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Behaviour of membrane transport proteins under high hydrostatic pressure

Holger Teichert

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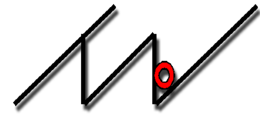

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Doctoral thesis

Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

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Vorwort

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Abbreviations

ABC	ATP binding cassette
ABS	ATP binding site
ATP	adenosintriphosphate
BGG	bovine gamma globulin
bp	base pair
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DNA	desoxyribonucleic acid
dNTP	desoxynucleotid phosphate
DOPC	1,2-dioleyl-sn-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSM	DSMZ, Braunschweig, Germany
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
Fig.	figure
g	gram
GP	generalized polarization
h	hour
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilo dalton
KOD	*Thermococcus kodakarensis* KOD1

Lb.	*Lactobacillus*
Lc.	*Lactococcus*
LUV	large unilamellar vesicles
M	Mega (10^6), molar
m	Milli (10^{-3}), meter
Mg	magnesia
min	minutes
MLV	multi lamellar vesicles
NICE	nisin-controlled gene expression
OD	optical density
p	pressure [Pa]
pK_a	negative decimal logarithm of the acid dissociation constant
RBS	ribosome binding site
PCR	polymerase chain reaction
SDS	sodium n-dodecylsulfate
s	second
Tab.	table
Taq	*Thermus aquaticus*
T_M	transition temperature
TMD	trans membrane domain
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})

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1 Introduction

Pressure - force per area applied to an object in a direction perpendicular to the surface. It doesn't matter, if the pressure mediating media is solid, fluid or gaseous. It must only contact the surface to transmit the force. This is a short physical definition of pressure. Mostly we do not realize this pressure in the normal course of life. The pressure, human beings are normally exposed to is named ambient pressure. However, what is understood as ambient pressure exists only in a minor part of our planet. Two thirds of our planet is covered with water from the oceans and the sea, where pressure is always higher than 0.1 MPa. This is the habitat of many known and unknown species, which reign the majority of this world. Living organisms can be found in regions of the deep sea, where temperature is quite stable at 4° C (except of points with volcanic activity). The pressure in these regions is up to 110 MPa. Organisms from the deep sea normally die rapidly, if they were brought to the surface. On the other hand, higher life forms from the surface of our planet won't resist a high pressure as it exists in the deep sea. And the only thermodynamic parameter that is changed is the pressure. This shows that high hydrostatic pressure (HHP) can be rather more than a technique used in chemistry industries: it is an excellent tool for changing conditions with only one thermodynamic parameter, which affects drastically the mechanisms of life. Bacteria can thereby play an excellent role in investigation of the impact of the high hydrostatic pressure to life, as bacteria often are the target of the application of HHP in alternative food preservation methods. Conventionally, foods were preserved in reducing water or oxygen during storage or with a process, which changes one thermodynamic parameter for a period of time: chilling for the complete storage period or a temperature increase during the period of a pasteurization treatment. Such thermal processes usually denature the proteins of the spoiling bacteria and enzymes, but also of the matrix of the food.

Alternative food processing and preservation technologies attract special interest of the food industry as a result for the consumers demand of high nutritional and sensory qualities as well as microbiological safety (Knorr *et al.* 2002). High hydrostatic pressure harbors the possibility to inactivate microorganisms, viruses, helminthes and enzymes while effects on the the flavor and nutrient content of food is low compared to usual thermal treatments (Kingsley *et al.* 2002; Molina-Garcia *et al.* 2002; San Martin *et al.* 2002). Since HHP was established as a processing step for food in Japan, it gained interest of the European food industry. 1995 Pernod Ricard (France) presented a HHP treated orange juice, sliced ham from Espuna (Spain) was brought to the market one year later (sausages since 2002) and Danone (France)

is since 2001 in possession of an accreditation according to novel food edict (EC) nr. 258/97 for HHP treated fruit based preparations (2001/424/EC). HHP is a useful tool to inactivate *Listeria* in milk (Koseki *et al.* 2008) or *E. coli* in soup (Munoz *et al.* 2007) or other pathogens in ham (Tassou *et al.* 2008). High hydrostatic pressure can even inactivate spores but can also induce their germination (Wuytack *et al.* 1998; Paredes-Sabja *et al.* 2007). Equally the history of the organisms affect their resistance to HHP (Scheyhing *et al.* 2004; Hormann *et al.* 2006). For this reason we have to learn more about the different impacts and effects in inactivation of microbes to understand the mechanisms, which lead to an inactivation or repression of microbes. In combination with additives, e. g. nisin, HHP is able to protect food against bacterial spoilage (Lopez-Pedemonte *et al.* 2003). Besides to nisin, HHP increases the bactericidal ability of compounds like lactoferrin and lactoferricin (Masschalck *et al.* 2001). Also, Lactoferrin and nisin modulated bactericidal effect is at least partially located to the membrane, (Ellison *et al.* 1990; Ellison 1994; Murdock *et al.* 2007). The membrane is hypothesized to be the main target of HHP (Hoover *et al.* 1989). Lactoferricin is assumed to reach the cytoplasmatic membrane, which will be perturbed and eventually disintegrated (Yamauchi *et al.* 1993). Pressure dependant loss of membrane integrity and loss of transport capacity at the membrane was shown earlier and the membrane phase affected by the lipid composition plays a role for pressure resistance of transport activity (Ulmer *et al.* 2000; Ulmer *et al.* 2002; Molina-Hoppner *et al.* 2004). It is long known, that membrane lipids and proteins form a unit, which is crucial for the existence of life by separating the cytoplasm from the exterior (Kleinschmidt 2003). Membrane lipids and proteins influence each other directly as a result of their biochemical nature and in reaction to environmental changes (Popot *et al.* 2000; Engelman *et al.* 2003), including extreme conditions such as high or low temperatures and high pressure.

