \rightarrow$ protein-COOH	+ 10.0
	Protein-NH_3^+ + $OH^- \rightarrow$ protein-NH2 + H_2O	+ 20.0
Hydrogen-bond formation	Poly (L-lysine) (helix formation)	- 1.1
	Poly (A + C) (helix formation)	+ 1.1[b]

Hydrophobic hydration	$C_6H_6 \rightarrow (C_6H_6)_{water}$	- 6.2
	$(CH_4)_{hexane} \rightarrow (CH_4)_{water}$	- 22.7
Hydration of polar groups	n-propanol → (n-propanol)$_{water}$	- 4.5
Protein dissociation / association	Lactate dehydrogenase (M4 → 4M) apoenzyme	- 500
	Holoenzyme (satured with NADH)	- 390
	Microtubule formation (tubulin propagation; ΔV per subunit)	+ 90
	Ribosome association (*E. coli* 70S)	≥ 200[c]
Protein denaturation	Myoglobin (pH 5, 20°C)	-98

[a] ΔV for each ionization step.
[b] for DNA denaturation: 0-3 ml/mol base pair.
[c] 200-850 ml/mol, depending on pressure and state of charging.

Covalent bonds are barely affected by HHP whereas some non covalent bonds are very sensitive to pressure (Boonyaratanakornkit *et al.*, 2002). It also follows from the principle of microscopic ordering, that an increase in pressure, at constant temperature, leads to more molecular ordering or a decrease in entropy of the system (Balny, 2004).

1.2 HHP in biological systems

High pressure plays an important role in the evolution and distribution of microorganisms (Yayanos, 1986). It is likely that the majority of prokaryotes lives in habitats with high pressure conditions (Whitman *et al.*, 1998). These habitats are the oceans, deep lakes and subsurface regions (Karl *et al.*, 1999; Kato *et al.*, 1998; Szewzyk *et al.*, 1994). The pressure in the biosphere reaches from – 2 MPa in the xylem of trees up to 110 MPa in the deep sea (Scholander *et al.*, 1965). More than half of the volume of the global biosphere consists of biotopes located deeper than 1000 m which means existing pressure above 10 MPa (Jannasch & Taylor, 1984). The oceans which are covering 70% of the earth surface have an average depth of 3800 m (38 MPa), this indicates that atmospheric pressure (0.1 MPa) is far from being the normal case for aquatic organisms (Gross & Jaenicke, 1994).

The first attempt to investigate the deep sea under microbiological aspects, which got some attention, was started in 1882 by Certes and Portier (Jannasch & Taylor, 1984). Certes, during the Travaillier Talisman Expeditions (1882-1883), examined sediment and water collected from depths to 5000 m and found microorganisms in almost every sample. He noted that microorganisms survived at great pressure and might live in a state of suspended animation (Certes, 1884). In 1904 scaled and autoclaved glass tubes were used as sampling

devices by Portier, to sample microorganisms at various depths and locations (Richard, 1907). However the work on the effects of hydrostatic pressure on microbial activities was really started in 1949 by Zobell and Johnson (ZoBell & Johnson, 1949). Zobell and Johnson also invented the term *barophilic* (now: piezophilic) which is used for microorganisms with optimal growth at pressures above 0.1 MPa, or the requirement of higher pressures to grow. Also the term hyperpiezophilic is used for microorganisms with optimal growth conditions at pressures above 60 MPa. In 1957 the first evidences for piezophilic microorganisms were found in deep sea sediments (ZoBell & Morita, 1957). Nevertheless the first pure culture of a piezophilic microorganism was isolated and described 1979 by Yayanos et al. (Yayanos *et al.*, 1979). Since quite a number of piezophilic microorganisms have been identified. Most of the identified bacteria are not only piezophilic but psychrophilic as well and will not grow at temperatures above 20°C. These piezophilic bacteria belong typically to closely related families of the γ-Proteobacteria class (Kato & Bartlett, 1997). In contrast to the piezophilic bacteria, the also identified piezophilic archaea, belong to various families of the Euryarchaeota and Crenarchaeota phyla. Most of these archaea are as well not psychrophilic instead they are hyperthermophilic and have their growth maxima at temperatures above 60°C. The archaea are typically found at deep sea hydrothermal vents. Interestingly the piezophilic bacteria and archaea in culture are closely related to shallow water microorganisms which are not piezophilic (Bartlett, 2002). Also non piezophilic bacteria have been found in isolates from the deepest sea mud of the Mariana Trench which is 10897 meters deep. These non piezophilic bacteria have been identified by 16S rDNA sequences (Takami *et al.*, 1997). This connoted that non piezophilic bacteria are able to survive in the deep-sea and as a result are able to adapt to pressure.

The ability of non piezophilic bacteria to adapt to pressure and exhibit a stress response is also of great interest for the food industry, since it has been shown that high pressure is a useful tool for the inactivation of microorganisms (Hoover *et al.*, 1989). The use of high pressure treatment for sterilizing food has advantages against the use of high-temperature treatment for this purpose. Because it does not provoke a Maillard reaction, it does not affect covalent bonds, hence cannot destroy natural flavors or colorants, and it allows the production of half-prepared food (Gross & Jaenicke, 1994). To understand how high pressure influences microorganisms, piezophilic as well as non-piezophilic organisms have to be analyzed (Abe *et al.*, 1999; Bartlett, 1999; Kato & Bartlett, 1997).

1.3 Effects of HHP on microorganisms

Generally almost every cell-process is influenced by a pressure change (Bartlett, 2002). Normally yeasts and fungi's are more pressure sensitive than bacteria, among whom as a general rule gram positive bacteria are more resistant than gram negative bacteria. And similar to other lethal environmental factors, pressure has stronger effects on cells in the logarithmic phase than on cells in the stationary phase (Pagan & Mackey, 2000). The pressure effect on the microorganisms is also influenced by other environmental factors like temperature, pH and the nutrients in the growth media (Abee & Wouters, 1999; Pagan & Mackey, 2000; ZoBell, 1970).

An interesting effect of HHP is that it affects the morphology of some microorganisms. For example some mesophilic bacteria like *E. coli* and *L. lactis* become filamentous when are incubated at elevated pressures, which are not high enough to prevent cell growth (Aertsen & Michiels, 2005; Molina-Hoppner *et al.*, 2003; ZoBell & Johnson, 1949; ZoBell & Oppenheimer, 1950; ZoBell & Cobet, 1964; ZoBell, 1970). In this case cell growth is less pressure-sensitive than cell division. Interestingly the same happens to piezophilic microorganisms when they are grown at pressures below or above their pressure optima (Jannasch, 1987; Yayanos & DeLong, 1987). The basis for this could be a possible pressure-sensitivity of several division proteins. Some of these proteins share similarities to cytoskeletal proteins of higher organisms which are known to be pressure-sensitive (Crenshaw & Salmon, 1996; Sato *et al.*, 1995). A well studied protein of this kind is FtsZ. FtsZ is a GTP-hydrolyzing tubulin-like protein which, during early stages of the septation process, polymerizes into a ring in the cell middle (Erickson & Stoffler, 1996; Lockhart & Kendrick Jones, 1998). This FtsZ rings are largely absent in cells incubated at high pressure which might be the reason for the filamentation (Ishii *et al.*, 2004; Kawarai *et al.*, 2004; Molina-Hoppner *et al.*, 2003; Sato *et al.*, 2002). This is also supported by in vitro studies of pressure effects on tubulin filaments. Like mentioned before HHP affects every reaction which is accompanied by a change in system volume (Gross & Jaenicke, 1994). These studies show that the dissociation of these polymers is accompanied by a volume change of -90 ml mol^{-1} and therefore enhanced by elevated pressure (Morild, 1981). In contrast to *E. coli* where the cells divide after pressure treatment, the same was not observed by cells from *L. lactis* (Ishii *et al.*, 2004; Kawarai *et al.*, 2004; Molina-Hoppner *et al.*, 2003).

Pressure also influences the DNA synthesis, it was shown for *E. coli* that pressures between 25 and 43 MPa are able to promote synchronization of cell division and DNA synthesis (Yayanos & Pollard, 1969). Furthermore the abundance of DNA per cell is reduced

in *E. coli* and *S. cerevisiae* at elevated pressures (Abe & Horikoshi, 2000; ZoBell & Cobet, 1964). In addition a decrease in DNA per cell after culture decompression was shown for the piezophilic bacterium *Colwellia* MT41 (Chastain & Yayanos, 1991). For *E. coli* it has been demonstrated that DNA-synthesis is inhibited at 50 MPa, RNA-synthesis at 77 MPa and protein-biosynthesis at 48 MPa (Gross *et al.*, 1993; Landau, 1967; Yayanos & Pollard, 1969). Especially the function of the ribosome seems to be highly pressure sensitive. It is assumed that the inhibition of the protein-biosynthesis, which is caused by the pressure sensitivity of the ribosome, is the main reason for the reduced growth of non piezophilic microorganisms under pressure (Pope & Berger, 1973). In vitro experiments have shown that ribosome's in combination with mRNA and tRNA are stable against dissociation at pressures up to 100 MPa, in contrast uncharged ribosome's have only been stable up to 60 MPa (Gross *et al.*, 1993). In *E. coli* a total inhibition of protein synthesis at 67 MPa was observed from Schwarz and Landau. They have been able to exclude aminoacyl transfer ribonucleic acid (AA-tRNA) formation, polysomal integrity, or amino acid permeability as the reason for the inhibition (Schwarz & Landau, 1972a). It is assumed that a step between the forming of the AA-tRNA and the forming of the peptide bond are affected by pressure. Translocation as well as the binding of AA-tRNA to the ribosome might be the target. In vitro experiments have shown that the only step in translation that is inhibited in an identical manner than protein-synthesis in living cells is the binding of AA-tRNA to the ribosome mRNA complex (Pande & Wishnia, 1986; Schwarz & Landau, 1972b). Moreover it has been observed, that the binding of AA-tRNA to the 30S subunit of the ribosome, leads to a conformational change. This conformational change leads to a volume increase, thus is inhibited by elevated pressure (Arnold & Albright, 1971; Gross *et al.*, 1993; Schulz *et al.*, 1976; Smith *et al.*, 1975). A defective translation leads not only to a decelerated protein synthesis it can also lead to a higher error rate (Hardon & Albright, 1974). This is the case by *E. coli* and *Listeria monocytogenes*, if ribosome function is there highly affected by HHP, the accumulation of defective proteins can lead to cell death (Isaacs *et al.*, 1995). By determining the effect of sublethal hydrostatic pressure on the transcriptome of *Lactobacillus sanfransciscensis* Pavlovic *et al.* have also been able to show that the translational machinery is a target for high pressure. From 750 spots that passed quality analysis 42 genes were induced, while six were repressed when cells were incubated at 45 MPa for 30 min. The nature of these genes and their differential expression has clearly indicated cellular efforts to counteract a decrease in translational capacity (Pavlovic *et al.*, 2005). A proteome approach to characterize the high pressure response of *L. sanfranciscensis* also supported the idea that the translational

machinery is a target for high pressure. In this approach nine proteins have been found to be induced by HHP (80 MPa), four of these proteins possessed possible chaperone activity, and the only specific HHP-increased protein found in this work was a Clp protease. This stress response was seen as an attempt to minimize the negative effects of misfolded, truncated and denatured proteins which were probably caused by HHP treatment. Because 80 MPa are to low for denaturation of intracellular proteins, a ribosomal dysfunction was the likely explanation for these results (Hörmann *et al.*, 2006).

Another big contact point for high hydrostatic pressure is the cell membrane. In *E. coli* and *L. lactis* the cell membrane can be damaged by HHP, that RNA and intracellular protein are released and the HHP influence can finally lead to a complete lysis of the cell (Malone *et al.*, 2002; Manas & Mackey, 2004). The opposite effect is observed for the piezophilic bacterium *Colwellia* MT41 which responds to decompression by forming intracellular vesicles and releasing membrane fragments into the medium, followed by cell lysis (Chastain & Yayanos, 1991). Beside complete damage of the cell membrane the influence of HHP on membrane fluidity is also a big factor. It was shown that changes in the membrane fluidity, like fluid-to-gel phase transitions, are able to inactivate membrane proteins at pressures above 200 MPa (Chong *et al.*, 1985; Ulmer *et al.*, 2000; Ulmer *et al.*, 2002). But also slighter changes in membrane fluidity seem to bee able to affect membrane bound proteins. So was reported that a decrease in the reaction rate of membrane-bound Na^+/K^+-ATPase, caused by increasing pressures, might be based on a decrease in fluidity of the lipid bilayer (Kato *et al.*, 2002). The HHP effects on the membrane and membrane-bound proteins are discussed as one of the major reasons for the inactivation of microorganisms through HHP (Gänzle & Vogel, 2001; Ulmer *et al.*, 2002; Wouters *et al.*, 1998). Under these circumstances it seems very important for piezophilic organisms to possess systems to maintain their membrane functionality under HHP (Bartlett & Bidle, 1999; Somero, 1992). For the most piezophilic bacteria not only HHP, also the low temperature, in their habitats (under 20°C), reduces the membrane fluidity. Therefore piezophilic bacteria possesses the ability to increase the amount of unsaturated fatty acids in their membrane, to maintain membrane functionality under pressure and lower temperature (Allen *et al.*, 1999; Balny *et al.*, 2002; Bartlett, 2002; DeLong & Yayanos, 1985; Kamimura *et al.*, 1993; Kato & Hayashi, 1999). Experiments with *P. profundum* SS9 have shown, that at elevated pressure, it integrates a higher amount of mono- and polyunsaturated fatty acids into its cell membrane (Allen *et al.*, 1999). Some piezophilic bacteria have high amounts of omega-3 polyunsaturated fatty acids in their membranes. These omega-3 polyunsaturated fatty acids are of high biomedical interest, especially their use in

reducing the risk of human cardiovascular diseases and certain cancers and their use as dietary supplements in marine culture and poultry farming (Yano *et al.*, 1997). It was shown for *P. profundum* SS9 that chemical mutants which produce diminished amounts of monounsaturated fatty acids are pressure sensitive (Allen *et al.*, 1999). The importance of monounsaturated fatty acids for HHP adaptation, was also confirmed with help of a Δ*fabF* mutant from SS9, which was also pressure sensitive (Allen & Bartlett, 2000). FabF encodes the enzyme β-ketoacyl-ACP synthase II, an enzyme catalyzing the monounsaturated fatty acid cis-vaccenic acid (Garwin *et al.*, 1980b). An interesting fact is that the polyunsaturated fatty acids seem not to be important for adaptation to HHP like monounsaturated fatty acids. This was shown with help of an other SS9 mutant, which was not able to produce the polyunsaturated fatty acid eicosapentaenoic acid (EPA) and was not altered in its growth as a function of either HHP or temperature (Allen *et al.*, 1999). The reason of unsaturated fatty acids production by these bacteria might lie in symbiotic interactions with higher deep-sea fauna, where they are needed as essentially fatty acids (Bartlett, 2002). So far there is no proof that non piezophilic bacteria are able to adapt their membrane fatty acid composition in response to HHP. However experiments with *E. coli* grown at 10°C and 37°C have shown that the membrane contains more unsaturated fatty acids at 10°C than at 37°C. The cells grown at 10°C are more resistant against pressure, in the exponential phase, than the cells grown at 37°C This indicates that the higher membrane fluidity is the reason for the higher resistance against HHP (Casadei *et al.*, 2002).

Another field of HHP influence is the gene expression. One example for this is the lactose repressor. It was shown that the repressor is destabilized at HHP after binding to operator DNA (Royer *et al.*, 1990). Also the expression of chloramphenicol acetyltransferase, under control of the *lac* promoter, in *E. coli*, is stimulated at HHP (Kato *et al.*, 1994). Furthermore an increased membrane proteins synthesis was observed in the deep-sea bacteria *Shewanella benthica* and *Shewanella violaceaea* under HHP (Qureshi *et al.*, 1998a; Qureshi *et al.*, 1998b; Tamegai *et al.*, 2005). It was possible to identify one HHP induced gene in *Shewanella sp.* DSS12, with high homology to *cydD* from *E. coli*. CydD is necessary for assembly of the cytochrome *bd* complex, inside the membrane. *E. coli* cells with a defect *cydD* gene are more pressure sensitive. It was possible to complement the defect with introduction of *cydD* from *Shewanella sp.* DSS12. The cytochrome *bd* complex can only be found in *Shewanella sp.* DSS12 cells if they were grown under pressure (Tamegai *et al.*, 1998). In *Shewanella benthica* strain DB172F two different types of c-type cytochromes were found, one membrane bound and one cytoplasmic (Qureshi *et al.*, 1998b). Only the membrane

bound c-type cytochrome was produced at high pressure (60 MPa). Moreover a *ccb* oxidase was identified, with increased abundance at HHP (Qureshi *et al.*, 1998a). Like *Shewanella* also the moderate piezophilic bacterium *P. profundum* SS9 modulates the amount of membrane proteins. Especially for the outer membrane proteins OmpH and OmpL, it was shown that the amount of OmpH increases at higher pressures and the amount of OmpL decreases (Bartlett *et al.*, 1989; Chi & Bartlett, 1993; Welch & Bartlett, 1996). The genes encoding these proteins are transcriptionally regulated by the ToxR and ToxS proteins (Welch & Bartlett, 1998). The function of ToxR and ToxS as some kind of pressure sensor, in *P. profundum*, makes them very interesting for pressure related experiments.

1.4 ToxR and ToxS

The inner membrane proteins ToxR and ToxS were first discovered in *Vibrio cholerae* (Miller & Mekalanos, 1984). However, both ToxR and ToxS homologs have been found in certain members of the *Vibrionaceae* family (Lee *et al.*, 2000; Li *et al.*, 2000; Osorio & Klose, 2000; Reich & Schoolnik, 1994; Welch & Bartlett, 1998). Some of these members are human or fish pathogens, like *Vibrio parahaemolyticus,* the major cause of gastroenteritis associated with seafood consumption (Joseph *et al.*, 1982). Other bacteria that containe ToxR and ToxS are *Vibrio fischeri*, a bioluminescent bacterium associated with the light organs of certain fish and squid, and *P. profundum* strain SS9, a deep-sea bacterium originally isolated from amphipod crustaceans (Bartlett *et al.*, 1989; Dunlap, 1999; Ruby, 1999). The ToxR regulated virulence genes in *V. cholerae* (not the *toxRS* operon) are largely acquired by horizontal gene transfer (Karaolis *et al.*, 1999; Waldor & Mekalanos, 1996). ToxR is a transmembrane DNA-binding protein oriented in the inner membrane with a sensor periplasmic carboxy- terminal domain and a cytoplasmatic amino-terminal domain that functions in DNA binding and transcriptional regulation. As a dimer ToxR is able to bind to specific DNA regions, like the *ctx* promoter. This dimerization is stabilized by ToxS (DiRita & Mekalanos, 1991; Miller *et al.*, 1987; Miller *et al.*, 1989). If ToxR is over expressed, ToxS is not necessary for dimerization (Ottemann & Mekalanos, 1996). In *V. cholerae* ToxR and ToxS are best known for their central role in the environmental regulation of virulence gene expression. They are regulated in response to changes in osmolarity, pH, temperature and to the levels of certain extra cellular amino acids (Miller & Mekalanos, 1984; Skorupski & Taylor, 1997). The *V. cholerae* ToxR virulence regulon is composed of a set of over 20 genes. Except for one gene all others require ToxR for their expression, the one remaining is repressed by ToxR. Some of these genes are also co-regulated by the transcription factor ToxT, whose gene expression is

activated by ToxR and ToxS along with TcpP and TcpH, another pair of membrane proteins related to ToxR and ToxS, respectively (Hase & Mekalanos, 1998). The co-regulation of ToxT includes expression of toxin and colonization genes associated with the CTXΦ genome and the Vibrio pathogenicity island, which has also been proposed to represent a filamentous phage genome (Karaolis *et al.*, 1999; Waldor & Mekalanos, 1996). The genes that are only regulated by ToxR are encoding two outer membrane proteins. The gene *ompU* is activated by ToxR and the gene *ompT* is repressed by ToxR (Crawford *et al.*, 1998; Li *et al.*, 2000; Miller & Mekalanos, 1988; Sperandio *et al.*, 1996). Like mentioned before, in *P. profundum* the expression of the genes *ompH* and *ompL* is also regulated by ToxR. The gene *ompH* is repressed by ToxR and the gene *ompL* is activated by ToxR (Bartlett *et al.*, 1993; Welch & Bartlett, 1996). Beside of *ompH* and *ompL* eight potentially ToxR regulated genes have been identified in *P. profundum*. They were identified by using RNA arbitrarily primed PCR (RAP-PCR) with wild-type and *toxR* mutant strains of SS9. Seven of the eight identified genes are activated by ToxR and one is repressed. Their gene's products belong to two functional categories, those whose products alter membrane structure and /or those that are part of a starvation response. It is likely that many of the identified genes are controlled by multiple regulatory factors (Bidle & Bartlett, 2001). By replacing different protein substructures of ToxR, from *V. cholerae*, Kolmar *et al*. (1995) were able to examine which parts are essential for the transcription activation of the *ctx* promoter. They could show that the trans-membrane region has no special function in signal transduction, and that the activation of the genes under control of the *ctx* promoter depends strictly on the dimerization of the periplasmic ToxR domain. Based on this results they discussed the possible application of ToxR as a technical tool to analyze protein-protein interactions between trans membrane domains (TMD) (Kolmar *et al.*, 1995). So far different systems for the detection of trans-membrane-interactions have been constructed with ToxR from *V. cholerae* (Brosig & Langosch, 1998; Gurezka & Langosch, 2001).