1.1 General principles of high hydrostatic pressure

“If a system at equilibrium experiences a change in concentration, temperature, volume or total pressure, then the equilibrium shifts to partially counteract the imposed change.” This short simplified sentence gives the main meaning of the Le Chatelier’s principle. If a system experiences a change via the thermodynamic parameter pressure it will respond with changes in other parameters (volume temperature or concentration) or equilibria (e.g. a chemical reactions, phase transitions or changes in molecular conformation). If the temperature and the total volume stays constant (e.g. in a tempered autoclave), the equilibrium of a reaction shifts to the side with a lower volume. A low reaction volume results in minor impact of the

parameter pressure to the reaction. A negative reaction volume facilitates a reaction under pressure, while positive reaction volumes impede reactions upon increase of pressure. Table 1.1 gives a few important reaction volumes with biochemical interest.

Table 1.1. Reaction volumes associated with selected biochemically important reactions at 25°C (Gross *et al.* 1994) in ΔV (mL/mol).

Reaction	Example	ΔV
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 → $(\text{n-propanol})_{water}$	- 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] V 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

1.2 Membrane lipids under high hydrostatic pressure

It is long known, that membrane lipids and proteins form a unit, which is crucial for the existence of life by separating the cytoplasm from the exterior (Kleinschmidt 2003). They are in sensitive macromolecular interaction, which determines their structure and function in transport, communication or energy metabolism (Kleinschmidt 2003). Membrane lipids and proteins influence each other directly as a result of their biochemical nature and in reaction to

environmental changes (Popot *et al.* 2000; Engelman *et al.* 2003), including extreme conditions such as high or low temperatures and high pressure.

Membrane lipids usually form a lamellar conformation referred to as lipid bilayer. In dependence to the thermodynamic parameters temperature and pressure, the bilayer takes different conformational orders of the head groups and the acyl chains. The respective lipid phases are labelled according to the nomenclature of Tardieu (Tardieu *et al.* 1973). A big "L" means a lamellar region and the annex "α" stands for liquid phases. A "β" means gel packed chains. An apostrophe to the annex indicates that the angle from the acyl chains to the membrane surface is lower than 90°. Figure 1.1 gives models of the lipid phases.

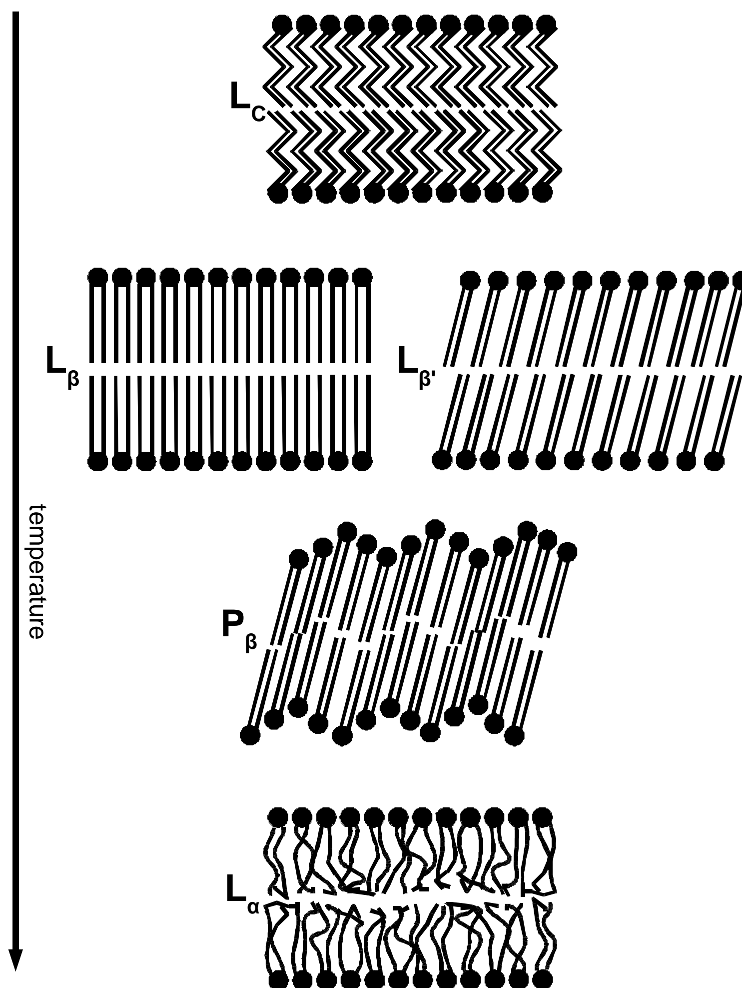

Figure 1.1. Schematic representation of the membrane phases of according to Tardieu (Tardieu *et al.* 1973). L_α shows the liquid crystalline phase, L_β and $L_{\beta'}$ represents the gel phase without and with an angle, respectively. L_c is the crystalline phase and $P_{\beta'}$ the interphase.