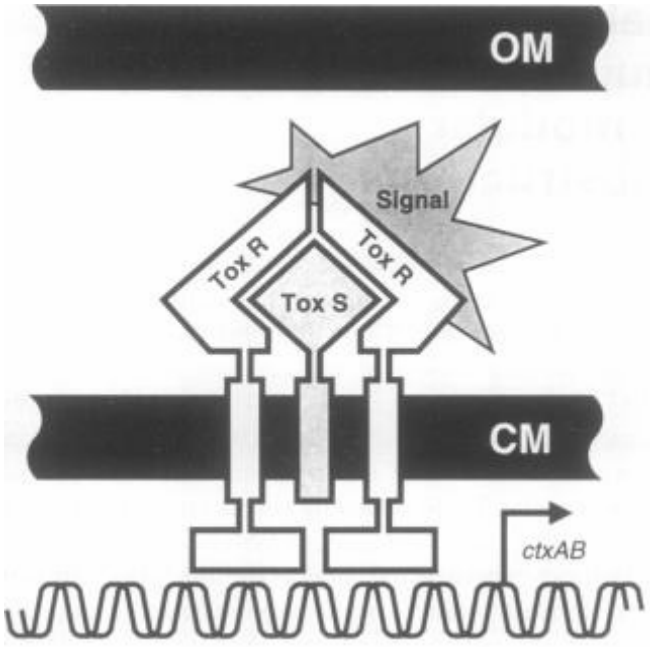

Figure 1.1: The ToxR/S system in *V. cholerae*. A Signal-mediated interaction between periplasmic domains of membrane proteins ToxR and ToxS leads to passive dimerization of cytoplasmic ToxR domains with the result of sequence-specific biding to *ctx* promoter DNA and, consequently activation of transcription. OM, outer membrane; CM, cytoplasmic membrane. (Kolmar *et al.*, 1995)

1.5 ToxR-systems

Based on ToxR from *V. cholerae*, the ToxR-transcription-activator-system was developed by Langosch et al. (Langosch *et al.*, 1996). The original system (ToxRI) consists of a fusion protein which is encoded on a high copy plasmid where it is constitutively expressed. The fusion protein consists of a cytoplasmic-domain of ToxR, a variable TMD and a periplasmic MalE (maltose binding protein) domain. The dimerization ability of the fusion protein in this system is only dependent on the TMD interactions. The fusion protein is used in combination with a specific *E. coli* reporter strain, where a *ctx*::*lacZ* fusion is integrated into the chromosome (Kolmar *et al.*, 1995). Inside the reporter strain a dimerized fusion protein is able to activate the transcription of the *lacZ* gene. The *lacZ* gene used in this construct is N-terminally truncated (starting at nucleotide pair 34 according to GenEMBL accession number V00296) and encodes a less active β-galactosidase than wild type *lacZ*. The dimerization can then indirectly be measured by β-galactosidase activity (Langosch *et al.*, 1996). Based on the ToxR-transcription-activator-system another system was developed. The POSSYCCAT (Positive Selection System Based on Chromosomally integrated CAT) system (ToxRIV) was created for the *in vivo* selection of high affine, homolog interacting TMDs from combinatory

libraries (Gurezka & Langosch, 2001). In this system the fusion protein is also encoded on a high copy plasmid, but is not constitutive expressed. The expression of the fusion protein is under control of the arabinose inducible *araBAD* promoter. Also a different reporter strain, Chr3, is used. For the construction of Chr3 a *ctx::cat* fusion was integrated into the chromosome of *E. coli* DH5α. In this system the dimerization will activate the transcription of the *cat* gene, which leads to a chloramphenicol resistance of the bacterium. The intensity of the resistance depends on the dimerization ability of the TMDs. This certainty allows the accumulation of cells, which contain high affine, homolog interacting TMDs. Both systems have often shown there usefulness in many TMD interaction experiments (Brosig & Langosch, 1998; Gurezka & Langosch, 2001; Langosch *et al.*, 1996; Langosch *et al.*, 2002; Li *et al.*, 2004; Mendrola *et al.*, 2002; Ridder *et al.*, 2005; Russ & Engelman, 2000; Sal-Man & Shai, 2005).

1.6 Proteins under pressure

Proteins are of high biological importance, they are involved in almost every step of biological activity. The protein structure can be defined by four levels of protein conformation the primary structure (amino acid sequence), the secondary structure (alpha helix, beta sheet), the tertiary structure (the sterically relations of the secondary structures to each other) and the quaternary structure (interactions of more then one protein molecule). It is long known that pressure affects proteins e.g.: it was shown in 1914 that HHP induces protein denaturation (Bridgman, 1914). Since then many experiments have been made about the influence of pressure on monomeric and oligomeric proteins. This experiments were normally performed in aqueous solutions or pure lipid bilayer systems (Balny *et al.*, 2002; Winter *et al.*, 2007). However, pressure-induced phase characteristics as well as folding kinetics of monomeric and oligomeric proteins in solution (e.g.: Snase, Rnase A, GFP, ubiquitin, Tryp-repressor, β-lactoglobulin, elastin, α-chymotrypsin, etc.) and the aggregation of proteins have been investigated mostly in aqueous environments (Balny *et al.*, 2002; Foguel & Silva, 2004; Herberhold & Winter, 2002; Herberhold *et al.*, 2003; Panick & Winter, 2000; Pappenberger *et al.*, 2000; Seemann *et al.*, 2001; Winter, 2002; Winter *et al.*, 2007; Woenckhaus *et al.*, 2001). A general result of these pressure experiments is that the four levels of protein conformation are differentially affected by HHP. The reason for the different sensitivity to pressure lies by the interactions that are involved in protein stabilization. These interactions are covalent bonds, electrostatic interactions, hydrogen bonds and hydrophobic interactions. Covalent bonds are strong interactions and the volume changes for exchanges in covalent bonds are

nearly zero. Therefore most covalent bounds participating in the protein primary structure are not sensitive to HHP as high as 1000 MPa (Balny, 2004). This is supported by several experiments e.g. it was shown that the primary structure of hen egg-white lysozyme is not affected by pressures greater than 1000 MPa (Gross & Jaenicke, 1994; Mozhaev *et al.*, 1996). In contrast weak interactions like electrostatic interactions, hydrogen bonds and hydrophobic interactions are sensitive to pressure (Boonyaratanakornkit *et al.*, 2002). Electrostatic interactions are weakened by HHP because the disruption of electrostatic interactions leads to a large volume decrease, caused by electrostriction of water molecules surrounding the unpaired charged residues. A good example for this is the pressure induced denaturation of α-chymotrypsin caused by dissociation of a salt bridge in the active site region (Heremans & Heremans, 1989). Also hydrophobic interactions are weakened by HHP, since the hydration of hydrophobic residues reduces the system volume, which is favored by HHP (van Eldik *et al.*, 1989). Moreover hydration of the protein core is suggested as the mechanism of pressure-induced unfolding (Hummer *et al.*, 1998). It was shown that pressure is able to cause water molecules to enter protein cavities (Collins *et al.*, 2005). Interestingly stacking interactions between aromatic rings have negative volume changes and therefore are favored by HHP (Sawamura *et al.*, 1989). Also Hydrogen bonds are stabilized by HHP a good example therefore is the pressure dependence of the infrared spectra of the α-helix in myoglobin. Only a very small volume change is observed for processes in which there is an exchange between the existing hydrogen bonds (van Eldik *et al.*, 1989). It is proposed that pressure promotes the hydrogen bond formation inside of proteins (Michels *et al.*, 1996). The tree levels of protein conformation that are defined by weak interactions, secondary structure, tertiary structure and quaternary structure are also different affected by pressure. Generally the secondary structure is less affected than tertiary structure and tertiary structure is less affected than quaternary structure (Boonyaratanakornkit *et al.*, 2002). Secondary structure changes were observed by pressures above 300 MPa, which were leading to non-reversible denaturation. However there are also proteins like GFP where the secondary structure is not affected by HHP up to 1300 MPa (Scheyhing *et al.*, 2002). The tertiary structure is affected by pressure over 200 MPa. And in many cases HHP above 100 MPa has been found to promote the dissociation of oligomeric proteins. The dissociation of oligomeric proteins is, as a rule, accompanied by negative and relatively large volume changes (Boonyaratanakornkit *et al.*, 2002). Until now, no *in vivo* experiments have been made on the influence of pressure on the behavior of membrane proteins in the membrane. Therefore, little is known abut the behavior upon

pressurization of membrane proteins integrated in their natural lipid bilayer environment, and their putative function in pressure sensing.

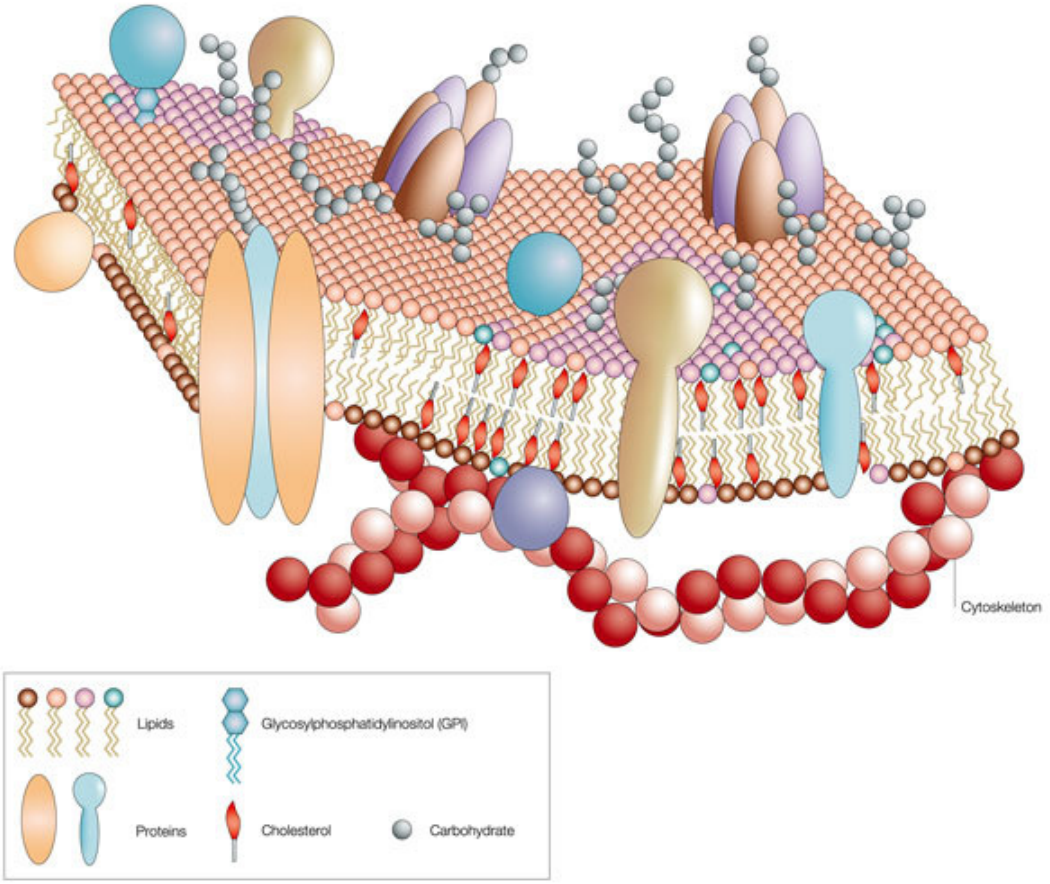

Figure 1.2: The fluid mosaic model of the cell membrane (Pietzsch, 2004)

1.7 Biomembranes under pressure

Biological membranes are necessary for biological systems they are building the outer boundaries of cells and providing the opportunity to build cell compartments. A biological membrane consists mainly of lipids and proteins, whose association with each other is largely defined by hydrophobic effects. However when the membrane is formed it is also stabilized by hydrogen bonds, electrostatic interactions and van der Waals forces. The “fluid mosaic model” is a simple model to explain the gross organization and structure of the proteins and lipids of biological membranes (Singer & Nicolson, 1972). According to this model cell membranes are two dimensional fluid bilayers composed of phospholipids in which the integral proteins are a heterogeneous set of globular molecules. The non-polar groups of the

protein are placed in the hydrophobic interior of the membrane, and the ionic and highly polar groups of the protein are protruding from the membrane into the aqueous phase. The globular molecules are partially embedded in a matrix of phospholipids. Therefore the majority of the phospholipids are organized as a discontinuous, fluid bilayer. The membrane is a dynamic system where proteins and lipids influence each other. Several experiments have shown that biomembranes are highly affected by HHP (Hoover *et al.*, 1989; Malone *et al.*, 2002; Ulmer *et al.*, 2002). One major effect of HPP on membranes is that it leads to a higher ordering of molecules. Therefore the membrane can undergo a phase transition under pressure, from a relatively disordered liquid-crystalline-phase to a relatively ordered gel-phase (Winter *et al.*, 2007). How the membrane fluidity will be affected by pressure depending on the membrane composition, like acyl chain length, saturation of fatty acids and phospholipid groups, also the reaction temperature has a huge influence (Kato & Hayashi, 1999; Winter *et al.*, 1996). Generally higher temperatures and more unsaturated fatty acids are switching the point of phase transition to higher pressures. Beside a change in membrane fluidity it has been shown that HHP leads also to an increase of fluid bilayer thickness (Kato *et al.*, 2002; Winter *et al.*, 2007). At the same time, the integrated proteins influence the biophysical state of the membrane, their structure and function is also affected by the biophysical state of the membrane (Lipowsky & Sackmann, 1995). It was shown that a phase transition from liquid-crystalline phase to gel-phase is able to inactivate membrane proteins (Chong *et al.*, 1985; Ulmer *et al.*, 2002). And it was also proposed that a decrease in membrane fluidity, below phase transition, is able to influence protein activity (Kato *et al.*, 2002). Moreover a significant increase in membrane thickness could lead to a hydrophobic mismatch between the transmembrane segment of a protein and the hydrophobic thickness of a membrane. It has been shown that a hydrophobic mismatch affects protein-lipid organization (Killian, 1998; Zein & Winter, 2000). So far little is known how these HHP induced membrane changes influence the behavior of integrated membrane proteins. And especially how important they are for the function of sensor proteins.

1.8 Objectives of the work

The aim of this work was to understand the initial steps of signal transduction and the function of membrane proteins and membranes as sensors for chemical and physical influences. Therefore the interactions between membrane proteins and the membrane should be investigated, which are dependent on membrane characteristics like fluidity and composition. HHP is suggested to influence bacterial physiology by changing structure and function of membranes and/or integrated proteins and was therefore used to study this interaction. The membrane composition was also influenced with temperature, addition of local anesthetics modifying membrane fluidity and use of a fatty acid synthesis mutant. These influences were quantified with help of model systems based on ToxR, *in vivo*. The ToxR protein was used because of its specific functions as sensor and transcription activator. Also its role as some kind of pressure sensor in *P. profundum* and its important part in virulence gene expression in *V. cholerae* were major reasons for its use. Through the ability of ToxR to bind as a dimer to specific promoter regions, it should be possible to follow its dimerization *in vivo*.

ToxR expression cassettes based on ToxR from *P. profundum* should be constructed. These constructs will be used at different membrane compositions where the ToxR dimerization ability in response to HHP will be quantified. The influence of membrane fluidity on ToxR dimerization should be investigated by the use of the expression cassettes in different *E. coli* and *P. profundum* strains and with use of different growing temperatures and addition of the local anesthetic phenethyl alcohol. To analyze the influence of protein structure on dimerization ability in response to HHP and membrane composition a ToxR system based on ToxR from *V. cholerae* should be used. This system had already proven its usefulness in the investigation of TMS interactions (Gurezka *et al.*, 1999; Langosch *et al.*, 1996).

2 Material and Methods

2.1 Material

2.1.1 Bacterial Strains

Table 2.1: Used bacterial strains and their genotypes

Strain	Genotype	Refrence
E. coli ToP10	F-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74 recA1, araD139, Δ(araleu)7697, galU galK rpsL (StrR), endA1 nupG	Invitrogen
E. coli DH5α	F-, deoR, endA1, gyrA96, hsdR17(rk-mk+), recA1, relA1, supE44, thi-1, del(lacZYA- argF)U169, (Phi80lacZdelM15)	Stratagen
E. coli FHK12	F', lacZΔM15, lacY $^{+}$, ProA $^{+}$ B $^{+}$ ara, Δ(lac-proAB), rpsl, ϕ80 dΔ (lacZM15), attB::ctxDsiglacZ	(Kolmar *et al.*, 1995)
E. coli PD28	F-, thiA, relA, araD139, ΔlacU169, rpsL, malT^{c} 1, ΔmalE444, Δ(srlR-recA)306::Tn10	(Duplay *et al.*, 1987)
E. coli JW1081-4	F-, DE(araD-araB)567, lacZ4787(del)(::rrnB-3), LAM-, fabF759(del)::kan, rph-1, DE(rhaD-rhaB)568, hsdR514	(Baba *et al.*, 2006)
E. coli JW1081-4_del_Kan	F-, DE(araD-araB)567, lacZ4787(del)(::rrnB-3), LAM-, ΔfabF, rph-1, DE(rhaD-rhaB)568, hsdR514	This work
E. coli JWPOLA_Δ*fab*F (TMW 2.830)	F-, DE(araD-araB)567, lacZ4787(del)(::rrnB-3), LAM-, ΔfabF759, rph-1, DE(rhaD-rhaB)568, hsdR514, attB::pompL-lacZ	This work
E. coli POLG	F-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74 recA1, araD139, Δ(araleu)7697, galU galK rpsL (StrR), endA1 nupG, attB::pompL-GFP	This work
E. coli POLA (TMW 2.705)	F-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74 recA1, araD139, Δ(araleu)7697, galU galK rpsL (StrR), endA1 nupG, attB::pompL-lacZ	This work
E. coli POHA (TMW 2.827)	F-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74 recA1, araD139, Δ(araleu)7697, galU galK rpsL (StrR), endA1 nupG, attB::pompH-lacZ	This work
P. profundum SS9	*P. profundum* wild type	(Vezzi *et al.*, 2005)
P. profundum TW10	*P. profundum* with ompL::lacZ fusion via mini-Mu insertion	(Welch & Bartlett, 1996)

2.1.2 Plasmids

Table 2.2: Plasmids

Plasmid	Description	Reference
pBAD/*Myc*-HisB	Vector for dose-dependent expression of recombinant proteins in *E. coli*: *amp*$^+$, pBR322 *ori*, myc-epitope, *araC* ORF, *ara*BAD-promoter, *C*-terminal 6xHis-tag	Invitrogen
pBAD/*Myc*-His/*lacZ*	pBAD/*Myc*-His containing *lacZ*	Invitrogen
pQBI63	Vector containing GFP	Qbiogene
pSTBlue1	Multi-purpose cloning vector: *amp*$^+$, *kan*$^+$, MCS, SP6 und T7 binding sites, pUC *ori*, f1 *ori, lacZ*	Novagen
pBADK/*Myc*-HisB	pBAD/*Myc*-HisB whose *amp*$^+$ was exchanged with the *kan*$^+$ from pSTBlue1.	This work
pBADK-ToxR-his	pBADK/*Myc*-HisB with ToxR from *P. profundum* SS9	This work
pLDR8	Helper plasmid contains *int* gene under the control of the λcI_{857} repressor, *kan*$^+$	(Diederich *et al.*, 1992)
pLDR10	Vector for Integration into the λ Attachment Site	(Diederich *et al.*, 1992)
pLDR10+pOmpL-GFP	pLDR10 with integrated *pompL*-GFP fusion	This work
pLDR10+pOmpL-*lacZ*	pLDR10 with integrated *pompL-lacZ* fusion	This work
pLDR10+pOmpH-*lacZ*	pLDR10 with integrated *pompH-lacZ* fusion	This work
pCP20	FLP$^+$, λ ci857$^+$, λ P_R Rep^{ts}, AP^R, Cm^R	(Cherepanov & Wackernagel, 1995)
pToxRIV	Vector for dimerization tests	(Gurezka & Langosch, 2001)
pFL190	Broad host-range expression vector	(Lauro *et al.*, 2005)
pFL190-ToxR	pFL190 with ToxR from *P. profundum* SS9	This work
pRK2073	Is a derivative of pRK2013 containing a Tn7 insert in the kanamycin resistance gene. A helper plasmid which carries the conjugal transfer genes of RK2	(Figurski & Helinski, 1979)

2.1.3 Primer

Table 2.3: Primer (all primers were ordered from MWG Biotech (Ebersberg))

Primer	Sequenz	Chapter
toxR-ex_for	TATATCCATGGAAATGCTTAAAATTTCCACCAA	2.2.3.7
toxR-ex_rev	TAAAGTCGACTTGGCATAGCTTCGAATTAT	2.2.3.7
kan-pstb_for	AGGATCTAGGTGAAGATCCT	2.2.3.7
kan-pstb_rev	ACGAAAACTCACGTTAAGGG	2.2.3.7
p-ompl_for	TTATAAGCTTTTTAAAGGGTTACTGTCATT	2.2.3.7
p-ompl_rev	TTCTCCTTTGCTAGCCATTGCCGAATCCTTTTTAAAAT	2.2.3.7
gfp_for	ATTTTAAAAAGGATTCGGCAATGGCTAGCAAAGGAGAA	2.2.3.7
gfp_rev	TTATTCTAGATTTCGGGCTTTGTTAGCA	2.2.3.7
pOmpL-L_rev	AACGACGGGATCTATCATTGCCGAATCCTTTTTAAAAT	2.2.3.7
lacZ-L_for	ATTTTAAAAAGGATTCGGCAATGATAGATCCCGTCGTT	2.2.3.7
lacZ-L_rev	TTATTCTAGATCATTTTTGACACCAGACCAAC	2.2.3.7
pOmpH-L_for	TTATAAGCTTCTAAATATACCAAAGCAGAA	2.2.3.7
pOmpH-L_rev	AACGACGGGATCTATCATGATAATCCACTGCCTTTTA	2.2.3.7
lacZ-LL_for	TAAAAGGCAGTGGATTATCATGATAGATCCCGTCGTT	2.2.3.7
pBAD-screen	CCATAGCATTTTTATCCATAAG	2.2.3.7
ToxR_ex_1	TATAGCTAGCATGCTTAAAATTTCCACCAA	2.2.3.7
ToxR_ex_2	TATAGAATTCTTATTGGCATAGCTTCGAA	2.2.3.7
pFL190checkF	GCAAGGCGATTAAGTTGGG	2.2.3.7
pFL190checkR	CGACCGACGGTGGCGAT	2.2.3.7
fabFcheckF	GTCTGCGTGGTTATGAG	2.2.3.7
fabFcheckR	GGTAAAACAACCATCACC	2.2.3.7

2.1.4 Chemicals and Enzymes

2.1.4.1 Chemicals

Unless otherwise stated the chemicals used in this work are from Amersham Biosciences (Freiburg), BioRad (München), Gerbu (Gaiberg), Merck (Darmstadt), Roche (Mannheim), Roth (Karlsruhe), Serva (Heidelberg) or Sigma-Aldrich (Taufkirchen).