Starting from the liquid cristalline state the membrane gets more and more ordered when the temperature is decreased corresponding to relatively ordered and more extended hydrocarbon chains. If the lipid bilayer contains only one sort of lipid, e.g. only one kind of phospholipid, the phase transitions will occur at a specific temperature called melting point or melting temperature T_m (transition from β to α and vice versa). A transition region with coexisting phases will appear with bilayers containing different types of lipids. Pure lipid systems are well studied regarding membrane phase transitions, also their behaviour under high pressure conditions (Cevc 1993; Lipowsky *et al.* 1995; Li *et al.* 2005; Allain *et al.* 2006; Barman *et al.* 2006; Nicolini *et al.* 2006a; Nicolini *et al.* 2006b). Also the influence of peptides and proteins on membrane structure and phase behavior has been described (Periasamy *et al.* 2006; Powalska *et al.* 2007). In these studies, high hydrostatic pressure (HHP) has proven to be an excellent tool to influence the phase behavior of lipid membranes. With increasing pressure, the conformational order of the acyl chains can be easily tuned and phase transitions can be induced, such as from the liquid-crystalline to the gel state, without changing the temperature or adding reagents.

1.3 Proteins under high hydrostatic pressure in aqueous solutions

The protein structure is defined by four structural levels. Primary structure is defined from the sequence of amino acids which is given from the DNA. Hydrogen bonds define the secondary structure in which the primary structure is forming alpha helixes or beta sheets. The three dimensional structure is given in the tertiary structure and the quaternary structure defines the coordinates of subunits in the case of a protein, which consists of different protein subunits. It has been known for a long time that the structure levels are affected from temperature effects as well as HHP.

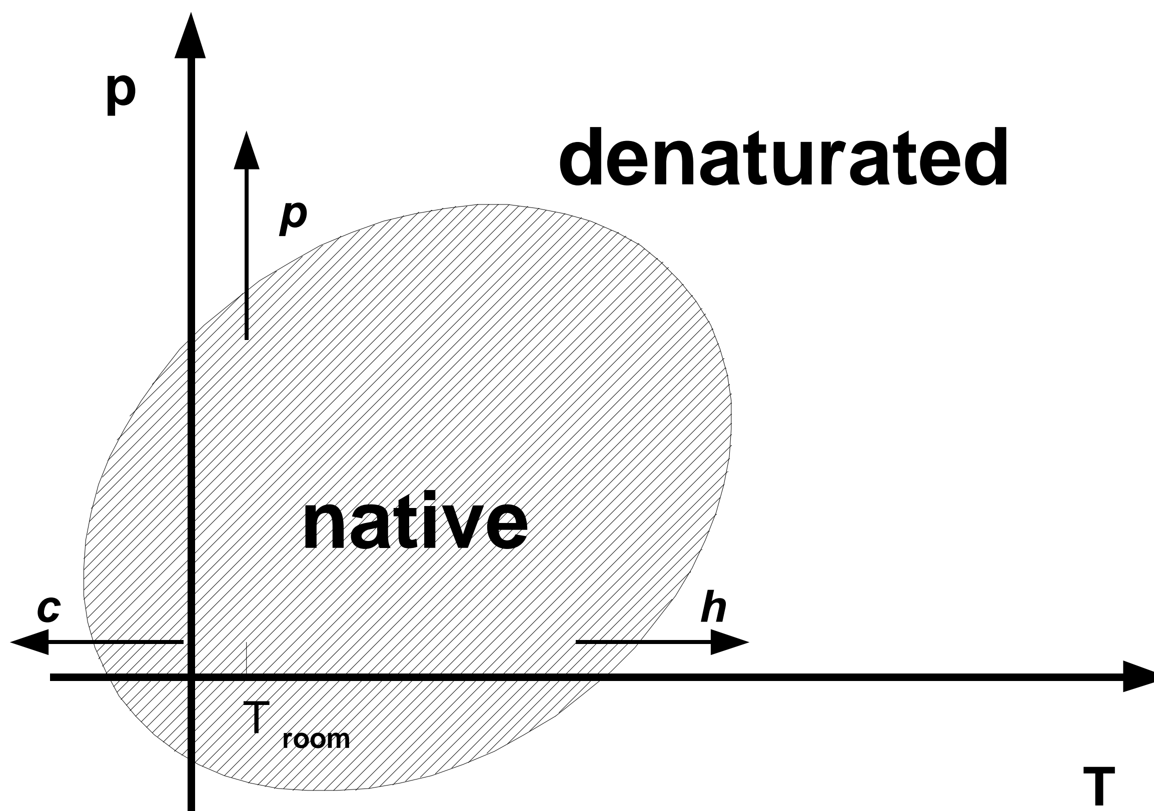

Figure 1.2. Schematic representation of the elliptic phase diagram of proteins according to Smeller (Smeller 2002). Arrows indicate the specific denaturation ways as (*p*) stands for pressure, (*h*) for heat and(*c*) for cold denaturation.