2.1.4.2 Enzymes

Restriction endonucleases, DNA ligase and Taq polymerase were from MBI-Fermentas (St. Leon-Rot). KOD polymerase was from Novagen. Further enzymes like lysozyme were from Serva (Heidelberg) and Sigma Aldrich (Taufkirchen).

2.1.4.3 Antibodies

The Goat-anti-Rabbit alkaline phosphatase conjugated antibody was from Dako (Hamburg) and the rabbit-anti-his6 antibody was from Rockland (Gilbertsville).

2.1.4.4 Kits

Kits were purchased from Qiagen (Hilden).

2.1.5 Equipment

Systec (Wettenberg): Autoclave Systec 2540 ELV
Tecan (Crailsheim): Micro plate reader (ELISA-reader) Tecan Sunrise
Hermle (Wehingen): Centrifuges
Pharmacia Biotech (Freiburg): Photometer Novaspec II
Dunze GmbH (Rosengarten): Pressure vessel

2.1.6 Software

Clone Manager: Virtual cloning and creation of genetic maps.
Clustal W: Multiple sequence alignments
Excel: Calculation
Sigma Plot: Graphs

2.1.7 Media

2.1.7.1 Growth media

All media were autoclaved, if not noted otherwise, at 121°C for 20 min. Heat-sensitive solutions were sterile filtrated. For solidification 1.5 % Agar-Agar (Difco) was added.

LB-medium (Sambrook *et al.*, 1989)

10 g tryptone, 5 g yeast extract, 5 g NaCl, add 1 l $aqua_{deion}$

SOC-medium (Hanahan, 1983)

20 g tryptone, 5 g yeast extract, 10 mM NaCl, 2.5 mM KCl, add 1 l $aqua_{deion}$

After autoclavation ad 20 mM glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$

Minimal medium

M9 salts (Sambrook *et al.*, 1989), 2mM $MgSO_4$, 0.4 % maltose

Marine broth (for conjugation)

18.75 g 2216 MB (Difco), 10 g tryptone, ad 1 l $aqua_{deion}$

Marine broth (for growth)

28 g 2216 MB (Difco)

2.1.7.2 Media additives

Ampicilin: 50-100 µg/ml

Kanamycin: 50 µg/ml

Chloramphenicol: 25 µg/ml

Isopropyl-β-D-thiogalactopyranosid (IPTG): 40 µg/ml

5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal): 25 µg/ml

2.1.8 Buffer

For all buffers, if not noted otherwise, $aqua_{deion}$ was used as solvent.

2.1.8.1 DNA and enzyme buffer

Buffer for restriction enzymes and ligation enzymes

The supplied buffers were used.

TE-buffer

10 mM Tris, 1mM EDTA, pH 7.5

E1-lysis-solution

25 % (w/v) sucrose, 10 % (w/v) Ficoll, after autoclavation add 0.4 g/l RNAseA, 1 g/l lysozyme

Na-acetate solution

3 M Na-acetate, pH 5.3

EDTA

25 mM EDTA, pH 8

RNAse-lysozyme-solution

1 g/l RNAseA, 2 g/l lysozyme in TE-buffer

Sucrose-solution

20 % (w/v) sucrose in TE-buffer

Protease K-stock-solution

20 g/l protease K in TE-buffer

N-lauroylsarcosin-Na-solution

5 % N-lauroylsarcosin-Na in TE-buffer

Protease K-lauroylsarcosin-solution

25 µl Protease K-stock-solution, 1.2 ml N-lauroylsarcosin-Na-solution

2.1.8.2 Buffer for agarose gel electrophoresis

TAE-buffer

40 mM Tris-acetat, 2 mM EDTA, pH 8

TBA-buffer

90 mM Tris, 90 mM boric acid

Agarose gel

0.5 – 2 % (w/v) agarose in TAE or TBE

2.1.8.3 Buffer for transformation

$CaCl_2$

100 mM $CaCl_2$

Hanahan

10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7 (with KOH), 55 mM $MnCl_2$

2.1.8.4 Buffer for proteins

HEPES-KOH-buffer

50 mM HEPES-KOH, pH 7.0

Buffer A

50 mM HEPES-KOH (pH 8.0), 0.3 M NaCl, 10 % (v/v) glycerol, 0.05 % (w/v) DDM, 40 mM imidazole

Buffer B

50 mM HEPES-KOH (pH 7.0), 0.3 M NaCl, 10 % (v/v) glycerol, 0.05 % (w/v) DDM, 280 mM imidazole

Buffer for SDS-PAGE

See methods

TBS-buffer

20 mM Tris-HCl, 50 mM NaCl, pH 7.5

TBS-T-buffer

20 mM Tris-HCl, 50 mM NaCl, 0.05% Tween 20, pH 7.5

Blocking-buffer

20 mM Tris-HCl, 150 mM NaCl, 3 % BSA

Developer-solution

100 mM Tris-base, 100 mM NaCl, 5mM $MgCl_2$, pH 9,5

NBT-stock-solution

75 g/l NBT (nitro blue tetrazolium) in 70 % dimethylformamide

BCIP-stock-solution

50 g/l BCIP (5-bromo-4-chloro-3-indolyl phosphate) in 70 % dimethylformamide

Blotting-buffer

10 mM CAPS, 10 % methanol, pH 11

Z-Buffer

16.1 g $Na_2HPO_4x7H_2O$, 5.5 g $NaH_2PO_4xH_2O$, 0.75 g KCl, 0.246 g $MgSO_4x7H_2O$, add 1 l $aqua_{deion}$, pH 7.0

2.2 Methods

2.2.1 Isolation and display of DNA

2.2.1.1 Plasmid isolation from *E. coli* with QIAprep Spin Miniprep Kit

The plasmids have been isolated with the QIAprep Spin Miniprep Kit from Qiagen. The Kits purification protocol is based on an alkaline lysis procedure, followed by a binding of plasmid DNA to an Anion-Exchange resin under special low salt and pH conditions. Proteins, RNA, dyes and low molecular weight impurities are removed by a medium salt wash. Plasmid DNA is then eluted in a higher salt buffer and then concentrated and desalted by isopropanol precipitation. All procedures were done as described in the manual, except that the bacteria were taken directly from an agar plate and resuspended in buffer P1.

2.2.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis allows the detection and displaying of DNA or RNA. The negatively charged nucleic acid molecules are moved to an agarose matrix with help of an electric field. The speed of the molecules is depending on their size and conformation (linear, supercoil). For linear DNA a big area exists where there is a dependency between the logarithm of the fragment length and its migration distance. This allows with help of molecular weight markers the determination of the fragment size. A change in agarose concentration changes the resolving area of the gel. With ethidium bromide staining the DNA becomes visible under UV light.

- Make a 0.8-2% agarose solution in TAE or TBA buffer, bring the solution to boil to dissolve the agarose, cool the solution down to approx. 60°C and pour it into the gel rack.
- When the gel is cooled down and solid, remove the comb
- Put the gel into a tank with TAE or TBA
- Mix DNA samples with loading buffer and inject to the wells
- Use a DNA ladder in the first well
- Run the gel between 50-100 V.
- Stain the gel with ethidium bromide
- Detect the DNA under UV light

2.2.1.3 Eckhard-lysis from *E. coli*

Via Eckhard lysis cells can be investigated for plasmid content (Eckhardt, 1978). Therefore the cells will be lysed directly in the wells of an agarose gel with lysozyme and SDS. Afterwards the released plasmids are separated in the gel.

- Make a 0.8-1% agarose solution in TAE buffer, bring the solution to boil to dissolve the agarose, cool the solution down to approx. 60°C.
- Add 1 ml 20% (w/v) SDS to the solution and pour it into the gel rack
- When the gel is cooled down and solid, remove the comb
- Put the gel into a tank with TAE
- Take a small amount of a bacterial colony and resuspend in 10 µl TE buffer
- Add 20 µl E1 lysis solution
- Put 20 µl on the gel
- Incubate the probes for 15 min in the wells
- Start 15-20 min by 20 V then 2-3 h at 80-100 V

- 2 h watering, change water periodically
- Stain the gel with ethidium bromide
- Detect plasmids with UV light

2.2.2 Polymerase chain reaction (PCR)

2.2.2.1 General PCR

PCR is an *in vitro* enzymatic replication of specific DNA regions. Therefore the region which should be amplified has to be flanked with short oligonucleotides (primers). These primers act as the starting points for a DNA polymerase (mostly heat-stable), which assembles a new DNA strand from nucleotides using single-stranded DNA as template. The new synthesized strand will be separated by heat; so that the primers can bind again (annealing) and a new synthesis (extension) can be started. The heat stability of the polymerase allows a manifold repeat of the reaction. Under optimal conditions an exponential amplification can be achieved. The used primers should have similar annealing temperatures and no strong secondary structures. The exact protocol for a PCR depends on the desired application and the used polymerase. The PCR is performed in a programmable Thermocycler.

Protocol for *Taq*-polymerase:

Taq-polymerase is named after the thermophilic bacterium *Thermus aquaticus*. The replication speed is 1 kb/min. A *Taq* DNA product has an Adenine overhang at its 3' end.

- 4 µl dNTPs (2,5 mM)
- 5 µl 10x *Taq*-buffer
- 1 µl primer for (10 µM)
- 1 µl primer rev (10 µM)
- 1 µl template-DNA
- 0.2 µl *Taq*-polymerase (10 U/µl)
- Fill up with $aqua_{deion}$ to 50 µl

Program for PCR with *Taq*-polymerase:

- Initialization step: 2 min, 92°C
- Denaturation step: 30 sec, 92°C
- Annealing step: depends on primer
- Elongation step: 1 min/1 kb, 72°C
- Repeat denaturation, annealing and elongation 30 times

- Final elongation: 10 min, 72°C

Protocol for KOD-hot-start-DNA-polymerase:

KOD-hot-start-DNA-polymerase (Novagen) is a polymerase from *Thermococcus kodakaraensis,* which builds a complex with monoclonal antibodies. The polymerase is inhibited by the antibodies, which makes it easier to handle (no ice). The antibodies are disconnected from the polymerase by heating up to 94°C for 2 min (hot-start). The polymerase has a 3'-5'-exonuclease activity (proof-reading), which is upgrading the accuracy of the polymerase reaction. KOD is quite faster than *Taq* with a replication speed of 20 sec/ 1 kb. KOD makes blunt ends.

- 5 µl 10 KOD-buffer
- 5 µl dNTPs (2 mM)
- 2 µl $MgSO_4$ (25 mM)
- 1 µl primer for (10 µM)
- 1 µl primer rev (10 µM)
- 1 µl template
- 1 µl KOD (1 U/µl)
- Fill up with $aqua_{deion}$ to 50 µl

Program for PCR with KOD:

- Initialization step: 2 min, 94°C
- Denaturation step: 15 sec, 94°C
- Annealing step: depends on primer
- Elongation step: 20 sec/1 kb, 68°C
- Repeat denaturation, annealing and elongation 30 times
- Final elongation: 10 min, 68°C

2.2.2.2 Cleaning of PCR products with QIAquick PCR purification Kit

The PCR purification Kit from Quiagen allows the cleaning of the PCR products from primers and nucleotides. The procedure is based on the binding of the PCR product to an Anion-Exchange Resin under special low salt and pH conditions. All procedures were done as in the manual described.

2.2.2.3 MOE (mutagenesis by overlap extensions)

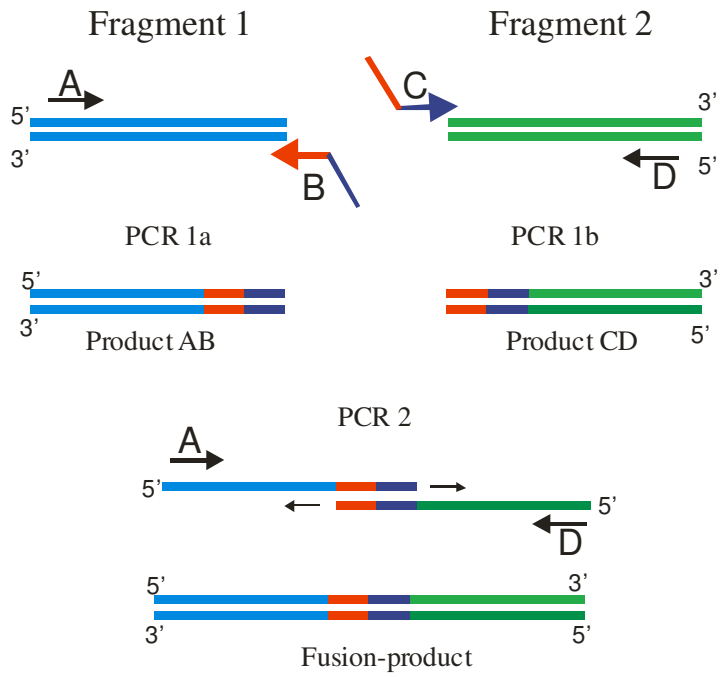

Figure 3.1: Schematic diagram of a MOE-PCR. A, B, C and D are the used primers, B and C are the complementary primers.

MOE (Ho *et al.*, 1989) can be used to combine DNA-sequences of almost any kind with each other. In a first step the DNA fragments are generated by PCR using complementary primers. Then the fragments are combined in a following reaction in which the overlapping ends anneal, allowing the 3' overlap of each strand to serve as a primer for the 3' extension of the complementary strand. The resulting fusion product is amplified further by PCR. It is possible to introduce specific alterations in the nucleotide sequence by incorporating nucleotide changes into the overlapping primers.

PCR1:

- 5 µl 10 KOD-buffer
- 5 µl dNTPs (2 mM)
- 2 µl $MgSO_4$ (25 mM)
- 1 µl primer for, fragment 1 or fragment 2 (10 µM)
- 1 µl primer rev, fragment 1 or fragment 2 (10 µM)
- 1 µl template
- 1 µl KOD (1 U/µl)

- Fill up with $aqua_{deion}$ to 50 µl

Program for PCR1:

- Initialization step: 2 min, 94°C
- Denaturation step: 30 sec, 94°C
- Annealing step: depends on primer
- Elongation step: 20 sec/1 kb, 68°C
- Repeat denaturation, annealing and elongation 30 times
- Final elongation: 10 min, 68°C

The products were cleaned via an agarose gel and were then used for PCR 2.

PCR 2:

- 5 µl 10 KOD-buffer
- 5 µl dNTPs (2 mM)
- 2 µl $MgSO_4$ (25 mM)
- 1 µl primer for, fragment 1 (10 µM)
- 1 µl primer rev, fragment 2 (10 µM)
- 1 µl fragment 1
- 1 µl fragment 2
- 1 µl KOD (1 U/µl)
- Fill up with $aqua_{deion}$ to 50 µl

Program for PCR 2:

- Initialization step: 2 min, 94°C
- Denaturation step: 30 sec, 94°C
- Annealing step: depends on primer and products 1 and 2
- Elongation step: 20 sec/1 kb, 68°C
- Repeat denaturation, annealing and elongation 30 times
- Final elongation: 10 min, 68°C

2.2.3 Cloning experiments

2.2.3.1 Cleavage of DNA by type II restriction enzymes

A type II restriction enzyme recognizes a specific palindromic sequence of DNA and cleaves the DNA at this site. Depending on the enzyme there will be an overhang (*sticky end*) after the cleaving or not (*blunt end*).

- 2-4 µl restriction-buffer (depending on the enzyme)
- 1-5 U/µg DNA restriction enzyme
- DNA
- Fill up with $aqua_{deion}$ to 20 µl
- Incubate at the ideal temperature (common 30°C or 37°C)
- If necessary inactivate by heat and clean with kit
- Control of the cleaving by gel electrophoresis

2.2.3.2 Ligation

The DNA ligase is an enzyme which forms two covalent phosphodiester bonds between 3' hydroxyl ends of one nucleotide with the 5' phosphate end of another. Therefore the ends have to be sticky ends or blunt ends. For blunt ends higher enzyme concentrations and different reaction conditions are required.

- cleaved vector DNA and insert DNA rate 1:5-15
- 2 µl 10x ligase buffer
- 2 units T4 DNA ligase
- Fill up with $aqua_{deion}$ to 20 µl
- 2-4 h by RT or over night by 4°C

2.2.3.3 Transformation of *E. coli* ($CaCl_2$)

With $CaCl_2$-treatment *E. coli* cells becoming competent. Competent cells are able to take up exogenous DNA (plasmids) from the environment.

Creation of competent *E.coli*:

- Carry *E. coli* from an o/n culture to 10 ml LB-broth, incubate through logarithmic phase ($o.D._{590}$ of approx. 0.5)
- Centrifuge 5 min by 5000 G and 4 °C
- Resolve pellet gently in 5 ml 100mM $CaCl_2$-solution (4°C)
- Incubate 30 min by 0°C (on ice)

- Centrifuge 5 min by 5000 G and 4 °C
- Resolve pellet gently in 1 ml 100 mM $CaCl_2$-solution (4°C)
- Add 175 µl cold glycerol (87%)
- Incubate for 2 h or o/n by 0°C
- Stock by -80°C (freeze with liquid nitrogen)

Transformation of competent *E. coli*:

- Give DNA to 200 µl competent *E. coli*-cells
- Incubate 30 min by 0°C
- Heat shock: 3 min by 42°C
- Add 800 µl LB and mix gently
- Incubate 45 min by 37°C or 30°C (depends on plasmid)
- Plate 100 µl on selective agar plates
- Centrifuge the cells and decant supernatant
- Resuspend cells in left over medium and plate on selective agar plates
- Incubate o/n

2.2.3.4 Transformation of *E. coli* according to Hanahan

The transformation according to Hanahan (Hanahan, 1983) is similar to $CaCl_2$-transformation but uses additionally manganese.

Creation of competent *E. coli*:

- Carry 5 ml *E. coli* from an o/n culture to 50 ml SOC-broth, incubate through exponential phase ($o.D._{590}$ of approx. 0.5)
- Centrifuge 5 min by 5000 G and 4 °C
- Resolve pellet gently in 15 ml transformation buffer (4°C)
- Incubate 15 min by 0°C (on ice)
- Centrifuge 5 min by 5000 G and 4 °C
- Resolve pellet gently in 5 ml transformation buffer (4°C)
- Add 175 µl DMSO, mix gently
- Incubate 5 min by 0°C
- Add 175 µl DMSO, mix gently
- Incubate 5 min by 0°C
- Stock by -80°C (freeze with liquid nitrogen)

Transformation of *E. coli*:

- Give DNA to 200 µl competent *E. coli*-cells
- Incubate 20 min by 0°C
- Heat shock: 90 sec by 42°C
- Add 800 µl SOC and mix gently
- Incubate 45 min by 30°C, 37°C or 42°C (depends on plasmid)
- Plate 100 µl on selective agar plates
- Centrifuge the cells and decant supernatant
- resuspend cells in left over medium and plate on selective agar plates
- Incubate o/n

2.2.3.5 Conjugation of *P. profundum*

The deep sea bacterium *P. profundum* cannot be transformed. For that reason conjugation has to be used to transfer exogenous DNA (plasmids) into *P. profundum* cells. Therefore a special conjugation protocol for *P. profundum*, based on the vector pFL190, has been used (Lauro *et al.*, 2005).

- Set up 4 ml *P. profundum* culture in 75% 2216 Marine broth and incubate at 16°C for 2days.
- Set up 4ml cultures of plasmid strain (*E. coli* + pFL190-fragment) and helper *E. coli* (pRK2073) in LB-broth and incubate at 37°C for 1 day.
- Centrifuge cultures 5 min by 5000 G.
- Decant supernatant and resuspend in 4 ml media to wash cells of antibiotics.
- Centrifuge cultures 5 min by 5000 G.
- Resuspend the *E. coli* cells in 1 ml 75% 2216 Marine broth.
- Resuspend *P. profundum* in left over 2216 medium in the tube and store on ice.
- Place 4 filters on conjugation plates and add: 60 µl donor strain *E. coli* (with your plasmid), 60 µl helper strain *E. coli* (pRK2073) and 120 µl *P. profundum*. Mix by pipetting on filter
- Store conjugations on bench under cover overnight.
- Prepare 4 ml tubes of 75% 2216 Marine broth and put sterile transfer filters into tubes on ice. Vortex tubes for 5 seconds, put on ice, vortex again 5 seconds. Remove filter from tubes.
- Centrifuge cultures 5 min by 5000 G.
- Decant supernatant and resuspend in left over medium.

- Plate out 60 µl onto 2216 plates with Kan 200 µg/ml and Strep 150 µg/ml.
- Incubate at 16°C for 5 days for growth of exconjugants.

2.2.3.6 Chromosomal integration of DNA

The background of this method is a two vector system (pLDR8 and pLD10) (Diederich *et al.*, 1992). This system allows the integration into the λ attachment site (*attB*) from *E. coli*. The cloning vector pLDR10 features the λ attachment site (*attB*). The helper plasmid pLDR8 features the λ *int* gene with λ P_R promoter, which is under control of the temperature sensitive cI_{857} repressor. The DNA-fragment in question has to be cloned into the multiple cloning site from pLDR10. Thereafter the origin cassette has to be cut (*NotI*) out of pLDR10 and the fragment be ligated. This leads to a closed circular DNA molecule lacking a replication origin. The *E. coli* strain of choice carrying pLDR8 will be then transformed with this fragment. After that the strain will be incubated by 42°C for the induction of the *int* gene expression. Additionally the high temperature leads, after a few cell cycles, to the loss of pLDR8, because its replication is blocked by 42°C (cI_{857} repressor).

- Cut pLDR10 carrying the fragment in question with *NotI.*
- Put the cleaved fragment for separation on an agarose gel.
- Cut the right fragment out of the gel and purify with a kit.
- Ligate the fragment.
- Transform competent (Hanahan by 30°C) ToP10-pLDR8 cells with the ligated fragment.
- Incubate the cells for 60 min by 37°C.
- Plate 100 µl on LB plates with Amp 50 µg/ml.
- Centrifuge the cells and decant supernatant.
- Resuspend cells in left over medium and plate on LB plates with Amp 50 µg/ml.
- Incubate o/n by 42°C
- The chromosomal integration was confirmed by Eckhard-lysis (plasmid contend) and PCR (amplification of the integrated plasmid)

2.2.3.7 Vector construction

<u>pBADK/*Myc*-HisB:</u>

The gene encoding kanamycine resistance was amplified from vector pSTBlue1 using the primers kan-pstb_for and kan-pstb_rev. The fragment and vector pBAD/*Myc*-HisB were

digested with *PagI*. The digested fragment was then ligated into the digested vector pBAD/*Myc*-HisB resulting in pBADK/*Myc*-HisB.

pBADK-ToxR-his:

The ToxR gene was amplified out of *P. profundum* SS9 using the primers toxR-ex_for and toxR-ex_rev. The fragment and vector pBADK/*Myc*-HisB were digested with *NcoI* and *SalI*. The digested ToxR fragment was then ligated into the digested vector pBADK/*Myc*-HisB resulting in pBADK-ToxR-His.

pLDR10-pOmpL-GFP:

The promoter region of *ompL* (The 400 bp upstream of *ompL*) was amplified out of *P. profundum* SS9 using the primers p-ompl_for and p-ompl_rev. The GFP gene was amplified from vector pQBI63 using the primers gfp_for and gfp_rev. Both fragments were fused by MOE-PCR. The fused fragments and vector pLDR10 were digested with *HindIII* and *XbaI*. The digested fragment was then ligated into the digested vector pLDR10 resulting in pLDR10-pOmpL-GFP.

pLDR10-pOmpL-*lacZ*:

The promoter region of *ompL* (The 400 bp upstream of *ompL*) was amplified out of *P. profundum* SS9 using the primers p-ompl_for and pOmpL-L_rev. The *lacZ* gene was amplified from vector pBAD/*Myc*-His/*lacZ* using the primers lacZ-L_for and lacZ-L_rev. Both fragments were fused by MOE-PCR. The fused fragments and vector pLDR10 were digested with *HindIII* and *XbaI*. The digested fragment was then ligated into the digested vector pLDR10 resulting in pLDR10-pOmpL-*lacZ*.

pLDR10-pOmpH-*lacZ*:

The promoter region of *ompH* (The 400 bp upstream of *ompH*) was amplified out of *P. profundum* SS9 using the primers pOmpH-L_for and pOmpH-L_rev. The *lacZ* gene was amplified from vector pBAD/*Myc*-His/*lacZ* using the primers lacZ-LL_for and lacZ-L_rev. Both fragments were fused by MOE-PCR. The fused fragments and vector pLDR10 were digested with *HindIII* and *XbaI*. The digested fragment was then ligated into the digested vector pLDR10 resulting in pLDR10-pOmpH-*lacZ*.

pFL190-ToxR:

The ToxR gene was amplified out of *P. profundum* TW10 using the primers ToxR_ex_1 and ToxR_ex_2. The fragment and vector pFL190 were digested with *NheI* and *EcoRI*. The digested ToxR fragment was then ligated into the digested vector pFL190 resulting in pFL190-ToxR.

2.2.4 Protein analyses

2.2.4.1 Purification of His6-ToxR

Histidine-tagged ToxR was purified using the protocol described from Bart van den Berg van Saparoea *et al.* (van den Berg van Saparoea *et al.*, 2005) with the following modifications: Top10 + pBADK-ToxR-his cells were grown at 37 °C to an OD_{590} of 0.6-0.8. ToxR expression was induced by addition of 0.0002 % arabinose and cells were grown for further 90 min. Cells were harvested, washed with 50 mM HEPES-KOH (pH 7.0) and resuspended in the same buffer. The cells were disrupted via ultrasonic treatment on ice (10 s, cycle 0,5, amplitude 80 %) for three times (with 6 cycles each) with 20 s intercooling on ice using a ultrasonic processor UP 200s (Hielscher Ultrasonics GmbH) followed from a 10 s treatment with a homogenizer Miccra D-8 DS-20/PG (stage A). Cell debris and unbroken cells were removed at 13.000 g at 4 °C for 30 min. Membrane vesicles were collected using an ultra centrifuge (125.000 g, 1 h, 4 °C), washed twice in 50 mM HEPES-KOH (pH 7.0) with 10% glycerol, centrifuged (280.000 g, 30 min, 4 °C), resuspended in the same buffer, frozen in liquid nitrogen and stored at -80 °C. Membrane lipids were solubilised and mixed with Ni Sepharose 6 Fast Flow beads, (~25 µl of beads/mg of protein) which was pre-equilibrated in buffer A (50 mM Hepes-KOH (pH 8.0), 0.3 M NaCl, 10 % (v/v) glycerol, and 0.05 % (w/v) DDM) containing 40 mM imidazole. The suspension was incubated for 1 h at 4 °C, transferred to a centrifuge tube, centrifuged for 2 min at 1000 g, washed 5 times with 4 volumes of buffer A and protein was eluted with buffer B (pH 7.0), having a composition as buffer A but containing 280 mM imidazole (all steps at 4 °C). The purified protein was stored at -80 °C.

2.2.4.2 SDS-PAGE

The heterologous expressed proteins were controlled with SDS-Page after Laemmli (Laemmli, 1970). It is a technique, which separates proteins according to their electrophoretic mobility. The acrylamide concentration in the resolving gel depends on the size of the proteins. The smaller the protein the greater the concentration has to be. A standard formula for a mini gel is:

Stacking gel:

- $aqua_{deion}$; 6.1 ml
- 1,5 M Tris*HCl, pH 8.8; 2.5 ml
- 25 % (w/v) SDS-solution; 40 µl
- Acrylamide / bisacrylamide (29/1); 1.3 ml
- 10 % ammonium persulfate; 50 µl
- TEMED; 5 µl

Resolving gel:

- $aqua_{deion}$; 3.35 ml
- 1,5 M Tris*HCl, pH 8.8; 2.5 ml
- 25 % (w/v) SDS-solution; 40 µl
- Acrylamide / bisacrylamide (29/1); 4 ml
- 10 % ammonium persulfate; 50 µl
- TEMED; 5 µl

5x running buffer:

- Tris*HCl; 9 g
- Glycerol; 43.2 g
- SDS; 3 g
- $aqua_{deion}$; add 600 ml, pH 8.3

Sample buffer:

- 0.5 Tris*HCl, pH 6.8; 1 ml
- 87 % Glycerol; 0.92 ml
- 25 % (w/v) SDS-solution; 0.7 ml
- 2-mercaptoethanol; 0.4 ml
- 1 % bromophenol blue; 0.4 ml
- $Aqua_{deion}$; 4.58 ml

The electrophoretic separations were done in a Mini-Protean III electrophoresis cell (BioRad, Munich) at approx. 160 V, 100 mA for 1 h.

2.2.4.3 Gel staining

The gels were stained with Coomassie brilliant blue (CBB). CBB is an anionic dye, which binds to proteins non-specifically. A gel will be stained for 30 min by slow heating. Afterwards it will be incubated in destaining solution till the background is destained and the protein bands are visible. The gels will be scanned and digitalized.

Coomassie staining solution:

- 0.25 g/l CBB
- 10 % (v/v) acetic acid
- 50 % (v/v) ethanol

Destaining solution:

- 10 % (v/v) acetic acid
- 50 % (v/v) ethanol

2.2.4.4 Western Blot

The via SDS-PAGE separated proteins were blotted on nitrocellulose membranes with a semi-dry-electro blotting procedure (Burnette, 1981).

- Store blotting buffer on ice.
- Prepare per gel one nitrocellulose membrane (mark one corner) and three filter papers.
- Check the wettability of the nitrocellulose membrane with methanol.
- Incubate gel for 20 min in blotting buffer.
- Wash the nitrocellulose membrane for 5 min in blotting buffer.
- Soak filter papers with blotting buffer.
- Stack the gel, membrane and filter papers in the following order: (cathode) filter, filter, gel, membrane, filter, filter (anode).
- Put the stack into a plotting apparatus.
- Transfer at approx. 0.8 mA/cm^2 for approx. 1.5 h.
- Wash membrane for 2 min in methanol.
- Wash membrane for 1 min in TBS-buffer.
- Incubate membrane for 1 h in blocking buffer containing 1 % BSA.
- Incubate membrane for 1 h with primary antibody.
- Wash membrane 3 x 5 min in TBS-T-buffer.
- Incubate membrane for 1 h with secondary antibody.

- Wash membrane 3 x 5 min in TBS-T-buffer.
- Wash membrane 5 min in TBS-buffer.
- Incubate membrane with staining solution till bands become visible (approx. 30 to 120 sec.)
- Stop reaction adding EDTA.
- Wash membrane in $aqua_{deion}$ and dry the membrane by RT and protected from light.
- The membrane will be scanned and digitalized

2.2.5 Determination of fatty acid composition

For the determination of fatty acid composition the *E. coli* strains were sent to Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig). There the cells were grown at 30°C and 37°C on LB agar plates. The fatty acids were extracted, transesterified and analyzed by gas chromatography.

2.2.6 Membrane phase state determination

2.2.6.1 Protoplast formation

E. coli POLA protoplasts were prepared similar as described before by R.L. Weiss (Weiss, 1976): *E. coli* POLA was grown in LB-medium to an optical density at 600 nm of approx. 0.9 at 37°C. The cells were harvested by centrifugation at 4°C at 10000 G for 5 min. The bacteria were washed twice at 23°C with 10 mM Tris buffer (pH8). The pellet was suspended by pipetting using the following formula according to the conventions of Osborn et al. (Osborn *et al.*, 1972): 500 ml culture x 0.9 = 450 optical density units, with a suspension of the cells to 10 optical density units/ml performed thereafter. Suspension in 45 ml of 0.1 M Tris (pH8) containing 20% (w/w) sucrose was done at 37°C directly in the centrifuge tube. Cells were then transferred into small flask, and the temperature was adjusted to 37°C. Within 1 min 2.25 ml of a 2 mg/ml solution of lysozyme in $aqua_{deion}$ was added for a final concentration of 100 µg lysozyme per ml. During the addition of lysozyme the suspension of the cells was stirred with a magnetic stirrer. After the addition the temperature was adjusted to 37°C. Incubation was carried out with stirring for 12 min at 37°C, after which EDTA was added by slow dilution using 0.1 M EDTA (pH7) (1:10 (v/v) EDTA/cells) with prewarmed (37°C) EDTA, added slowly over 2.5 min with continuous stirring to avoid lysis. EDTA, pH8, may be substituted. The temperature will drop during this addition, and it should be adjusted back to 37°C. Within 8 to 10 min more than 99% of the cells become spherical; they can be checked for roundness in the phase microscope.

2.2.6.2 Determination of the pressure-dependent phase state of the membrane

Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) fluorescence spectroscopy was used to study the polarity of the lipid interface and to detect potential phase changes of the lipid membrane. Laurdan is an amphiphilic fluorescence probe which allows the determination of phase transitions in model and biological membranes (Parasassi *et al.*, 1990; Parasassi *et al.*, 1991). The spectral changes of the emission spectrum of Laurdan is generally quantified by the so-called generalized polarization function, witch is defined as $GP = (I_B\text{-}I_R) / (I_B+I_R)$, where I_B and I_R are the fluorescence intensities at 440 nm (characteristic for a gel (ordered) phase environment) and 490 nm (characteristic for a fluid, liquid-crystalline lipid state), respectively. Hence, *GP* values range from -1 to +1. The fluorescence spectroscopic measurements were performed using a K2 multifrequency phase and modulation fluorometer (ISS inc., Champaign, Ill). The temperature of 37°C was controlled by a circulating water bath and 1 ml of sample (Laurdan embedded in *E. coli* POLA protoplast) was pressurized using a home-built high pressure cell with optical windows using deionized water as pressurizing agent (Herberhold *et al.*, 2003). The measurements of the GP values were performed at Dortmund University of Technology by Nagarajan Periasamy.

2.2.7 Control of integration into the inner membrane

Only right oriented (N-terminus in cytoplasm, C-terminus in periplasm) and integrated ToxR fusion proteins can be used for an interaction analysis. Therefore *E. coli* PD28 cells have been transformed with the ToxR fusion proteins. These cells have a genetic MalE deletion which leads to the fact that they are unable to grow in minimal medium, with maltose as the only carbon source (Duplay *et al.*, 1987). But if they are transformed with plasmids of the ToxR fusion proteins, and the protein is integrated into the membrane in the right orientation, the MalE part of the protein is in the periplasm. Now the cells are able to take up maltose and are able to grow.

- *E. coli* PD28 was transformed with ToxR vectors.
- The cells were grown o/n in LB medium.
- The cells were centrifuged for 5 min at 5000 G.
- The pellets were washed in PBS-buffer 3 times.
- M9 medium was inoculated with the washed cells.
- The cells were incubated at 37°C for 72 h.

2.2.8 Determination of ToxR dimerization

The β-galactosidase activities were quantified in crude cell lysates of the cells transformed with the different plasmids. Therefore, in case of FHK12, POLA and JWPOLA_Δ*fabF*, fresh LB medium containing Amp, Kan and L-arabinose were inoculated with an overnight culture of the different transformed cells. For *P. profundum* 2216 MB containing Kan, Strep and L-arabinose was inoculated with an o/n culture. The inoculated medium was then transferred to 3 ml transfer pipettes. The transfer pipettes were heat sealed, transferred into a pressure vessel and the probes were incubated. *E. coli* cells were incubated 22 h at 37°C respectively 30°C, and *P. profundum* cells were incubated 72 h at 15°C, all under different pressure conditions. For the measurement of β-galactosidase activity, 20 - 30 µL of the sample were transferred into a 90 well micro titer plate. The OD_{590} was before measured with a Novaspec II photometer (Pharmacia Biotech). After addition of 100 µL chloroform-saturated Z-buffer (100 mM sodium phosphate, 1 mM KCl, 1 mM $MgSO_4$, 10 g/l DTT, pH 7.0) the cells were lysed by addition of 100 µL of Z-buffer containing 0.4% SDS and incubation at 28°C for 30 min. 50 µL of Z-buffer containing 40 mg/ml o-nitrophenyl-β-D-galactopyranoside (ONPG) were added to the cell lysate and the OD_{405} was monitored for a period of 35 min at 28°C in intervals of 60 seconds with an Tecan Sunrise ELISA-reader (Tecan). The specific β-galactosidase activities are expressed as Miller units (Miller, 1972) and were calculated as follows:

$$1 \text{ Miller Unit} = 1000 * \frac{Abs_{405}}{Abs_{590} * v * t}$$

Where:

- Abs_{405} is the absorbance of the yellow o-nitrophenol.
- Abs_{590} reflects cell density.
- t = reaction time in minutes.
- v = volume of culture assayed in milliliters.

3 Results

3.1 ToxR from *P. profundum* SS9

3.1.1 Construction of an expression cassette in *E. coli* ToP10

For the expression cassette construction only ToxR from *P. profundum* was used. ToxS was not included in the construction, since it was shown before in *Vibrio cholerae* that, if ToxR is over expressed, ToxS is not necessary for dimerization (Ottemann & Mekalanos, 1996). Later the results of this work confirmed this also for ToxR from *P. profundum*. Beside ToxR the promoter regions from *ompL* and *ompH* were used for the construction. For *ompL* a dimerized ToxR acts as an activator and for *ompH* as a repressor (Bidle & Bartlett, 2001). The promoter regions (300 bp upstream of the ORF) from *ompL* and *ompH* were fused to reporter genes (GFP and *lacZ*) and integrated into the *E. coli* ToP10 (resulting in reporter strains *E. coli* POGA for *ompL* and GFP, *E. coli* POLA for *ompL* and *lacZ* and, *E. coli* POHA for *ompH* and *lacZ*) and *E. coli* JW1081-4 (resulting in reporter strain *E. coli* JWPOLA_Δ*fabF*) chromosome. In a first attempt the promoter region of *ompL* was fused to a GFP reporter gene. After a few experiments with this construct it was clear that not enough GFP was produced to get a strong enough signal. Therefore a *lacZ* reporter gene instead of a GFP reporter gene was used. With *lacZ* as reporter gene it was possible to get a strong enough signal to perform these experiments. Beside the construct with promoter region from *ompL* also a construct with the promoter region of *ompH* was used in combination with *lacZ*. The *ompH* promoter region was used because of *ompH* positive regulation under pressure in *P. profundum*. Still it was not possible to get a strong enough signal to perform experiments with this construct. To integrate the plasmids into *E. coli* JW1081-4 it was necessary to eliminate the kanamycin resistance gene (kan^R), because the kanamycin resistance was used in the integration system as a marker gene. This was manageable because the mutant was constructed using a technique, for one-step inactivation of chromosomal genes in *E. coli*, developed by Datsenko and Wanner (Datsenko & Wanner, 2000). The basic strategy of this technique is the replacement of a chromosomal sequence with a selectable antibiotic resistance, where the antibiotic resistance gene is flanked by FRT (FLP-recombinase recognition target) sites. Therefore after selection it is possible to eliminate the resistance gene by using the helper plasmid pCP20 encoding the FLP-recombinase. The mutant without kanamycin resistance was named *E. coli* JW1081-4_del_Kan and used for integration. The ToxR protein was expressed from the vector pBADK-ToxR-His under control of an arabinose inducible promoter (*ara*BAD). An overview of the expression cassette is shown in figure 3.1.

The vector pBADK-ToxR-His was also used for the over expression and purification of His6-ToxR, like described in chapter 2.2.4.1. The His6-ToxR was purified out of the membrane phase, which proofs that ToxR was integrated into the membrane. By using also plasmid pBAD/ *Myc*-HisB as a negative control it was possible to show that the signal was mainly caused by ToxR and not by the vector or basal activity. The negative control had an average β-galactosidase activity from approx.: 40 Miller units at 0.1 MPa. To moreover analyze if the protein expression from vector pBAD/ *Myc*-HisB is pressure sensitive, plasmid pBAD/*Myc*-His/*lacZ* was used in *E. coli* strain Top10. A possible pressure sensitivity of the promoter would have influenced the results and therefore made the system not useful for the experimental approach.