Pressure/temperature diagrams of a number of proteins are published so far and their elliptic shape shows an optimum temperature at which proteins are quite resistant to pressure (Smeller 2002). Except the primary structure, all structural levels are affected by HHP (Cordeiro *et al.* 2006). Denaturation of proteins normally does not take place at pressures under 400 MPa (Gross *et al.* 1994; Kato *et al.* 2002) while membrane transport is already inhibited at lower pressures (Ulmer *et al.* 2002; Kilimann *et al.* 2005). Denaturation of the secondary structure of red shifted GFP could be shown with a decrease of the amount of beta-sheets at pressures over 900 MPa at room temperature (Herberhold *et al.* 2003) and for transthyretin at pressures of 1200 MPa (Cordeiro *et al.* 2006). Remarkably, some proteins show a reversibility of pressure induced changes of the secondary structure as shown for ubiquitin (Herberhold *et al.* 2002). This leads to the conclusion, that HHP induced changes in the secondary structure are not a main factor for protein enzyme inhibition as the observed pressure influence at much lower pressures. Multiple reviews deal with the observation, that HHP influences the quaternary structure in a stronger way than the tertiary and the secondary

structure (Heremans *et al.* 1998; Boonyaratanakornkit *et al.* 2002; Marchal *et al.* 2005). Tertiary structures of proteins, e.g. prions are affected by HHP in the range of 400 – 600 MPa (Marchal *et al.* 2005). For the human ataxin-3 protein, structural changes of the tertiary structure in the pressure range of 300 – 650 MPa were determined, while the secondary structure seemed to be unaffected (Marchal *et al.* 2003). Partial unfolding over the pressure increase indicates that unfolding procedures only take place in special regions the protein at their respective pressures. For example, they hypothesized that polyglutamine poor regions of the protein were less sensitive to unfolding effects from HHP. Two monomers from the enzyme enolase, which occurs as a homodimer, were obtained with pressure treatment up to 150 MPa without loosing the activity of the protein (Kornblatt *et al.* 2004). Tertiary structure was not affected and originally conformation was maintained. Dissociation into monomers and/or subunits is the first expected effect, followed by tertiary and later denaturation secondary structure changes with increasing the pressure. These effects were ascribed to hydrophobic and ionic interactions where water plays a crucial role for hydrogen bond formation and hydrophobic interaction (Gross *et al.* 1994). All these observations were made with proteins in aqueous solutions, but until now, little is known about membrane proteins residing in a phospholipids bilayer. Even though membrane can protect the protein against changes in the third structure level resulting from HHP (lack water in the membrane) (Kangur *et al.* 2008).

1.4 Membrane proteins

Membrane proteins can constitute about 30 % of the entire protein content of the cell and so, a large number of drugs are targeting the membrane (Kleinschmidt 2003). Membrane proteins rest to a varying extent in a lipid environment, where they act as anchors, enzymes or transporters. Therefore, membrane lipids and proteins influence each other directly as a result of their biochemical nature and in reaction to environmental changes (Popot *et al.* 2000; Engelman *et al.* 2003). The length of the hydrophobic part of the membrane protein should match the thickness of the membrane if the protein should be anchored perpendicular to the membrane (Mouritsen *et al.* 1984). If the trans membrane segment is longer, this hydrophobic mismatch leads to an angle which can affect the activity of membrane bound ATPases (Froud *et al.* 1986a; Froud *et al.* 1986b). As the membrane describes a barrier, which forces a gradient of many substances, membrane proteins usually play a role in using this gradient or in preventing it. Membrane bound ATPases for example, catalyze ATP / ADP conversions in

both directions, to either gain biochemical energy from an ion gradient across the membrane or drive enzymatic reactions or transport functions by ATP consumption. The ATP binding cassette is located in the cytoplasmic region of the protein (Richter *et al.* 2000). The trans membrane domain (TMD) of an F_oF_1-ATPase is involved in proton transport through the membrane (Boyer 1993). Analogous transport functions are known for other hetero oligomeric ATPases, such as potassium transporting ATPases (Sachs *et al.* 1989; Barwe *et al.* 2007). Along with their different functions, ATPases differ not only in structure. For example, the membrane bound enzyme F_oF_1-ATPase is a multi subunit protein with about 9 different subunits (Richter *et al.* 2000), while the proton motive force transporter LmrP from *Lactococcus lactis* is a homodimer in its functional state (Bolhuis *et al.* 1995; Souza *et al.* 2004). Multiple drug resistance transporters (MDRs) usually exhibit TMDs ranging through the lipid bilayer and mediate resistance to several drugs by active transport (Higgins 1992). This transport can be driven by two different stimulations: primary MDR transport under the consumption of ATP and proteins from the secondary class usually are driven from the protein/sodium motive force (McKeegan *et al.* 2003; Venter *et al.* 2005). Having a closer look on the TMD of secondary- and primary-active MDR, often twelve (or two times six as in the case of dimmeric proteins) trans membrane segments TMS are found (Venter *et al.* 2005). The global structure and the TMD and similarities of the drug binding sites goes along with overlapping substrate specificity.(Poelarends *et al.* 2002b; Reuter *et al.* 2003; Venter *et al.* 2003).

1.4.1 The ABC transporter LmrA

The MDR transporter LmrA from *Lactococcus lactis* is one of the best investigated membrane transporters with respect to substrate spectrum, functionality and structure (van Veen *et al.* 2000; Vigano *et al.* 2000; Grimard *et al.* 2001; Poelarends *et al.* 2002a; Reuter *et al.* 2003; Balakrishnan *et al.* 2004; Pleban *et al.* 2004; van den Berg van Saparoea *et al.* 2005; Federici *et al.* 2007). Unlike other bacterial MDRs, LmrA was one of the first ABC-(ATP binding cassette) transporters found in bacteria (Bolhuis *et al.* 1994). It is hypothesized to promote resistance to chemotherapeutics by expelling amphiphilic compounds from the inner leaflet of the cytoplasmic membrane in an ATP-dependend process (Bolhuis *et al.* 1996; van Veen *et al.* 1996). Its consists of two identical subunits (van Veen *et al.* 2001b; Federici *et al.* 2007) whose dimerisation to a homodimer is necessary to form the functionally active transporter (van Veen *et al.* 2001a; McKeegan *et al.* 2003). The LmrA monomer is a 590 amino acid protein with an N-terminal hydrophobic domain, consisting of six trans-membrane helices,

followed by the C-terminal hydrophilic domain, including the ATP-nucleotide binding site. The TMD is directly connected with the nucleotide binding domain (NBD) and therefore directly influences the ATPase activity when it undergoes conformational changes (Bolhuis *et al.* 1996; van Veen *et al.* 2000; Vigano *et al.* 2000; Grimard *et al.* 2001; Balakrishnan *et al.* 2004).

1.4.2 The ABC transporter HorA

Lactobacillus brevis possesses an LmrA like protein called HorA from the ABC MDR family (Sami *et al.* 1997). The amino acid sequence shows 53 % accordance with the translated *lmrA* gene from *Lactococcus lactis* (Sakamoto *et al.* 2001). It is described to contribute to the resistance against hop compounds (Sakamoto *et al.* 2001; Sakamoto *et al.* 2003; Suzuki *et al.* 2005; Haakensen *et al.* 2008) and was also found in other beer spoiling bacteria (Suzuki *et al.* 2006; Haakensen *et al.* 2008), but HorA is not necessarily part of the resistance of beer spoiling bacteria to hops (Behr *et al.* 2006). Like LmrA, its substrate spectrum also contains ethidium bromide (Sakamoto *et al.* 2001; Suzuki *et al.* 2006).

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1.5 Objectives of the work

It was the aim of this thesis to investigate the behavior of bacterial membrane proteins in their environment of lipid phases and in dependency of the lipid composition and to check the thesis, that membrane states influence the activity of the membrane proteins. Up to 200 MPa, increasing pressure has a huge impact to the lipid order of membrane lipids. In most cases, higher pressures only have minor effects to the membrane, because especially biological membranes nearly have reached the gel phase already. Denaturizing effects on the proteins are not expected in this pressure range. Only dissociation will occur and can lead to inhibition of activity as shown in aqueous solution. Certainly, lipid phases and membrane protein systems were affected from each other, remembering, that HHP up to 200 MPa causes inhibition of membrane transport in some cases, while altering the lipid composition may have preventing effects. LmrA and HorA offer a wide range for finding an adequate and similar method of measuring the activity, as both represent ATPases with an ATPase activity coupled with transport of substrates through the membrane. Hence these proteins are a good models to check the behavior of membrane proteins under HHP, because membrane transport is affected in that pressure range, but cells are not inactivated (Ulmer *et al.* 2000; Ulmer *et al.* 2002; Perrier-Cornet *et al.* 2005a). Adequate variances in the structure of HorA compared to LmrA is useful for testing in parallel the role of the protein / protein interactions of a homodimer compared to the impact of the lipid environment to a dimeric protein system. *Lb. plantarum* possesses many transport systems, for example, LmrA, which is coded in the genome and HorA activity of *Lb. plantarum* has been described previously (Ulmer *et al.* 2000; Kleerebezem *et al.* 2003). The membrane of *Lb. plantarum* therefore seems as a good model and natural environment for many membrane transport systems and therefore, this strain was chosen as a source for natural membrane lipids.

As interactions always work on both subjects and always influences each other, the impact of the protein concentration to the lipid order and membrane phase arrangement should be investigated.

Further on, to realize these subjects, an adequate measuring system should be established first, which allows the determination of the enzyme activity in a convenient way. This system needs to be simple and without many additional parameters like accompanying enzymes or substrates, which can be affected by the HHP. On the other hand, the measuring system must not affect the membrane integrity and protein activity. For this aim, several measurement techniques like fluorescence measurement or colorimetric measurements should be tested.

2 Material and Methods

Unless otherwise stated, chemical were obtained from major suppliers like Merck KGaA (Darmstadt, Germany), Sigma-Aldrich, Serva or Gerbu (Gaiberg, Germany). 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were provided by Lipoid GmbH (Ludwigshafen, Germany). The vector pNH*lmrA* with an N-terminal His-Tag was kindly provided by Driessen and van den Berg van Saparoea (van den Berg van Saparoea *et al.* 2005).