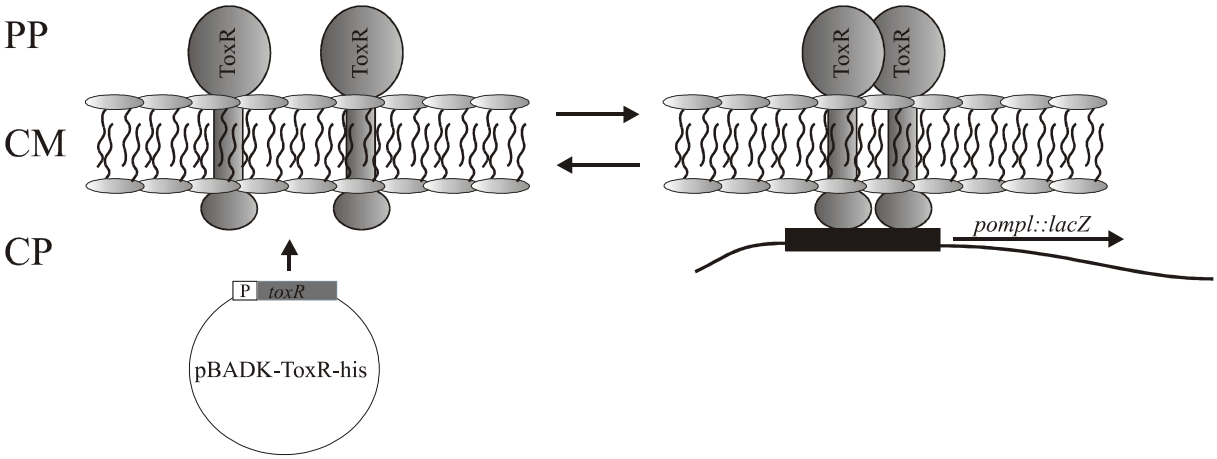

Figure 3.1: Expression construct with ToxR from *P. profundum*. ToxR is encoded on the plasmid pBADK-ToxR-his, where its transcription is controlled by the arabinose promoter. The ToxR protein integrates into the cytoplasmatic membrane (CP). Dimerized ToxR binds to the *pompL* promoter thus initiating *lacZ* transcription in the indicator cells. PP, periplasm; CP, cytoplasm.

3.1.2 Pressure sensitivity of the araBAD promoter

To analyze the pressure sensitivity of the araBAD promoter on pBAD/ *Myc*-HisB, *E. coli* Top10 was transformed with vector pBAD/*Myc*-His/*lacZ* (Invitrogen). The experiments with transformed strain *E. coli* ToP10+pBAD/*Myc*-His/*lacZ* were performed, like described in chapter 2.2.8. The β-galactosidase activities were determined after incubation for 22 h at 37°C and pressures of 0.1, 20, 40 and 50 MPa. The results are shown, as alterations in % compared to the β-galactosidase activity at 0.1 MPa, in figure 3.2. No significant changes in β-

galactosidase activity were observed up to 50 MPa revealing the araBAD promoter as pressure insensitive within this pressure range.

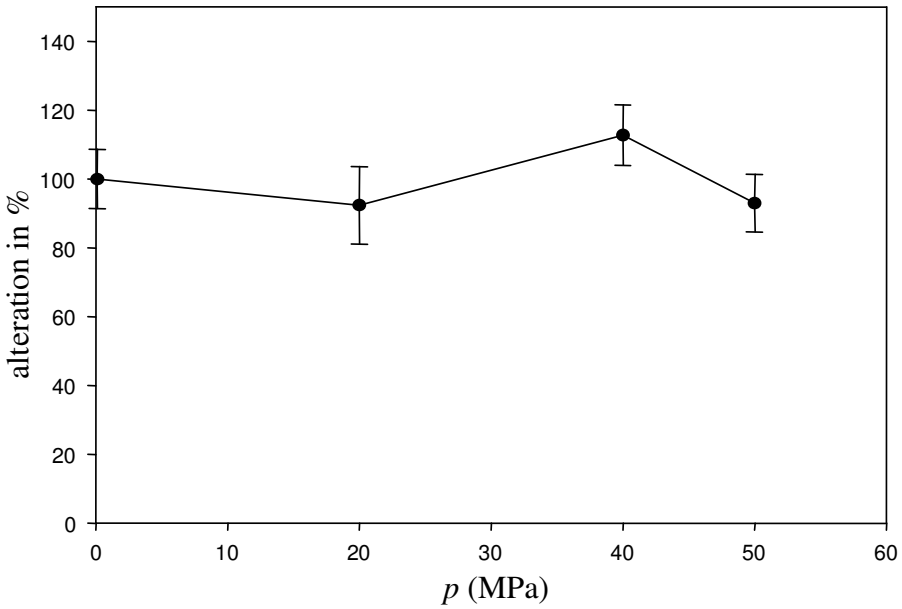

Figure 3.2: Alteration of the β-galactosidase activity from the strain *E. coli* ToP10+pBAD/*Myc*-His/*lacZ* (—●—) after incubation for 22 h at 37°C and pressures from 0.1 MPa – 50 MPa. 100% enzyme activity is equivalent to control incubation under atmospheric pressure.The error bars represent the standard deviation (n=3).

3.1.3 Dimerization experiments with ToxR from *P. profundum*

Beside HHP as the major tool to affect membrane organization, also different approaches were made to change the membrane organization otherwise. This was done to see the effects of various lipid environments on the protein behavior under different pressures. One approach was to perform the experiments at different temperatures (37 °C and 30°C), since it is known that temperature influences the membrane organization. Another approach was the use of phenethyl alcohol, which is also able to influence the membrane organization, by affecting the ordering of the phospholipid chains (Jordi *et al.*, 1990). It was shown before for *P. profundum* that the addition of 3 mM phenethyl ethanol reversed the high-pressure regulation of OmpL and OmpH in $ToxR^+$ cells (Welch & Bartlett, 1998). The dimerization experiments were performed in the reporter strain *E. coli* POLA. Additional a Δ*fabF* reporter strain, *E. coli* JWPOLA_Δ*fabF*, was used. The *fabF* gene encodes the enzyme β-ketoacyl-ACP synthase II, which catalyses the unsaturated fatty acid *cis*-vaccenic acid (18:1 *cis*-11). Experiments have shown before that *E. coli* Δ*fabF* mutants are deficient in *cis*-vaccenic acid (18:1 *cis*-11)

synthesis and are not able to regulate the amount of *cis*-vaccenic acid (18:1 *cis*-11) in response to temperature (Garwin *et al.*, 1980b; Gelmann & Cronan, 1972; Ulrich *et al.*, 1983). Also experiments with a *P. profundum* Δ*fabF* mutant have shown before that this mutant is pressure-sensitive. Interestingly it was shown in the same work that an *E. coli* Δ*fabF* mutant (*E. coli* MR86 (Magnuson *et al.*, 1995)) was not more pressure sensitive than its parental strain (Allen & Bartlett, 2000). Last but not least the ToxR protein was over expressed in its origin strain *P. profundum* (*P. profundum* TW10 (Welch & Bartlett, 1996)). All Dimerization experiments were performed, like described in chapter 2.2.8.

3.1.3.1 Dimerization of ToxR at 37°C

The β-galactosidase activities were determined after incubation for 22 h at 37°C and pressures of 0.1, 10, 20, 25, 30, 35, 40 and 50 MPa. The results are shown in figure 3.3 where the β-galactosidase activity is expressed in Miller units. By noticing of the standard deviation, there were no significant changes in β-galactosidase activity to observe up to 20 MPa. At 25 MPa the activity decreased to almost half the activity observed at 0.1 MPa and stayed constant till 30 MPa. Past 30 MPa the β-galactosidase activity decreased to a complete loss of activity at approx.: 45 MPa.

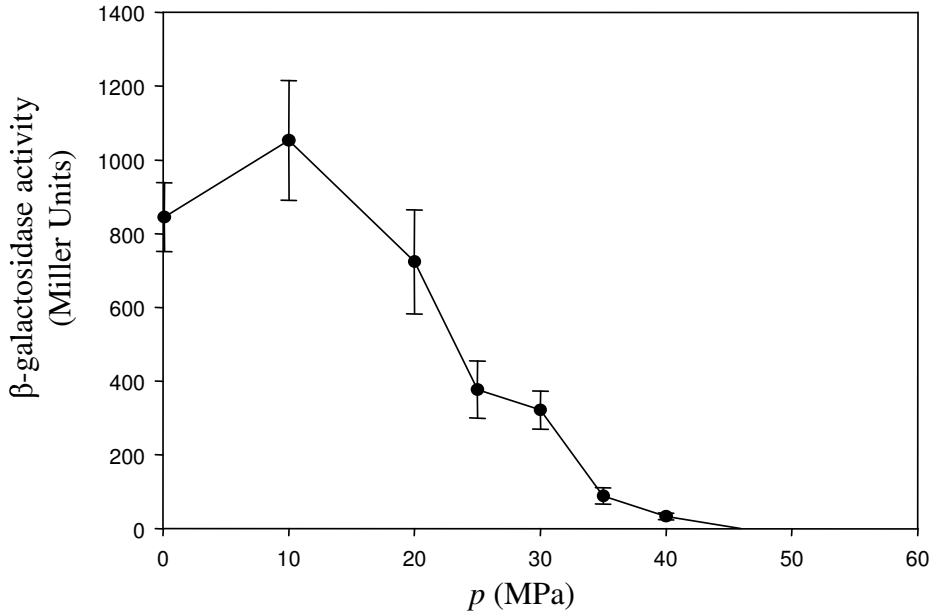

Figure 3.3: Maximal values of the β-galactosidase activity in Miller units from the strain *E. coli*-POLA+pBADK-ToxR-his (—●—) after incubation for 22 h at 37°C and pressures from 0.1 MPa – 50 MPa. The error bars represent the standard deviation (n=10).

3.1.3.2 Dimerization of ToxR at 30°C

The β-galactosidase activities were determined after incubation for 22 h at 30°C and pressures of 0.1, 10, 20, 25, 30, 35, 40 and 50 MPa. The results are shown in figure 3.4 where the β-galactosidase activity is expressed in Miller units. The dimerization at 30°C showed almost no significant changes compared to the activity at 37°C, seen in Figure 3.3. Also here no significant changes in β-galactosidase activity were to observe up to 20 MPa. And the same decrease in β-galactosidase activity was observed at 25 MPa. However the β-galactosidase activity decreased from 25 MPa to 30 MPa and was constant until 35 MPa. Past 35 MPa the β-galactosidase activity decreased to an almost complete loss of activity at 50 MPa.

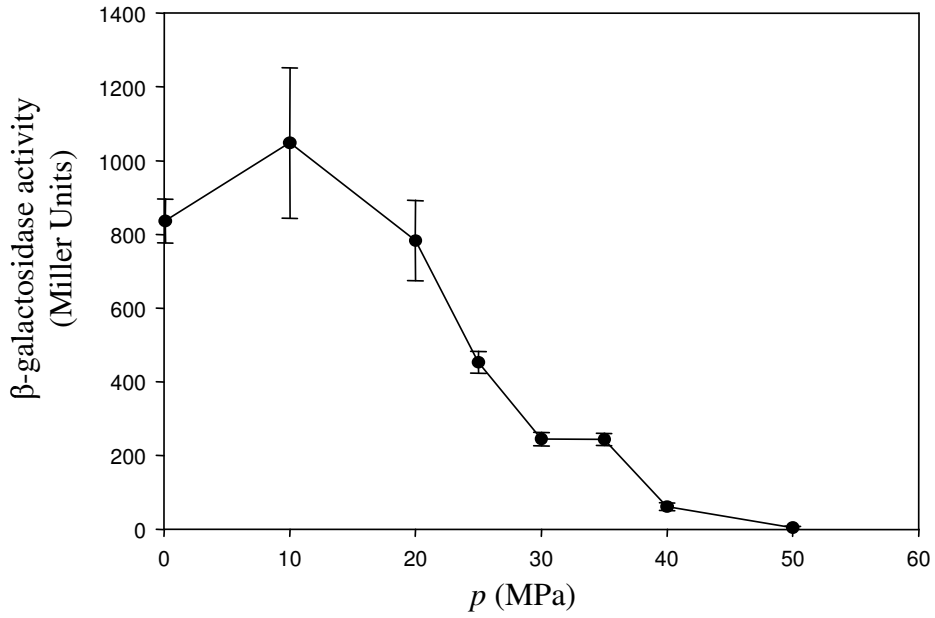

Figure 3.4: Maximal values of the β-galactosidase activity in Miller units from the strain *E. coli*-POLA+pBADK-ToxR-his (—●—) after incubation for 22 h at 30°C and pressures from 0.1 MPa – 50 MPa. The error bars represent the standard deviation (n=10).

3.1.3.3 Dimerization of ToxR at 37°C with addition of phenethyl alcohol

The β-galactosidase activities were determined after incubation for 22 h at 37°C and pressures of 0.1, 20, 25, 30, 35 and 40 MPa. Except the addition of 3mM phenethyl alcohol, the cells were grown exactly like described in chapter 2.2.8. The results are shown in figure 3.5 where the β-galactosidase activity is expressed in Miller units. The β-galactosidase activity was generally weaker than in the previous experiments without addition of phenethyl alcohol. The β-galactosidase activity at 0.1 MPa was equal to the β-galactosidase activity at 25 MPa in the previous experiments at 37°C (figure 3.3) and 30°C (figure 3.4), without addition of phenethyl alcohol. By noticing the standard deviation no significant change in β-galactosidase activity was to observe until 30 MPa. However, disregarding the standard deviation, a decrease in β-galactosidase activity about approx. 30 % compared to 20 MPa was observed at 25 MPa. Past 25 MPa the β-galactosidase activity decreased to an almost complete loss of activity at 30 MPa.

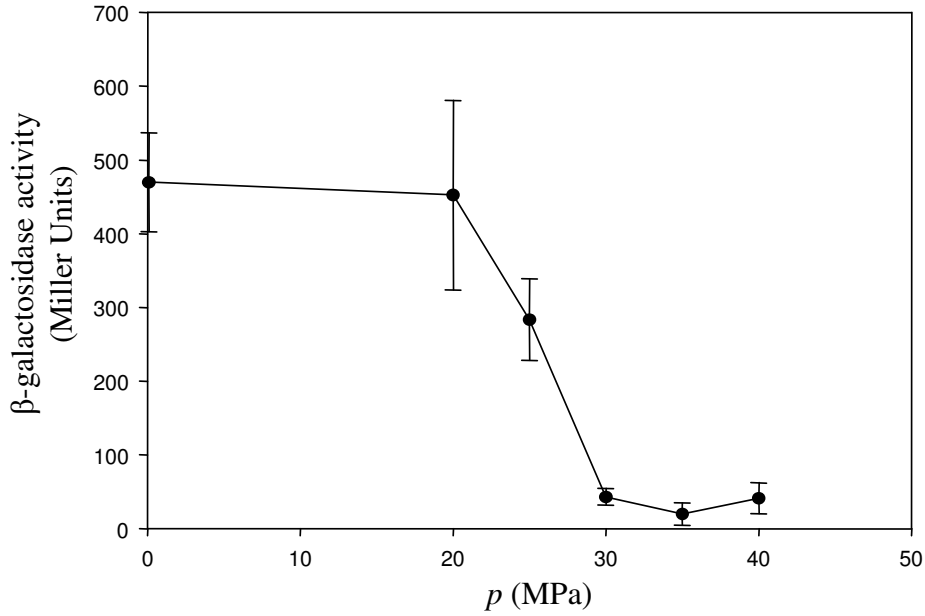

Figure 3.5: Maximal values of the β-galactosidase activity in Miller units from the strain *E. coli*-POLA+pBADK-ToxR-his (—●—) after incubation for 22 h at 37°C and pressures of 0.1 MPa – 50 MPa and addition of 3mM phenethyl alcohol. The error bars represent the standard deviation (n=5).

3.1.3.4 Dimerization of ToxR at 37°C in a Δ*fabf* mutant

The β-galactosidase activities were determined after incubation for 22 h at 37°C and pressures of 0.1, 20, 25, 30, 35, 40 and 50 MPa. The results are shown in figure 3.6 where the β-galactosidase activity is expressed in Miller units. The β-galactosidase activities were similar to the activities in the previous experiments at 37°C (figure 3.3) and 30°C (figure 3.4). No changes in β-galactosidase activity were to observe up to 20 MPa. At 25 MPa the β-galactosidase activity decreases to approx. 40 % compared to β-galactosidase activity at 20 MPa. Past 25 MPa almost no change in β-galactosidase activity happened until 40 MPa. Past 40 MPa the β-galactosidase activity decreases to approx. 10 % compared to 0.1 MPa.

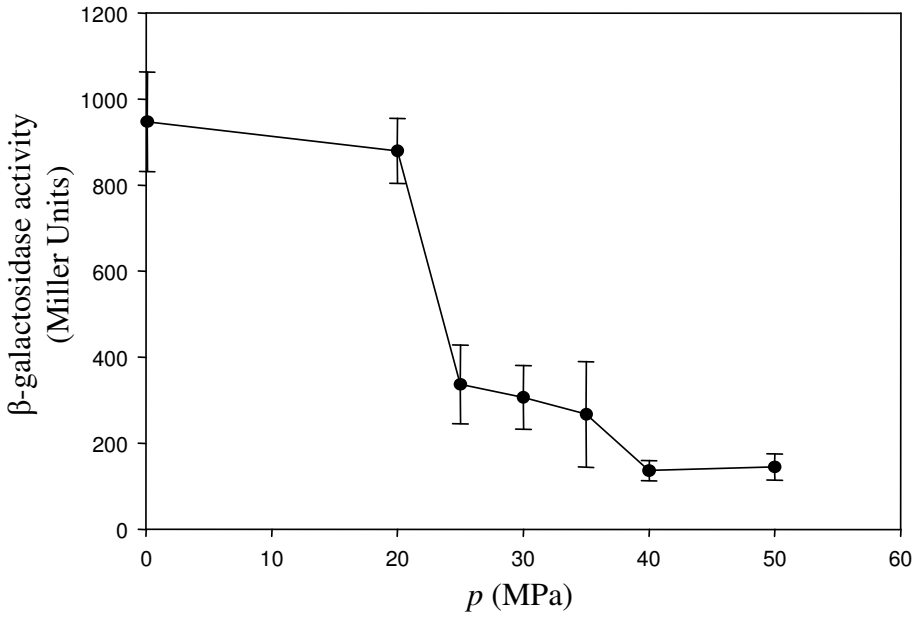

Figure 3.6: Maximal values of the β-galactosidase activity in Miller units from the strain *E. coli*-JWPOLA_Δ*fabF*+pBADK-ToxR-his (—●—) after incubation for 22 h at 30°C and pressures from 0.1 MPa – 50 MPa. The error bars represent the standard deviation (n=5).

3.1.3.5 Determination of the pressure-dependent phase state of the inner membrane from *E. coli* POLA

To determine the pressure-dependent phase state of the inner membrane *E. coli* POLA protoplast were produced. They were then analyzed by Laurdan fluorescence spectroscopy to study the polarity of the lipid interface and to detect potential phase changes of the lipid membrane. The effect of pressure on the generalized polarization data of the Laurdan labeled *E.coli* POLA protoplast is shown in figure 3.7, which exhibits *GP* data at 37°C as a function of pressure from 0.1 MPa up to 50 MPa. The *GP* values increase steadily with increasing pressure, starting from a relatively high value at ambient pressure (*GP* = 0.34), which is characteristic of a membrane of rather rigid conformational order of the lipid chains, already. Typically *GP* values range from about -0.2 to 0.2 for pure fluid-like, disordered phases of lipid bilayers to values of *GP* ≈ 0.55 - 0.60 in all solid-ordered, gel-like lipid phases (Nicolini *et al.*, 2006; Periasamy & Winter, 2006). Upon pressurization up to 50 MPa, *GP* values of 0.43 are reached, which is close to the tight packing of membranes reached for all-ordered conformational states of lipid bilayers.

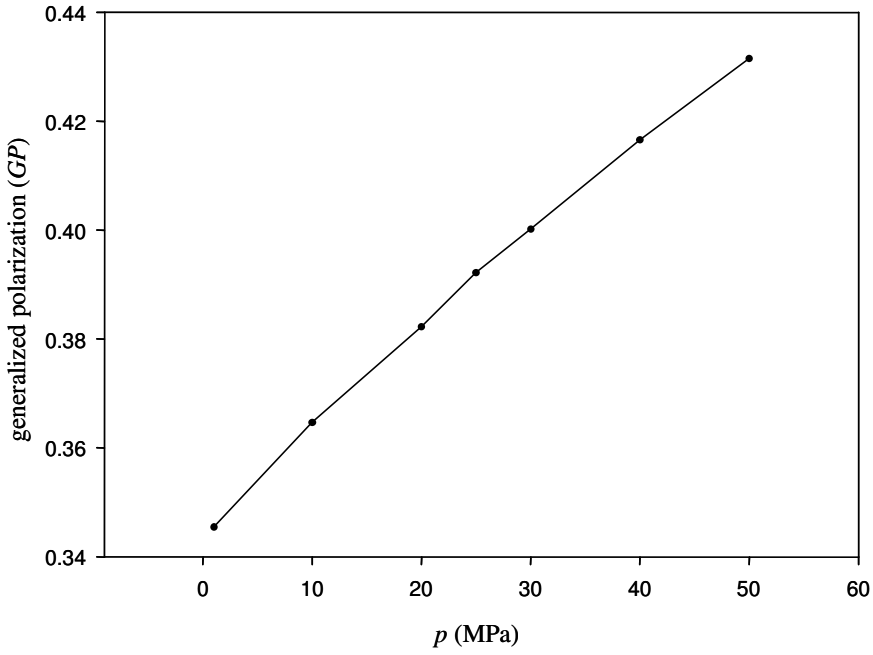

Figure 3.7: *GP* values of *E. coli* protoplasts stained with Laurdan at 37°C and pressure conditions ranging from 0.1 to 50 MPa.