2.1 Strains, media and growth conditions

Lactobacilli were cultivated anaerobically, usually at 30° C in MRS media (containing per litre: meat extract 2 g, peptone from casein 10 g, yeast extract4g, Tween 80 1 ml, KH_2PO_4 1,8 g, Na-acetate 5 g, $(NH_4)_2$ citrate 2 g, glucose 22 g). Lactococci were cultivated in GM17 media (M17, Difco with 0,5 % (w/v) glucose) at 30° C. *E. coli* cells were used for cloning procedures and were cultured aerobe using lysogeni broth (LB) with peptone from casein 10 g/L, yeast extract 5 g/l and 5 g/l sodium chloride at 37° C and shaken with approximately 180 rotations per minute. Where appropriate, chloramphenicol (24 µg/ml for *E. coli* and 5 µg/ml for *Lactococcus*) or ampicillin (100 µg/ml for *E. coli*) was added. Media were solidified with the addition of 15 g/l agar when needed for plates. Table 2.1 gives an overview to the strains and their application in this study.

Table 2.1. Strains used in this study. (A) gives the strains, which were used for cloning experiments as hosts. (B) gives the strains which were used determining the change of the membrane fluidity and the strains of *Lactobacillus brevis,* which were used for the horA-screening are given in (C): TMW stands for “Technische Mikrobiologie Weihenstephan”.

A: Strains used for cloning experiments

Strain	Genotype
Escherichia coli Top Ten	F’, (Φ80d*lacZ*.M15).(*lacZYAargF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rK-, mK+), *supE*44, λ-*thi*-1, *gyrA*96, *relA*1
Escherichia coli NovaBlue™	*endA*1 *hsdR*17 (r_{K12}^- m_{K12}^+) *supE*44 *thi-1 recA*1 *gyrA*96 *relA*1 *lac* F'[*proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M*15::Tn*10*] (Tet^R)
Escherichia coli BL21(DE3)	F – *ompT hsdSB* (*rB– mB–*) *gal dcm* (DE3)
Lactococcus lactis MG1363	Wildtype, plasmid free
Lactococcus lactis NZ9000 *ΔlmrA*	MG1363 with *nisRK* integrated into chromosome, *ΔlmrA*

B: Strains used for membrane fluidity tests

Strain	Application
Lactococcus lactis TMW 1.1085	Measurement of membrane fluidity
Lactobacillus plantarum TMW 1.460	Measurement of membrane fluidity and source for mebrane lipids
Lactobacillus brevis TMW 1.465	Measurement of membrane fluidity

C: Strains used for *horA*-screening

Lactobacillus brevis TMW	1.1282, 1.1283, 1.1284, 1.1326, 1.310, 1.311, 1.315, 1.317, 1.465, 1.483, 1.485, 1.599,.1.6

2.2 General molecular techniques

DNA from lactobacilli was prepared according to Lewington (Lewington *et al.* 1987). DNA and amplification products were separated on 1% agarose gels, stained with ethidium bromide, and visualized by UV transillumination. Plasmid DNA from *E. coli* was isolated with the Plasmid Mini Kit from Quiagen (Hilden, Germany). Plasmid DNA from Lactococci was isolated using the E.Z.N.A. plasmid Midi-Kit (Pequlab Biotechnologie GmbH, Erlangen, Germany). PCR product was purified as well as single cuts or restriction cuts of DNA with small “waste”ends (smaller then 50 base pairs (bp)) using the QIAquick PCR purification kit (Quiagen, Hilden, Germany) or isolated from agarose gels using an E.Z.N.A. gel extraction kit (Pequlab, Germany). Restriction enzymes and T4-DNA ligase were purchased from Fermentas (MBI Fermentas GmbH, St. Leon-Rot, Germany) and were performed following the recommendations of the supplier. DNA was amplified using a Thermo Cycler (Primus96 plus, MWG-Biotech AG, Ebersberg, Germany). For the amplification of DNA, *Taq*-DNA polymerase (MBI Fermentas GmbH, St. Leon-Rot, Germany or from Qbiogene, MP Biomedicals, Heidelberg, Germany) was used for verifying PCRs and *KOD*-DNA polymerase (Novagen, EMD Chemicals Inc., Merck KGaA, Darmstadt, Germany) was used for cloning PCR steps and sequencing works in concentrations according to the recommendations of the suppliers. DNA was sequenced at commercial labs like MWG (MWG-Biotech AG, Ebersberg, Germany) or GATC (GATC Biotech AG, Konstanz, Germany). Nucleotide and amino acid sequence analysis was carried out using CloneManager5 for Windows (Sci Ed, Scientific & Educational Software, USA). Table 2.2 gives an overview to the used primers and their application.

Table 2.2. List of the used primers in this study. Shown are the names, sequences and applications. All primers were purchased from MWG biotech, Ebersberg, Germany.

Primer	Sequenz	Application
pNZ8048_rev	5’-GCTGTAATTTGTTTAATTGCC-3’	Clone screening and sequencing
pNZ8048_for	5’-GATTAAATTCTGAAGTTTGTTAG-3’	Clone screening and sequencing
horAvectorsequ5’	5’-AATCGAACAACATTGGTGAT-3’	Sequencing of the plasmid
pGKV259screen5’	5’-GAGAGATAGGTTTGATAGAAT-3’	Sequencing of the plasmid
pBADscreen3’	5’-AAATTCTGTTTTATCAGACCG-3’	Clone screening and sequencing
lmrA_screen_for	5’-AATTACTTGATGAAGAAC-3’	*horA*/*lmrA* screening
horA_screen_for	5’-TGTCCGCCAATCCCAC-3’	*horA*/*lmrA* screening
ScreenGen_rev	5’-GTTGCTTCATCCAGCAT-3’	*horA*/*lmrA* screening
lmrA_NcoI2_for	5’-ATATATCAATGGAAAGAGGTCCACAAATGGC-3’	Cloning
lmrASalI_rev	5’-ATATATGTCGACTTGACCAACAGTCAATTGTTC-3’	Cloning
horAXhoI_for	5’-ATATATATCTCGAGTATGCAAGCTCAGTCCAAGAAC-3’	Cloning
lmrASalI_for	5’-GCATGCGTCGACATGGAAAGAGGTCCACAAATG-3’	Cloning
lmrAHisTPstI_rev	5’-ATAGACCTGCAGTCAATGATGATGATGATGATGTTGACCACCAGTCAATTGTTCTGA-3’	Cloning with C-terminal His-Tag
horAHisTHindIII_rev	5’-TTTAAGCTTTCAATGATGATGATGATGATGCCCGTTGCTCGTCGCGCTCTGATTTTT-3’	Cloning with C-terminal His-Tag
horAPstI_for	5’-GGGCTGCAGATGCAAGCTCAGTCCAAGAAC-3’	Cloning
lmrAXbaI_rev	5’-ATATCTAGATTTTGACCAACAGTCAATTG-3’	Cloning
lmrANcoI2_for	5’-ATATATCCATGGAAAGAGGTCCACAAATGGC-3’	Cloning
lmrASalI_rev	5’-ATATATGTCGACTTGACCAACAGTCAATTGTTC-3’	Cloning
horAXhoI_for	5’-ATATATATCTCGAGTATGCAAGCTCAGTCCAAGAAC-3’	Cloning
horASalI_rev	5’-ATATATGTCGACCCCGTTGCTCGTCGCGCTC-3’	Cloning
horARBSPstI_for	5’-ATATATCTGCAGGAGGATATAATATGCAAGCTCAGTCCAAGAAC-3’	Cloning with RBS
lmrAHisTHindIII_rev	5’-ATATATAAGCTTAATGATGATGATGATGATGTTGACCAACAGTCAATTG-3’	Cloning with C-terminal His-Tag
lmrAPmeI_for	5’-ATATATGTTTAAACTAATGGCCAATCGTATCGAAGG-3’	Cloning
horAPmeI_for	5’-ATATATGTTTAAACTAATGCAAGCTCAATCCAAGAAC-3’	Cloning

lmrAHisTPmeI_rev	5'-ATATATGTTTAAACTAATGATGA TGATGATGATGATGTTGACCAACA GTCAATTG-3'	Cloning with C-terminal His-Tag
lmrARBSPstI_for	5'-ATATATCAGCAGGAGGAATATAT ATGGAAAGAGGTCCACAAATG-3'	Cloning with RBS
horA&RBSPstI_for	5'-ATATATCTGCAGTAGAAGCATTT AGGGGATAATTTTG-3'	Cloning with RBS
sigX_for	5'-GATATAAGCTTGAGTATCCTGTA CGG-3'	Knock-out of *lmrP*
sigX_rev	5'-GACTGGTTTTCAAGCATATTATT C-3'	Knock-out of *lmrP*
yweA_for	5'-GAATAATATGCTTGAAAAGGAGT C-3'	Knock-out of *lmrP*
yweA_rev	5'-CATATGTTCGAATGTTGCTAAAG C -3'	Knock-out of *lmrP*
horAHisTPscI_for	5'-GATATACATGTTACATCATCATC ATCATCATCAAGCTCAGTCCAAG -3'	Cloning with N-terminal His-Tag
horAHindIII_rev	5'-CATATAAGCTTCACCCGTTGCTC GTCGC -3'	Cloning
nisR_for	5'-GACAGCATTAGAAATGAGAAAC -3'	Host screening
nisK_rev	5'-CTTAGAAAATTCTCTAATGAGAT G-3'	Host screening

Electrocompetent cells of *E. coli* were prepared according to Tieking (Tieking *et al.* 2005) and electrocompetent cells of *Lactococcus lactis* were prepared according to the protocol from Holo and transformed as described (Holo *et al.* 1989) with a glycine concentration of 1.25 % (w/v)growing in the growing media. For the transformation of *E. coli* cells also a BioRad gene pulser (BioRad Laboratories, Hercules, USA) was used with the following settings: voltage 2.5 kV, resistance 200 Ω, capacity 25 µF. E. coli cells were incubated for 1 h at 37° C and intense shaking (approximately 200 rotations per minute). In Table 2.3 the plasmids are listed which found application in this study.

Table 2.3. List of the used plasmids in this study. Shown are the names, description, applications and the source of the plasmids.