3.1.4 Construction of an expression cassette in *P. profundum*

To see if an over expressed ToxR behaves similar in its native membrane environment compared to its behavior in *E. coli* an expression cassette in *P. profundum* was constructed. For the construction of the expression cassette the *P. profundum* mutant TW10 was used. The *P. profundum* mutant TW10 featured already a chromosomal integrated *pompL::lacZ* fusion (Welch & Bartlett, 1996). The ToxR protein was expressed from the vector pFL190-ToxR under control of an arabinose inducible promoter. The TW10 mutant featured also a naturally regulated ToxR/ToxS system. Therefore it was possible to compare the β-galactosidase activities caused by native expressed ToxR (TW10) with β-galactosidase activities caused by over expressed ToxR (TW10+pFL190-ToxR). The experiments were performed like described in chapter 2.2.8.

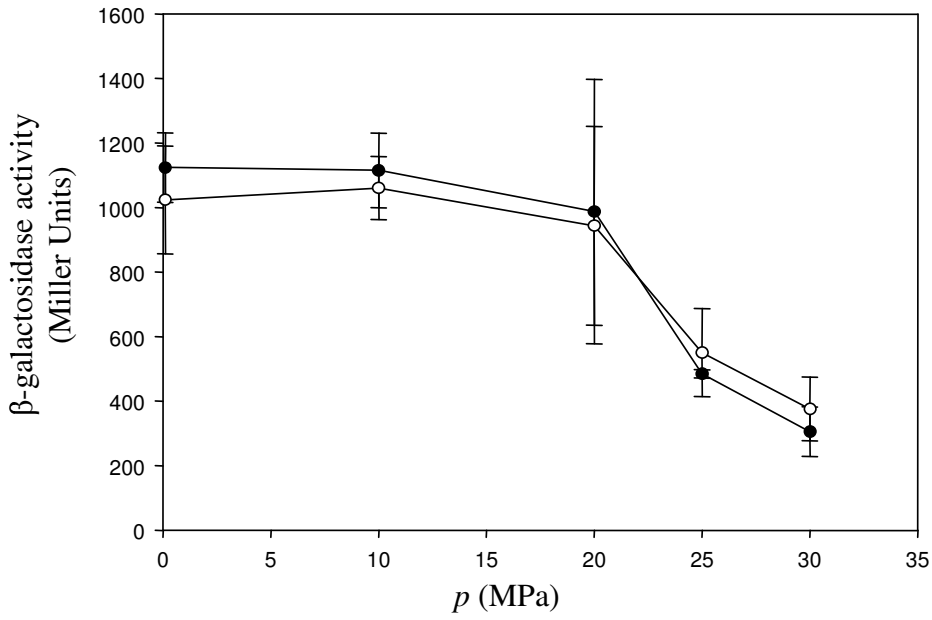

Figure 3.8: Maximal values of the β-galactosidase activity in Miller units from the strains *P. profundum* TW10 (—●—) and *P. profundum* TW10+pFL190-ToxR (—○—) after incubation for 72 h at 15°C and pressures of 0.1 MPa – 30 MPa. The error bars represent the standard deviation (n=10).

3.1.4.1 Dimerization of ToxR in *P. profundum* at 15°C

The β-galactosidase activities were determined after incubation for 72 h at 15°C and pressures of 0.1, 10, 20, 25 and 30 MPa. The β-galactosidase activities are shown in figure 3.8 where the β-galactosidase activity is expressed in Miller units. There were no significant differences to observe between the β-galactosidase activities from TW10 and TW10+pFL190-ToxR. For both no significant changes in β-galactosidase activity was observed up to 25 MPa where the activity was approx. 50% compared to 0.1 MPa. Past 25 MPa the activity decreased to approx. 25 % at 30 MPa. Despite the systems in *E. coli* and *P. profundum* being not 100% comparable as a result of different vector systems, dimerization behavior of ToxR in *P. profundum* was similar to the dimerization behavior in *E. coli* (figures 3.3 and 3.4).

3.2 ToxR with different transmembrane segments

3.2.1 The ToxR system

To investigate the pressure dependent behavior of different TMS a ToxR-transcription-activator-system was used. This system had already proven its usefulness in the investigation of transmembrane segment interactions (Langosch *et al.*, 1996). The original system (ToxRI) consists of a fusion protein which is encoded on a high copy plasmid where it is constitutive expressed. In a newer version the expression of the fusion protein is under control of the arabinose inducible *ara*BAD promoter (ToxRIV) (Gurezka & Langosch, 2001). The fusion protein is composed of a ToxR cytoplasmic-domain from *V. cholerae*, a variable TMD and a periplasmic MalE (maltose binding protein) domain. The dimerization ability of the fusion protein in this system depends only on TMD interaction. The fusion protein is used in combination with a specific *E. coli* reporter strain, where a *ctx*::*lacZ* fusion is integrated into the chromosome (FHK12 (Kolmar *et al.*, 1995)). When the fusion protein is expressed in the reporter strain a dimerized protein is able to activate the β-galactosidase transcription from the *lacZ* gene. Therefore the dimerization ability can be indirectly measured by β-galactosidase activity. The *lacZ* gene used in this construct is N-terminally truncated (starting at nucleotide pair 34 according to GenEMBL accession number V00296) and encodes a less active β-galactosidase than wild type *lacZ*. An overview of the used system is given in figure 3.9. Four different TMS and one construct without TMS have been used. The fusion proteins were expressed from the ToxRIV plasmid in combination with the reporter strains FHK12 and POLA_Δ*fabF*. The four TMS were: AZ2 a simplified version of a membrane-spanning leucine zipper interaction domain (AAS: LLAALLALLAALLALL), EG4 a mutation of the simplified version of a membrane-spanning leucine zipper (AAS:

LLAALAAALAALAAAL), GpA13 a wild type glycophorin A transmembrane segment (AAS: LIIFGVMAGVIGT) and GpAG83A a mutant of the wild type glycophorin A transmembrane segment (AAS: LIIFGVMAAVIGT). The construct without TMS consisted only of the ToxR cytoplasmic domain and MalE (ΔTM). All TMS have previously been characterized with respect to their ability to dimerize in the *E. coli* reporter strain FHK12 (Gurezka *et al.*, 1999; Langosch *et al.*, 1996). This system also provided the possibility to check the membrane integration of the constructs, this was achievable in combination of the MalE periplasmic domain and ΔMalE *E. coli* strain PD28 (Duplay *et al.*, 1987).

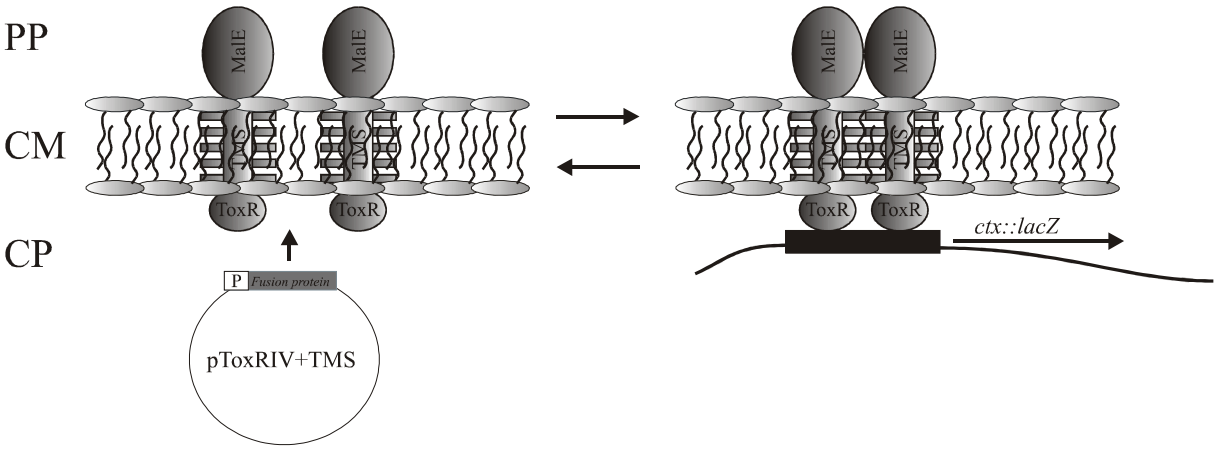

Figure 3.9: ToxR chimeric proteins obtained from *Vibrio cholerae*. ToxR is encoded on the plasmid ToxRIV+TMS, where its transcription is controlled by the promoter (P). The cytoplasmic domain of ToxR (ToxR) is linked to the periplasmic maltose binding protein (MalE) domain via a trans membrane segment (TMS) of choice. Dimerized ToxR binds to the ctx promoter thus initiating *lacZ* transcription in the indicator cells. PP, periplasm; CP, cytoplasm; CM, cytoplasmic membrane.

3.2.2 Control of integration into the membrane

The integration test was performed as described in chapter 2.2.7. All transformed strains except *E. coli* PD 28 + pToxRIV-ΔTM were able to grow under the test conditions (figure 3.10). Therefore all constructs, except of ΔTM, were able to complement the MalE deletion from *E. coli* strain PD28. This had proved that, except of fusion protein ΔTM, all fusion proteins were integrated into the inner membrane. These results also confirmed the previously published data (Gurezka *et al.*, 1999; Langosch *et al.*, 1996). Since the growing conditions under pressure were already limiting, it was only possible to perform this test by 0.1 MPa.

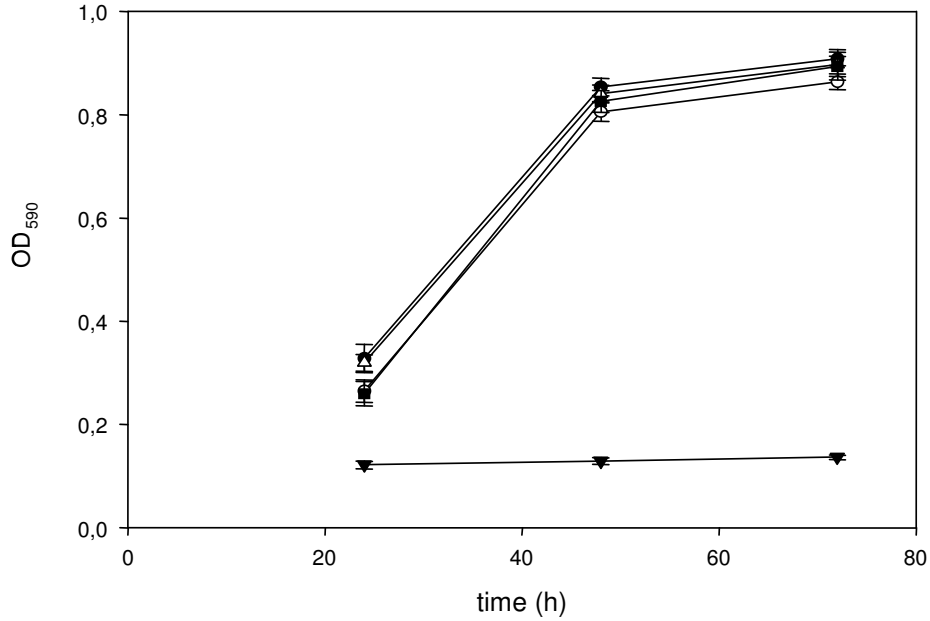

Figure 3.10: control of membrane integration. The plasmids pToxRIV+AZ2 (—●—), pToxRIV+EG4 (—○—), pToxRIV+GpA13 (—△—), pToxRIV+GpAG83A (—■—) and pToxRIV+ΔTM (—▼—) have been transformed into *E. coli* PD 28 cells and the cells were then grown in minimal medium with maltose as the only carbon source the OD_{590} was measured every 24 h. The error bars represent the standard deviation (n=3).

3.2.3 Dimerization experiments with different TMS

The experiments were performed, like the experiments with ToxR from *P. profundum,* as described in chapter 2.2.8. The experiments at different temperatures (37°C and 30°C) and use of phenethyl alcohol were performed in combination with reporter strain *E. coli* FHK12 and additionally experiments have been made at 37°C in reporter strain *E. coli* POLA_Δ*fabF*. The results from reporter strain *E. coli* FHK12 and *E. coli* POLA_Δ*fabF* are not 100% comparable, because of the different promoter regions and *lacZ* genes used for the chromosomally integrated fusion. *E. coli* FHK12 possessed a *ctx::lacZ* fusion where the *lacZ* gene was a truncated version of the wild type gene, and *E. coli* POLA_Δ*fabF* possessed a *pompL::lacZ* fusion where the lacZ gene was the wild type version. Therefore a stronger β-galactosidase activity in *E. coli* POLA_Δ*fabF* does not necessarily mean that the dimerization there is stronger than in *E. coli* FHK12.

3.2.3.1 Dimerization of ToxR with different TMS at 37°C

The β-galactosidase activities were determined after incubation for 22 h at 37°C and pressures of 0.1, 10, 20, 25, 30, 35, 40 and 50 MPa. The β-galactosidase activities from the strains, expressed in Miller units, are shown in figure 3.11. The β-galactosidase activity of all constructs was not significantly affected by pressures up to 20 MPa. Generally up to 20 MPa the β-galactosidase activities of the constructs carrying the wild type leucine zipper (AZ2) and glycophorin A segment were approx. twice as much as the activities of the constructs carrying the mutated leucine zipper (EG4) and glycophorin A segment (G83A). The constructs carrying the mutated leucine zipper (EG4) and glycophorin A segment (G83A) showed no significant differences in their response to pressure. Both showed a decrease in β-galactosidase activity with increasing pressure above 20 MPa, leading to an almost complete loss of activity at 50 MPa (approx. 20% of the activity at 0.1 MPa). However the constructs carrying the wild type leucine zipper (AZ2) and glycophorin A segment (GPA) showed only no significant differences in their response to pressure up to 20 MPa. The β-galactosidase activity of the construct with the leucine zipper TMS AZ2 drastically decreased at higher pressures above 20 MPa, resulting in only approx. 25% β-galactosidase activity at 50 MPa compared to the activity at 0.1 MPa. Most interestingly, the β-galactosidase activity of the construct GPA was affected to a much lesser extent between 20 MPa and 40 MPa than the activity of the other constructs. Its β-galactosidase activity at 40 MPa was still around 66% of the activity at ambient pressure. However, at 50 MPa, GPA shows a similar reduced activity as AZ2. For the construct without a TMS (ΔTM), only a weak β-galactosidase activity was measured with almost no change in activity up to 50 MPa.

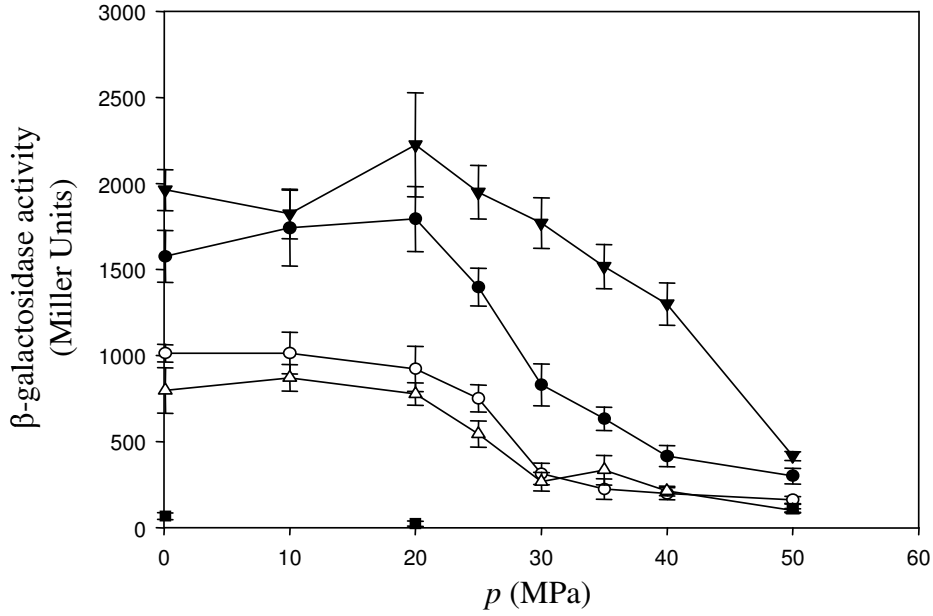

Figure 3.11: Maximal values of the β-galactosidase activity in Miller units from the strains *E. coli*-FHK12+pToxRIV+AZ2 (—●—), *E. coli*-FHK12+pToxRIV+EG4 (—○—), *E. coli*-FHK12+pToxRIV+GpA13 (—▼—), *E. coli*-FHK12+pToxRIV+GpAG83A (—△—) and *E. coli*-FHK12+pToxRIV+ΔTM (—■—) after incubation for 22 h at 37°C and pressures from 0.1 MPa – 50 MPa. The error bars represent the standard deviation (n=10).

3.2.3.2 Dimerization of ToxR with different TMS at 30°C

The experiments at 30°C were performed at pressures of 0.1, 10, 20, 25, 30, 35, 40 and 50 MPa. The β-galactosidase activities from the strains, expressed in Miller units, are shown in figure 3.12. There have been no significant differences in the β-galactosidase activities by 30 °C compared to 37°C (figure 3.12). The β-galactosidase activity of all constructs was not significantly affected by pressures up to 20 MPa. Also the β-galactosidase activities of the constructs carrying the wild type leucine zipper (AZ2) and glycophorin A segment were approx. twice as much as the activities of the constructs carrying the mutated leucine zipper (EG4) and glycophorin A segment (G83A). And the β-galactosidase activity of the construct GPA was also affected to a much lesser extent between 20 MPa and 40 MPa than the activity of the other constructs.

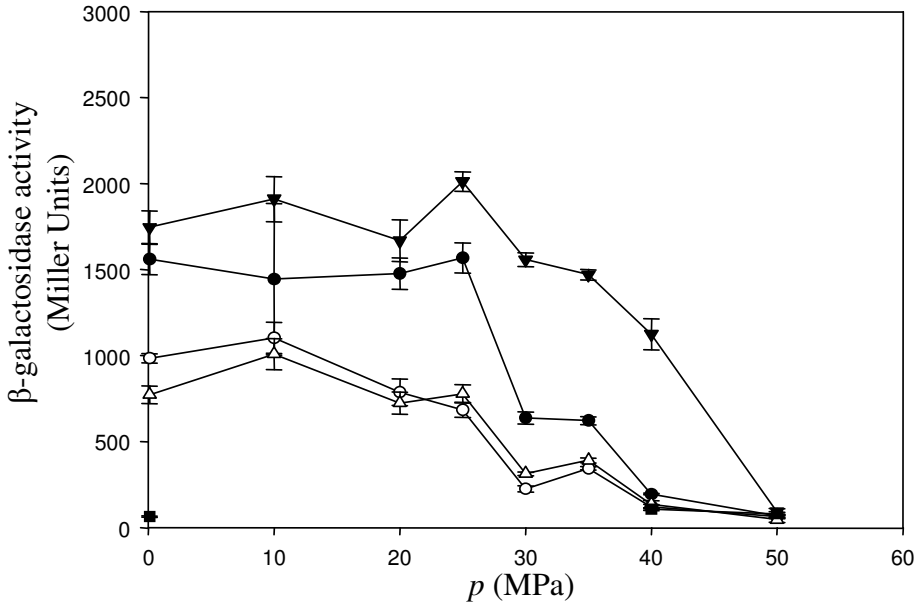

Figure 3.12: Maximal values of the β-galactosidase activity in Miller units from the strains *E. coli*-FHK12+pToxRIV+AZ2 (—●—), *E. coli*-FHK12+pToxRIV+EG4 (—○—), *E. coli*-FHK12+pToxRIV+GpA13 (—▼—), *E. coli*-FHK12+pToxRIV+GpAG83A (—△—) and *E. coli*-FHK12+pToxRIV+ΔTM (—■—) after incubation for 22 h at 30°C and pressures from 0.1 MPa – 50 MPa. The error bars represent the standard deviation (n=10).

3.2.3.3 Dimerization of ToxR with different TMS at 37°C with addition of phenethyl alcohol

The experiments at 37°C were performed by pressures of 0.1, 20, 25, 30, 35 and 40 MPa. Except the addition of 3mM phenethyl alcohol, the cells were grown exactly like described in chapter 2.2.8. The β-galactosidase activities from the strains, expressed in Miller units, are shown in figure 3.13. The β-galactosidase activity was generally weaker than in the previous experiments without addition of phenethyl alcohol. This effect was also observed in the experiments with *E. coli* strain POLA + pBADK-ToxR-his. Beside of the weaker activity, the same differences in β-galactosidase activity, from the different constructs, were observed. Particularly the β-galactosidase activity of the construct GPA was lesser affected by HHP up to 40 MPa than the β-galactosidase activity of the other constructs.

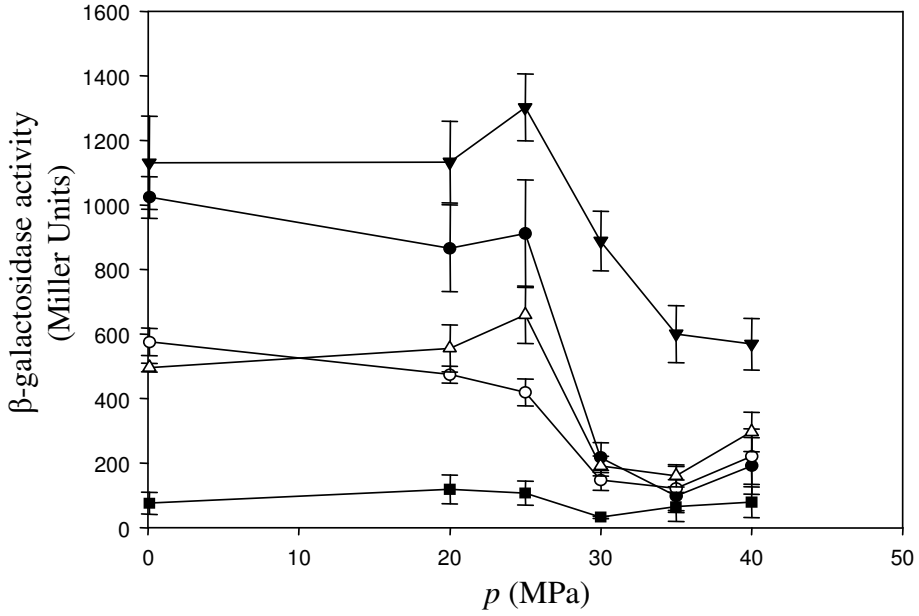

Figure 3.13: Maximal values of the β-galactosidase activity in Miller units from the strains *E. coli*-FHK12+pToxRIV+AZ2 (—●—), *E. coli*-FHK12+pToxRIV+EG4 (—○—), *E. coli*-FHK12+pToxRIV+GpA13 (—▼—), *E. coli*-FHK12+pToxRIV+GpAG83A (—△—) and *E. coli*-FHK12+pToxRIV+ΔTM (—■—) after incubation for 22 h at 37°C and pressures of 0.1 MPa – 50 MPa and addition of 3mM phenethyl alcohol. The error bars represent the standard deviation (n=5).

3.2.3.4 Dimerization at 37°C in a Δ*fabf* mutant

The experiments at 37°C were performed by pressures of 0.1, 20, 25, 30, 35, 40 and 50 MPa. The β-galactosidase activities from the strains, expressed in Miller units, are shown in figure 3.14. Generally a much stronger β-galactosidase activity was observed in this experiment compared to the previous performed experiments. This was probably caused by the different used promoter and reporter gene. However beside of this the behavior of the constructs to each other was the same like observed in the previous experiments. The β-galactosidase activity of the construct GPA was lesser affected by HHP up to 40 MPa than the β-galactosidase activity of the other constructs.

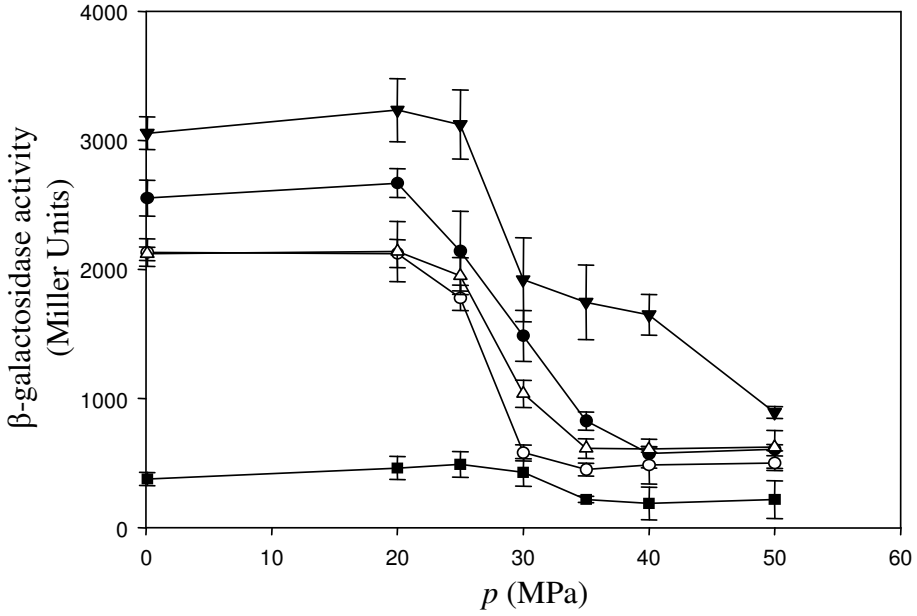

Figure 3.14: Maximal values of the β-galactosidase activity in Miller units from the strains *E. coli*-JWPOLA_Δ*fabF*+pToxRIV+AZ2 (—●—), *E. coli*-JWPOLA_Δ*fabF*+pToxRIV+EG4 (—○—), *E. coli*-JWPOLA_Δ*fabF*+pToxRIV+GpA13 (—▼—), *E. coli*-JWPOLA_Δ*fabF*+pToxRIV+GpAG83A (—△—) and *E. coli*-JWPOLA_Δ*fabF*+pToxRIV+ΔTM (—■—) after high pressure treatment in the range of 0.1 MPa – 50 MPa at 37°C. The error bars represent the standard deviation (n=5).

3.3 Fatty acid composition

3.3.1 The fatty acid composition

The fatty acid composition was analyzed from *E. coli* strains POLA, FHK12 and JW1081-4, grown at 30°C and 37°C, on LB agar plates. The fatty acid composition for every tested strain at 30°C and 37°C is shown in Figure 3.15. Generally the fatty acid composition at 30°C showed for every tested strain overall a slightly higher amount of unsaturated fatty acids compared to 37°C. The increase of unsaturated fatty acids was the biggest for *E. coli* JW1081-4, especially by 16:1 unsaturated fatty acids and not so much by 18:1 unsaturated fatty acids. The biggest increase in the amount of 18:1 unsaturated fatty acids was observed for *E. coli* ToP10. Strain *E. coli* FHK12 showed only small increases in the amount of both 16:1 and 18:1 unsaturated fatty acids. Also its overall amount of 16:1 fatty acids was generally small compared to the other strains. This was probably compensated by a higher amount of 19:0 CYCLO w8c unsaturated fatty acids. Especially the amount of 16:1 unsaturated fatty acids was very high in strain *E. coli* JW1081-4 and even higher as the

amount of 16:0 saturated fatty acids at 30°C. Except of this 16:0 saturated fatty acids were under every condition and in every strain the biggest fatty acid group. Surprisingly the amount of 18:1 unsaturated fatty acids was similar for every strain, even for JW1081-4, which was proposed to produce smaller amounts of these unsaturated fatty acids.

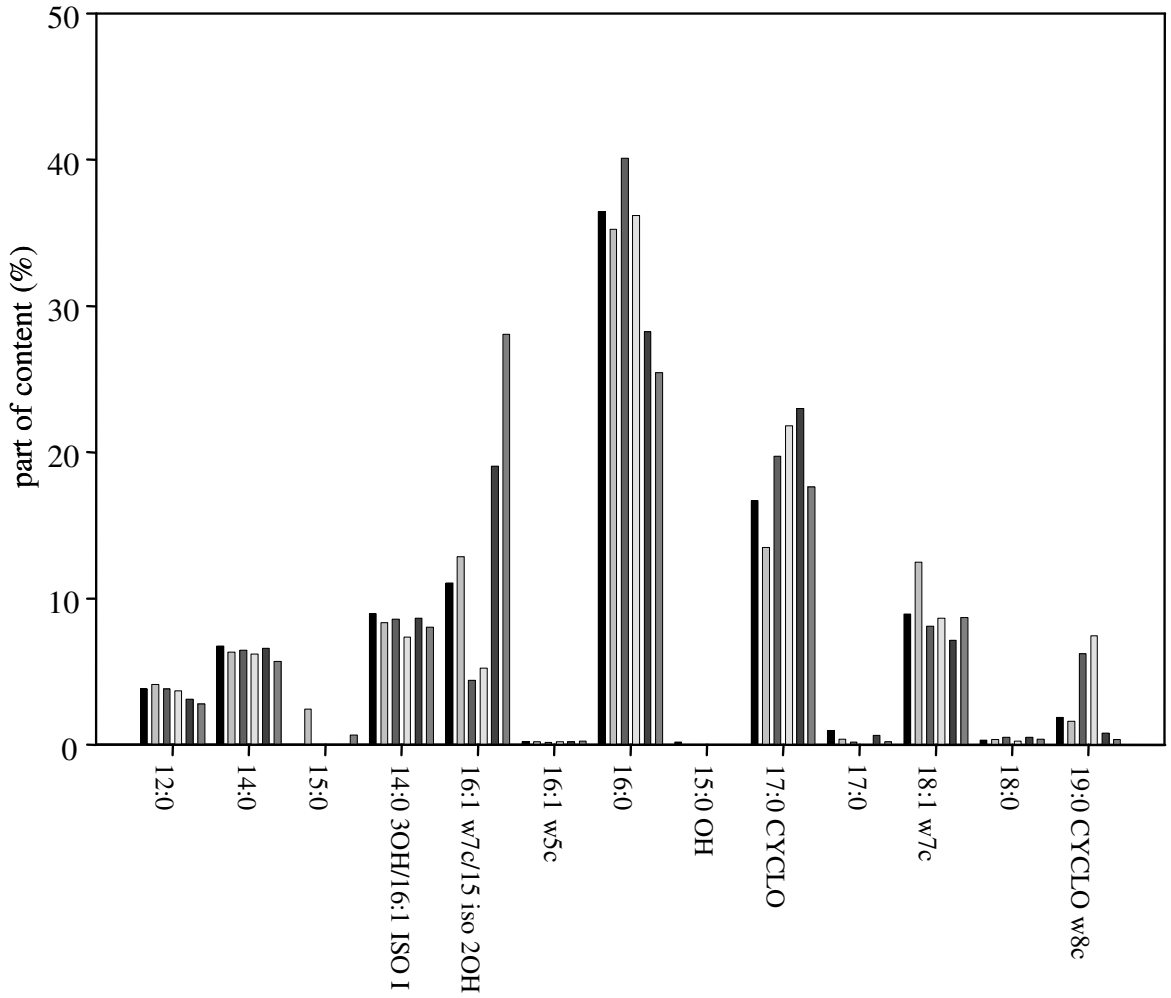

Figure 3.15: Fatty acid composition of *E. coli* ToP10 at 37°C (■) and 30°C (□), *E. coli* FHK12 at 37°C (■) and 30°C (□) and, *E. coli* JW1081-4 at 37°C (■) and 30°C (■). The numbers indicate acyl length : # of unsaturated bonds.

4 Discussion

In bacteria, reversible dimerization and oligomerization of transmembrane proteins is often essential in signal transduction (e.g., ToxR/S in *V. cholerae* and *P. profundum*), or direct regulation of enzyme activity (e.g., outer membrane phospholipase A in *E. coli*) (Bidle & Bartlett, 2001; Dekker *et al.*, 1997; Miller & Mekalanos, 1984). In this work, different ToxR systems were successfully used to examine the effects of mild HHP up to 50 MPa on the dimerization abilities of wild type ToxR (*P. profundum*) and of various TMS, in different membrane surroundings. Using these approaches, pressure induced membrane protein interaction was demonstrated for the first time *in vivo*. It was shown that pressures between 20 MPa and 50 MPa a sufficient to promote the dissociation of membrane proteins, to different extents depending on the protein nature. These pressures are too low to induce major membrane change like membrane phase change or major thickness change. Therefore the results of this work suggest that the dimerization ability of a membrane protein under high pressure is rather controlled by its transmembrane structure than by changes in the lipid bilayer environment. This indicates that in the case of ToxR, the initial step of signal transduction is the pressure effect on the protein and not an effect on the surrounding membrane.

4.1 The ToxR systems

It was possible to show for the ToxR systems used in *E. coli* that the wild type ToxR proteins and ToxR fusion proteins were integrated into the membrane at 0.1 MPa. The integration of the wild type ToxR into the inner membrane was proven by isolating it from the membrane phase after cell disruption. A test to prove the inner membrane integration of the fusion proteins was part of the used ToxR system (Langosch *et al.*, 1996). If integrated in the right orientation into the inner membrane the cytoplasmic MalE domain of the fusion protein was able to complement the lack of the *malE* gene in *E. coli* strain PD28 (Duplay *et al.*, 1987; Kolmar *et al.*, 1995). Moreover the membrane integration of the used TMS was already shown with the same test, thereby was also shown that the TMS constructs were similarly strong expressed (Gurezka *et al.*, 1999; Langosch *et al.*, 1996). Because of the already limiting growing conditions under pressure the tests in this work were only performed at 0.1 MPa. However it seems unlikely that the membrane integration of the proteins was affected by pressure in the used pressure range. The results from the experiments with *E. coli* ToP10+pBAD/*Myc*-His/*lacZ* have shown that the araBAD promoter was not affected by

pressures up to 50 MPa, like shown before for the *lacZ* promoter (Kato *et al.*, 1994). This made a pressure influence onto the expression of the ToxR proteins, which were under control of the araBAD promoter, relatively unlikely. The different behavior of the various TMS also indicates that the pressure induced inhibition of other cellular functions described as pressure sensitive, including ribosomal synthesis or transcription elongation, does not interfere with the *in vivo* reporter system in this pressure range. This is also supported by the fact that most of these effects appear at pressures above 50 MPa only (Gross *et al.*, 1993; Pagan & Mackey, 2000; Yayanos & Pollard, 1969). Moreover, it appears that the *ctx* promoter or the ability of a dimeric ToxR to bind to the promoter was not affected by pressures up to 50 MPa. This indicates that the measured expression changes are referred to changes in the dimerization abilities of the various constructs and wild type ToxR.

4.2 Fatty acid composition

The analysis of the fatty acid composition had shown that every one of the test strains was able to modulate its fatty acid composition in response to temperature changes. Also the modulation done by every strain was similar, in the way that every strain increased slightly the amount of unsaturated fatty acids at 30°C compared to 37°C. This increase of unsaturated fatty acids mainly 16:1 and 18:1 unsaturated fatty acids was accompanied by a decrease in the amount of saturated fatty acids, generally 16:0 fatty acids. This is a general response to temperature downshifts from *E. coli* strains (Magnuson *et al.*, 1993). Different mechanisms for *E. coli* to change the amount of unsaturated fatty acids in its membrane, in response to temperature, were previously found. It was shown that when supplemented with saturated and unsaturated fatty acids at lower growth temperature, *E. coli* preferred the incorporation of unsaturated fatty acids into its membrane (Cronan, 1975) . In another experiment it was shown that the increase in the amount of 18:1 unsaturated fatty acids and the decrease in the amount of 16:0 saturated fatty acids, incorporated into the membrane, was an intrinsic property of the fatty acid biosynthetic enzyme β-ketoacyl-ACP synthase II (encoded by *fabF*) (Garwin *et al.*, 1980b). *E. coli* β-ketoacyl-ACP synthase II is one of three isoenzymes that catalyze the elongation of fatty acid acyl chains. The enzyme β-ketoacyl-ACP synthase II functions specifically in the elongation of palmitoleoyl-ACP (16:1) to form *cis*-vaccenoyl-ACP (18:1) (D'Agnolo *et al.*, 1975). Neither mRNA nor protein synthesis is required for increased *cis*-vaccenic acid (18:1 *cis*-11) production at reduced temperature, indicating that, in this case, the thermal modulation of fatty acid production is controlled at the level of β-ketoacyl-ACP synthase II activity (Garwin & Cronan, 1980). This is supported by the fact that

the elongation activity of β-ketoacyl-ACP synthase II is temperature dependent, exhibiting decreased K_m for palmitoleoyl-ACP and increased relative V_{max} at reduced temperature (Garwin *et al.*, 1980a). It was shown that *E. coli fabF* mutants possess a deficiency in *cis*-vaccenic acid (18:1 *cis*-11) synthesis as well as a loss of *cis*-vaccenic acid (18:1 *cis*-11) thermal regulation (Garwin *et al.*, 1980b). However also β-ketoacyl-ACP synthase I is able to produce *cis*-vaccenic acid (18:1 *cis*-11), but has a higher K_m and lower V_{max} with palmitoleoyl-ACP than β-ketoacyl-ACP synthase II (D'Agnolo *et al.*, 1975). It was shown that over expression of β-ketoacyl-ACP synthase I leads to an over production of *cis*-vaccenic acid (18:1 *cis*-11) and is also able to restore the *cis*-vaccenic acid (18:1 *cis*-11) production in Δ*fabF* strains (de Mendoza *et al.*, 1983). The similar amount of 18:1 unsaturated fatty acids in the fatty acid composition of Δ*fabF* strain *E. coli* JW1081-4, compared to strains *E. coli* ToP10 and FHK12, might be explained by *cis*-vaccenic acid production through β-ketoacyl-ACP synthase I or incorporation of exogenous 18:1 unsaturated fatty acids. Generally, the temperature difference between 37°C and 30°C was not so high that a major increase in unsaturated fatty acids had to occur. Because of the already limiting growing conditions under pressure the experiments were not performed at lower temperatures or in minimal medium. This could probably have shown a different fatty acid composition for strain *E. coli* JW1081-4. However it seems likely that the slight increase in fatty acid composition was enough to provide similar membrane fluidity at 30°C than at 37°C, for every tested strain.

4.3 Effect of phase transition on protein dimerization

It has been shown before that changes in membrane fluidity, like fluid-to-gel phase transitions, are able to inactivate membrane proteins at pressures above 200 MPa (Chong *et al.*, 1985; Ulmer *et al.*, 2000; Ulmer *et al.*, 2002). Also the Laurdan measurements of the *E. coli* POLA protoplast have shown a continuously progressing ordering of the acyl-chains of the lipid membrane as indicated by a *GP* value, which steadily increases with increasing pressure. However, up to pressures of 50 MPa no solid-like (s_o) overall ordered phase state was reached. The continuous increase of the *GP(p)* values measured excluded the possibility that a phase transition was the reason for the dissociation of the dimmers in this particular pressure range.

4.4 Effect of membrane fluidity on protein dimerization

Beside an effect of phase transition can be excluded as the reason for the dissociation, the data could indicate that elevated pressure induced decrease in membrane fluidity decreases the

dimerization ability of membrane proteins however to different extends. A decrease in the reaction rate of membrane-bound Na^+/K^+-ATPase was previously proposed to be caused by an elevated pressure induced decrease in membrane fluidity (Kato *et al.*, 2002). Four different approaches were made to change the membrane composition and therefore the membrane fluidity for the HHP experiments:

1. The cells were grown by different temperatures which is known to affect the composition of the membrane (Magnuson *et al.*, 1993).
2. Phenethyl alcohol was added to the growth medium, which affects the ordering of the phospholipid chains (Jordi *et al.*, 1990).
3. A Δ*fabF* mutant was used, which should have been deficient in *cis*-vaccenic acid (18:1 *cis*-11) synthesis and not able to regulate the amount of *cis*-vaccenic acid (18:1 *cis*-11) in response to temperature (Garwin *et al.*, 1980b).
4. Tests were performed in the deep sea bacterium *P. profundum* (only ToxR from *P. profundum*).

The results for the experiments at 37°C and 30°C have shown no significant differences in the β-galactosidase activities at these two temperatures. This might be explained by the fact that all strains have been able to adjust their membrane composition, so that they were able to provide the same membrane fluidity at each temperature. The addition of phenethyl alcohol to the growth medium brought an overall weaker β-galactosidase activity. Beside of that weaker activity, the differences in β-galactosidase activity between the ToxR constructs were similar to differences in the other performed experiments. Since it is know that phenethyl alcohol is able to inhibit the DNA synthesis in *E. coli* (Berrah & Konetzka, 1962), it might be possible that this ability influenced our reporter system. This influence could have been the reason for the weaker β-galactosidase activities and furthermore, could have overshadowed possible positive effects on dimerization, like proposed for *P. profundum* (Welch & Bartlett, 1998). The results for the experiments performed in the *E. coli fabF* mutant JWPOLA_Δ*fabF* showed stronger β-galactosidase activities, for the ToxR fusion proteins, compared to the experiments performed in *E. coli* strain FHK12. A stronger β-galactosidase activity was not observed for the ToxR wild type protein, compared to the experiments performed in *E. coli* strain POLA. Moreover no significant changes in the β-galactosidase activity of the wild type ToxR protein were observed compared to the experiments performed at 37°C and 30°C in *E. coli* strain POLA. The stronger activity for the ToxR fusion proteins is explained by the fact that the *E. coli* strains POLA and JWPOLA_Δ*fabF* had the same promoter (*pompL*) and reporter gene (wild type *lacZ*) in contrast to *E. coli* strain FHK12 which had a different

promoter (*ctx)* and a different reporter gene (truncated *lacZ*), than the other strains. However beside of the stronger β-galactosidase activity the differences in β-galactosidase activity between the ToxR constructs were similar to differences in the other performed experiments. The fatty acid analysis for strain *E. coli* JW1081-4 (the parental strain of POLA_Δ*fabF*) had shown that it had a similar membrane composition under the experimental conditions. Therefore the expected lowering of the amount of unsaturated fatty acids (especially 18:1) in the membrane was not achieved with this strain. Since *E. coli* is able to incorporate exogenous supplied unsaturated fatty acids it might be possible that the growth medium was the source of the found 18:1 unsaturated fatty acids. Beside of the relatively low amount of 18:1 unsaturated fatty acids, it might also be possible that they were produced by β-ketoacyl-ACP synthase I (de Mendoza *et al.*, 1983). The similarity in the membrane compositions of the Δ*fabF* mutant and the other used *E. coli* strains explains the similar behavior of the ToxR proteins. The experiments in *P. profundum* were only performed with ToxR from *P. profundum*. It was shown that the β-galactosidase activities were similar to the β-galactosidase activities measured in *E. coli*. Since *P. profundum* is able to modulate its membrane composition in response to pressure and *E. coli* is not (Allen & Bartlett, 2000), the similar β-galactosidase activities in both bacteria might be a hint that membrane composition, to a specific point, is not important for the dimerization abilities of proteins under physiological conditions. Beside it is to say that the used systems in *P. profundum* and *E. coli* were not 100% comparable (e.g., the *P. profundum* strain TW10 had a functional ToxS protein). It was also shown that an over expression of ToxR in *P. profundum* strain TW10, which possessed a functional ToxR/S system, brought no stronger β-galactosidase activities. Generally it was not possible to show an influence of membrane composition and fluidity on the dimerization ability of the ToxR proteins under the used experimental conditions. This might also be caused by the required long incubation times and possible cellular adaptation. Thus, an influence of membrane composition and fluidity of the dimerization of the investigated proteins can not be completely excluded.

4.5 Effect of membrane thickness on protein dimerization

Beside a change in membrane fluidity it has also been shown that HHP leads to an increase of fluid bilayer thickness (Kato *et al.*, 2002; Winter, 2002; Winter & Dzwolak, 2005). A significant increase in membrane thickness could lead to a hydrophobic mismatch between the transmembrane segment of the protein and the hydrophobic thickness of the lipid membrane. Actually, a hydrophobic mismatch has been shown to strongly affect protein-lipid

organization (Killian, 1998; Zein & Winter, 2000). However the thickness change is probably very small in the pressure range covered (~1 Å/50 MPa for fluid bilayers (Winter, 2003)), and this would also hardly explain the stronger pressure resistance of the wild type GpA TMS (Gpa13), because the only differences to the GpA mutant TMS (GpAG83A) was an alanine on position 83 instead of a glycine, which should not lead to a significant difference in the TMS length.

4.6 Influence of protein structure on dimerization

While it was not possible to show an effect of membrane composition on the dimerization abilities of the examined ToxR proteins, it was possible to show an influence from protein structure. This was shown by the differences in the pressure sensitivity of the different TMS among each other and to wild type ToxR. Specifically, the dimerization of the glycophorin A TMS used in our model system was markedly less pressure sensitive than the dimerization of the other used TMS and the wild type ToxR. Therefore the data suggests that the elevated pressure affected directly the proteins and thus had caused their dissociation. It is known that at sufficiently high pressures, hydrophobic interactions, which are also responsible for the dimerization of proteins, are weakened (Winter *et al.*, 2007). Generally it has been found that pressures of 100 MPa – 200 MPa promote the dissociation of water soluble oligomeric proteins (Boonyaratanakornkit *et al.*, 2002; Gross & Jaenicke, 1994). This dissociations are typically accompanied by negative and relatively large volume changes ΔV (-50 to -200 ml/mol). The lesser pressure sensitivity of the glycophorin A TMS might therefore probably depend on a closer packing at the dimer interface and, hence, a larger activation volume for dissociation of the dimer.

4.7 Outlook

The usefulness of ToxR systems for the investigation of membrane protein dimerization under HHP was shown in this work. Beside their usefulness the use of these systems were also limited to pressures below 50 MPa. The reason therefore is that the β-galactosidase has to be expressed under pressure and this expression would be influenced by pressure sensitive, cellular functions like ribosomal synthesis or transcription elongation above 50 MPa. Therefore it would be the best to have a system where all parts are expressed before pressure treatment. An approach that would solve this problem could be the use of fluorescents resonance energy transfer (FRET). FRET was already used for the investigation of TMS dimerization in liposomes (You *et al.*, 2005). Beside of the vantages, the use of a FRET

system to determine membrane protein interaction, *in vivo,* might be far more tedious and laborious than the use of the ToxR systems used in this work.

5 Summary

The aim of this work was to understand the initial steps of signal transduction and the function of membrane proteins and membranes as sensors for chemical and physical influences. The structure and function of membrane proteins are affected by the biophysical state of the membrane (Cevc, 1993; Lipowsky & Sackmann, 1995). And at the same time, the integrated proteins influence the phase state and the lateral organization of the membrane. Therefore the interactions between membrane proteins and the membrane have been investigated, dependent on membrane characteristics like fluidity, composition, and protein structure. High hydrostatic pressure (HHP) is a powerful tool to influence bacterial physiology by changing structure and function of membranes and/or integrated proteins (Boonyaratanakornkit *et al.*, 2002) and was used in this work to study these interactions. The influence of the membrane proteins was quantified with help of model systems based on ToxR, *in vivo*. The ToxR protein plays an important role in virulence gene expression in *Vibrio cholerae* and acts also as some kind of pressure sensor in *Photobacterium profundum* (Bidle & Bartlett, 2001; Miller & Mekalanos, 1984). The ability of ToxR to bind as a dimer to specific promoter regions has made possible to follow its dimerization *in vivo* (Kolmar *et al.*, 1995; Ottemann & Mekalanos, 1996). For the experiments two ToxR expression cassettes based on ToxR from *P. profundum* were constructed and one already existing system based on ToxR from *V. cholerae* was used. These constructs, were investigated for dimerization, which is required for signal transduction, at conditions where the ToxR dimerization ability in response to HHP was quantified. Four different approaches were made to change the membrane composition for the experiments under HHP: 1. The cells were grown at different temperatures which is known to affect the composition of the membrane (Magnuson *et al.*, 1993). 2. Phenethyl alcohol was added to the growth medium, which affects the ordering of the phospholipid chains (Jordi *et al.*, 1990). 3. A *E. coli* Δ*fabF* mutant was used, which should have been deficient in *cis*-vaccenic acid (18:1 *cis*-11) synthesis and not able to regulate the amount of *cis*-vaccenic acid (18:1 *cis*-11) in response to temperature (Garwin *et al.*, 1980b). 4. Tests were performed in the deep sea bacterium *P. profundum*. Additional the fatty acid composition of the used *E. coli* strains at 30°C and 37°C was analyzed and the membrane fluidity of an *E. coli* strain in response to HHP was investigated. Generally it was not possible to show an influence of membrane composition on the dimerization abilities of the ToxR proteins under the used experimental conditions. However it was also not possible to exclude this possibility. It was shown that pressures between 20 MPa and 50 MPa are sufficient to promote the dissociation of membrane proteins. And also that the pressures up to 50 MPa

were to low to induce a membrane phase change in *E. coli* and therefore a phase change could not have been the reason for the loss of dimerization ability. It was shown before that membrane phase changes caused by pressures above 200 MPa are able to inactivate proteins (Chong *et al.*, 1985; Ulmer *et al.*, 2000; Ulmer *et al.*, 2002). However, a steady increase in ordering of the acyl-chains in the lipid membrane, based on increasing pressure, was observed, which might have influenced the dimerization ability. A decrease in the reaction rate of membrane-bound Na^+/K^+-ATPase was previously proposed to be caused by an elevated pressure induced decrease in membrane fluidity (Kato *et al.*, 2002). Also a change in membrane thickness, caused by HHP, was unlikely to have been the reason for the dissociation of the proteins. Because the thickness change was probably very small in the pressure range covered (~1 Å/50 MPa for fluid bilayers (Winter, 2003)). To analyze the influence of protein structure on dimerization ability in response to HHP and membrane composition the ToxR system based on ToxR from *V. cholerae* was used. This system had already proven its usefulness in the investigation of trans-membrane segment (TMS) interactions (Gurezka *et al.*, 1999; Langosch *et al.*, 1996). Four different TMS and one construct without TMS were used in this system. The four TMS were: AZ2 a simplified version of a membrane-spanning leucine zipper interaction domain (AAS: LLAALLALLAALLALL), EG4 a mutation of the simplified version of a membrane-spanning leucine zipper (AAS: LLAALAAALAALAAAL), GpA13 a wild type glycophorin A transmembrane segment (AAS: LIIFGVMAGVIGT) and GpAG83A a mutant of the wild type glycophorin A transmembrane segment (AAS: LIIFGVMAAVIGT). The construct without TMS consisted only of the ToxR cytoplasmic domain and MalE (ΔTM). All TMS have previously been characterized with respect to their ability to dimerize in an *E. coli* reporter strain (Gurezka *et al.*, 1999; Langosch *et al.*, 1996). The constructs were tested under the same conditions as ToxR from *P. profundum* (except in *P. profundum*). The effect of HHP on the dimerization abilities of the proteins was different for every protein. Especially the dimerization ability of the glycophorin A TMS used in the system was markedly less pressure sensitive as the ability of the other TMS or the wild type ToxR from *P. profundum*. This was also an argument against the possibility that a change in membrane thickness, caused by HHP, was the reason for the dissociation of the proteins, because a thickness change would hardly explain the stronger pressure resistance of the wild type GpA TMS (Gpa13), since the only difference to the GpA mutant TMS (GpAG83A) was an alanine on position 83 instead of a glycine, which should not lead to a significant difference in the TMS length. It is known that at sufficiently high pressures, hydrophobic interactions, which are also responsible for the

dimerization of proteins, are weakened (Winter *et al.*, 2007). Generally it has been found that pressures of 100 MPa – 200 MPa promote the dissociation of water soluble oligomeric proteins (Boonyaratanakornkit *et al.*, 2002; Gross & Jaenicke, 1994). This dissociations are typically accompanied by negative and relatively large volume changes ΔV (-50 to -200 ml/mol). The lesser pressure sensitivity of the glycophorin A TMS might therefore probably depend on a closer packing at the dimer interface and, hence, a larger activation volume for dissociation of the dimer. In general the results of this work suggest that the dimerization ability of a membrane protein under high pressure is rather controlled by its own body than by changes in the lipid bilayer environment. This indicates that in the case of ToxR, the initial step of signal transduction is the pressure effect on the protein and not an effect on the surrounding membrane.

6 Zusammenfassung

Das Ziel dieser Arbeit war es, initiale Schritte der Signalübertragung zu verstehen, insbesondere die Funktion von Membranproteinen und Membranen als Sensoren für chemische und physikalische Einflüsse. Die Struktur und Funktion von Membranproteinen wird beeinflusst vom biophysikalischen Zustand der Membran (Cevc, 1993; Lipowsky & Sackmann, 1995). Zur selben Zeit beeinflussen die in die Membran integrierten Proteine aber auch den Zustand und die laterale Organisation der Membran. Aus diesem Grund wurden die Interaktionen zwischen Membranproteinen und Membran untersucht, abhängig von Membrancharakteristiken wie Phase, Komposition und Proteinstruktur. Hoher hydrostatischer Druck (HHP) ist ein nützliches Werkzeug, um die bakterielle Physiologie durch Änderungen von Struktur und Funktion von Membranen und/oder integrierten Proteinen zu beeinflussen (Boonyaratanakornkit *et al.*, 2002) und wurde deshalb in dieser Arbeit benutzt, um diese Interaktionen zu studieren. Der Einfluss der Membranproteine wurde mit Hilfe von Modelsystemen, welche auf dem ToxR Protein basieren, *in vivo* quantifiziert. Das ToxR Protein spielt eine entscheidende Rolle bei der Virulenz Genexpression von *Vibrio cholerae* und fungiert auch als eine Art Drucksensor in *Photobacterium profundum* (Bidle & Bartlett, 2001; Miller & Mekalanos, 1984). Die Fähigkeit von ToxR als Dimer an eine spezifische Promoter Region zu binden, macht es möglich, seine Dimerisierung *in vivo* zu verfolgen (Kolmar *et al.*, 1995; Ottemann & Mekalanos, 1996). Zur Durchführung der Experimente wurden zwei auf ToxR von *P. profundum* basierende Expressions-Kassetten konstruiert und auf ein bereits existierendes System zurückgegriffen, das auf ToxR von *Vibrio cholerae* basiert. Die Konstrukte wurden in verschiedenen *E.coli* Stämmen und zum Teil in *P. profundum* eingesetzt. Mit diesen Konstrukten lies sich die ToxR Dimerisierung, welche für eine Signalübertragung notwendig ist, unter Einfluss von HHP und anderen Konditionen quantifizieren. Vier verschiedene Ansätze wurden durchgeführt, um die Membranzusammensetzung für die Versuche unter Druck zu verändern: 1. Die Zellen wurden bei verschiedenen Temperaturen inkubiert, welches Einfluss auf die Komposition der Membran hat (Magnuson *et al.*, 1993). 2. 2-Phenylethanol wurde zum Wachstumsmedium hinzugegeben, welches die Ordnung der Phospholipid Ketten beeinflusst (Jordi *et al.*, 1990). 3. Eine *E. coli* Δ*fabF* Mutante wurde benutzt, welche in der Produktion von *cis*-Vaccensäure (18:1 *cis*-11) eingeschränkt sein sollte und nicht fähig den *cis*-Vaccensäure Gehalt an Temperaturwechseln anzupassen (Garwin *et al.*, 1980b). 4. Experimente wurden in dem Tiefsee Bakterium *P. profundum* durchgeführt. Zusätzlich wurde die Fettsäurezusammensetzung der verwendeten *E. coli* Stämme bei 30°C und 37°C analysiert

und die Membranphasen von *E. coli* in Abhängigkeit von HHP bestimmt. Generell war es nicht möglich, unter den getesteten experimentellen Bedingungen eine Beeinflussung der Dimerisierungseigenschaften von ToxR durch die Membrankomposition festzustellen oder auszuschließen. Allerdings konnte gezeigt werden, dass Drücke zwischen 20 und 50 MPa ausreichend sind um die Dissoziation von Membranproteinen zu verursachen. Gleichzeitig wurde gezeigt, dass Drücke bis 50 MPa zu gering sind, um einen Membranphasenwechsel bei *E. coli* zu induzieren. Damit konnte gezeigt werden, dass ein Membranphasenwechsel in diesem Fall nicht der Grund für die Dissoziation der Proteine sein konnte. In anderen Experimenten wurde bereits gezeigt, dass durch Drücke von 200 MPa verursachte Membranphasenwechsel Proteine inaktivieren können (Chong *et al.*, 1985; Ulmer *et al.*, 2000; Ulmer *et al.*, 2002). Allerdings konnte ein ständiger Anstieg in der Ordnung der Acylketten beobachtet werden, welcher möglicherweise die Dimerisierungseigenschaften beeinflusste. Bereits in einem früheren Experiment wurde die Verringerung in der Reaktionsrate einer Membran gebundenen Na^+/K^+-ATPase, auf einen durch Druck verursachten Anstieg in der Ordnung der Acylketten, zurückgeführt (Kato *et al.*, 2002). Auch eine durch HHP verursachte Veränderung in der Dicke der Membran, als Grund für die Dissoziation, kann in diesem Druckbereich höchstwahrscheinlich ausgeschlossen werden. Da in diesem Druckbereich nur eine sehr geringe Dickenänderung zu erwarten wäre (~1 Å/50 MPa für flüssig kristalline Doppelmembranen). Um den Einfluss der Proteinstruktur auf das Dimerisierungsverhalten der Proteine unter Druck zu untersuchen, wurde das ToxR System basierend auf ToxR von *V. cholerae* verwendet. Dieses System hatte bereits in zahlreichen Experimenten seine Nützlichkeit bei der Untersuchung von Transmembransegment (TMS) Interaktionen bewiesen (Gurezka *et al.*, 1999; Langosch *et al.*, 1996). Es wurden vier verschiedene TMS und ein Konstrukt ohne TMS in diesem System verwendet. Bei den vier TMS handelte es sich um folgende: AZ2 eine vereinfachte Version einer Transmembran Leucin-Zipper Interaktionsdomäne (AAS: LLAALLALLAALLALL), EG4 eine Mutante der vereinfachten Version der Transmembran Leucin-Zipper Interaktionsdomäne (AAS: LLAALAAALAALAAAL), GpA13 ein Wildtyp Glycophorin A Transmembransegment (AAS: LIIFGVMAGVIGT) und GpAG83A eine Mutante des Wildtyp Glycophorin A Transmembransegments (AAS: LIIFGVMAAVIGT). Das Konstrukt ohne TMS bestand nur aus der Zytoplasmatischen ToxR Domäne und MalE (ΔTM). Alle TMS wurden bereits in früheren Experimenten bezüglich ihrer Neigung zur Dimerisierung, in einem *E. coli* Reporterstamm, charakterisiert (Gurezka *et al.*, 1999; Langosch *et al.*, 1996). Die Konstrukte wurden unter denselben Bedingungen getestet wie die Konstrukte basierend auf ToxR von *P.*

profundum (außer, dass sie nicht in *P. profundum* eingesetzt wurden). Der HHP Effekt auf die Dimerisierungseigenschaften war für jedes Protein verschieden. Insbesondere die Dimerisierungseigenschaft des Glycophorin A TMS war merklich geringer durch HHP beeinflusst als die Dimerisierungseigenschaften der anderen TMS oder des Wildtyp ToxR von *P. profundum*. Dieses stellt auch ein weiteres Argument gegen die Möglichkeit da, dass eine Veränderung in der Membrandicke der Grund für die Dissoziation der Proteine unter HHP sein könnte. Denn dieses würde nicht das unterschiedliche Dimerisierungsverhalten des Wildtyp Glycophorin A TMS gegenüber der Mutante erklären, da der einzige Unterschied darin besteht, dass die Mutante an Position 83 ein Alanin anstelle eines Glycin besitzt, was zu keinem größeren Längenunterschied führen sollte. Es ist bekannt, dass ausreichend hoher Druck hydrophobe Interaktionen, welche für die Dimerisierung von Proteinen verantwortlich sind, schwächt (Winter *et al.*, 2007). Generell wurde festgestellt das Drücke zwischen 100 bis 200 MPa die Dissoziation von wasserlöslichen oligomeren Proteinen begünstigen (Boonyaratanakornkit *et al.*, 2002; Gross & Jaenicke, 1994). Diese Dissoziationen werden typischer Weise von negativen und relativ großen Volumenänderungen begleitet ΔV (-50 zu -200 ml/mol). Die geringere HHP Sensitivität des Glycophorin A TMS lässt sich wahrscheinlich auf eine dichtere Packung des Dimer Interfaces und daraus resultierend auf ein größeres Aktivierungsvolumen für die Dissoziation des Dimers zurückführen. Im Allgemeinen legen die Ergebnisse dieser Arbeit nahe, dass die Dimerisierungseigenschaften von Membranproteinen unter HHP eher durch ihre Proteinstruktur als durch Änderungen in der sie umgebenden Doppelmembran bestimmt werden. Generell lässt sich jedoch ein Einfluss nicht komplett ausschließen. Für die Funktion von ToxR als Signalprotein bedeutet dieses, dass die direkte HHP Einwirkung auf das Protein höchstwahrscheinlich den initialen Schritt der Signalübertragung darstellt und seine Funktion nicht entscheidend von den HHP Einwirkungen auf die es umgebende Membran beeinflusst wird.

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8 Appendix

List of publications

Original paper

Linke, K., Periasamy, N., Ehrmann, M. A., Winter, R., and Vogel, R. F. (2008). Influence of high pressure on the dimerization of ToxR, a protein involved in bacterial signal transduction. *Appl Environ Microbiol* **74**, 7821-7823.

Vogel, R. F., Linke, K., Teichert H., Ehrmann, M. A. (2008). High pressure modulated transport and signalling functions of membrane proteins in models and *in vivo*. Joint 21st AIRAPT and 45th EHPRG Int. Conf. on High Pressure Science and Technology, Catania, Italy, 17 - 21 September 2007, *Journal of Physics: Conference Series* **121**, 112005.

Oral presentations

Vogel, R. F., Linke, K., Teichert, H., Periasamy, N., Ehrmann, M. A., Winter, R. (2008). High pressure modulated transport and signalling functions of membrane proteins. High Pressure Bioscience and Biotechnology, San Diego, USA, 14.-17 September 2008.

Linke, K., Teichert, H., Ehrmann, M. A., Vogel, R. F. (2005). Models to study high pressure tuning of membrane transporters and signal cascades in mesophilic and deep sea bacteria. Meeting of the COST Working Group, project D30/007/04. School of Food Biosciences, University of Reading, UK, 13 - 15 October 2005.

Poster Presentations

Vogel, R. F., Teichert, H., Linke, K., Periasamy, N., Winter, R., Ehrmann, M. A. (2008). High pressure modulated transport and signalling functions of membrane proteins in model systems and *in vivo*. VAAM Jahrestagung, Frankfurt, Germany, 09 - 11 March 2008.

Linke, K., Kleemann R., Ehrmann, M. A., Lindner E., Langosch D., Vogel, R. F. (2006). Potential of high pressure to quantify protein-protein interaction of membrane proteins in vivo. Symposium of the European High Pressure Group, Prag, Czech Republic, 04 - 08 September 2006.

Lebenslauf

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