Plasmid	Application	Description	Origin
pSTBlue-1	Sub cloning and sequencing	Plasmid with blue/white selection	Novagen
pET3a-HisT	Expression in *E. coli*	pET3a expression vektor with 6 - Histidine-Tag after	TMW
pBAD/Myc-His (B)	Expression in *E. coli*	Arabinose controlled expression in *E. coli* with a c-terminal *myc*-epitope and 6 - Histidine-Tag	Invitrogen
pGKV 259	Expression in Laktobazillen	constitutive expression in lactic acid bakteria	van den Vossen

pNZ8048	Expression in *Lactococcus lactis* NZ9000	Nisin controlled expression in NICE-hosts	van den Berg van Saparoea & Driessen
pNHLmrA	Expression in *Lactococcus lactis* NZ9000	Expression of LmrA with N-terminal Histidine-Tag	van den Berg van Saparoea & Driessen
pNZhorAH	Expression in *Lactococcus lactis* NZ9000	Plasmid with deleted *horA* as control	This work
pNHhorA	Expression in *Lactococcus lactis* NZ9000	Expression of HorA with N-terminal Histidine-Tag	This work

2.3 Preparation of membrane vesicles from *Lactococcus lactis* and *E. coli*

Membrane vesicles from *Lactococcus* are prepared as described elsewhere (Margolles *et al.* 1999; van den Berg van Saparoea *et al.* 2005) with the following modifications: cells were grown at 30 °C to an OD 660 of 0.6-0.8. LmrA/HorA expression was induced by addition of 0.5 ng/mL Nisin A 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. After a lysozyme treatment of at least 6 h at 30° C in the water bath with a concentration of Membrane vesicles were prepared 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, Teltow, Germany) followed by 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.

For *E. coli* the cells were grown at 37° C and approximately 180 rotations per minute to a OD 590 of 0.6-0.8 and expression was induced with the addition of arabinose/IPTG to an end concentration of 1 mM for pBAD-vector systems or pET3a-vector systems, respectively. After an incubation of further 90 min the protocol was the same as for *Lactococcus* except the lysozyme treatment, which was omitted.

2.4 Purification of His-Tagged membrane protein

Membrane lipids were solubilised as described (van den Berg van Saparoea *et al.* 2005) 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 the same composition as buffer A but containing 280 mM imidazole (all steps at 4 °C). The purified protein was used immediately for reconstitution in proteoliposomes or frozen in liquid nitrogen and stored at -80 °C.

2.5 Determining the protein concentration of purified protein and membrane vesicles

Protein-Assay (BioRad), which bases on the method of Bradford (Bradford 1976) was used for determining the protein concentration of the fractions of the membrane protein purification steps and the content of membrane protein in the membrane vesicles. Protein-Assay solution was diluted with $H_2O_{bidest.}$ in a ratio of 1:5. 990 µl were mixed with 10 µl sample and extinction at 595 nm against elution buffer B or water (containing glycerol and DDM in the same concentration as dissolved membrane vesicles) was detected. Membrane vesicles were preincubatred with 1 % (w/v) of DDM to solute the proteins. To exclude interferences of the detergent DDM, calibration curves were established in the presence of DDM in the same concentrations. The dye Coomassie brilliant blue binds to primarily basic and aromatic amino acid residues, especially arginine (Sedmak *et al.* 1977). Table 2.4 gives an overview of the amino acid composition of LmrA and some common used proteins for protein standards in science laboratories.

Table 2.4. List of the rates of amino acids in quantitiy and relative to the total number of amino acids in percentage. The Bradford factor was a fictive factor calculated as the sum of the percentage fraction of the basic amino acids Lys, His and Arg. The important fraction of arginine is marked with bold signs.

Amino acid	LmrA		BSA		BGG		Proteinase K		Lysoyzme		Trypsine	
Ala(A)	48	8,03%	48	7,91%	3	9,41%	48	12,50%	14	6,64%	15	6,64%
Arg(R)	23	**3,85**%	26	**4,28**%	0	**3,53**%	17	**4,43**%	17	**8,06**%	4	**8,06**%
Asn(N)	22	3,68%	14	2,31%	2	0,00%	21	5,47%	8	3,79%	18	3,79%
Asp(D)	25	4,18%	40	6,59%	2	2,35%	18	4,69%	11	5,21%	10	5,21%
Cys(C)	0	0,00%	35	5,77%	5	2,35%	5	1,30%	6	2,84%	12	2,84%
Gln Q)	27	4,52%	20	3,29%	6	5,88%	9	2,34%	8	3,79%	12	3,79%
Glu (E)	34	5,69%	59	9,72%	4	7,06%	14	3,65%	8	3,79%	5	3,79%
Gly(G)	49	8,19%	17	2,80%	1	4,71%	38	9,90%	28	13,27%	25	13,27%
His(H)	16	2,68%	17	2,80%	5	1,18%	6	1,56%	5	2,37%	4	2,37%
Ile(I)	52	8,70%	15	2,47%	18	5,88%	16	4,17%	16	7,58%	16	7,58%
Leu(L)	64	10,70%	65	10,71%	5	21,18%	26	6,77%	17	8,06%	16	8,06%

Lys(K)	29	4,85%	60	9,88%	1	5,88%	14	3,65%	14	6,64%	11	6,64%
Met(M)	27	4,52%	5	0,82%	1	1,18%	9	2,34%	6	2,84%	2	2,84%
Phe(F)	26	4,35%	30	4,94%	4	1,18%	9	2,34%	3	1,42%	5	1,42%
Pro(P)	17	2,84%	28	4,61%	3	4,71%	14	3,65%	4	1,90%	10	1,90%
Ser(S)	46	7,69%	32	5,27%	7	3,53%	44	11,46%	11	5,21%	27	5,21%
Thr(T)	32	5,35%	34	5,60%	0	8,24%	24	6,25%	10	4,74%	11	4,74%
Trp(W)	5	0,84%	3	0,49%	5	0,00%	2	0,52%	3	1,42%	4	1,42%
Tyr(Y)	13	2,17%	21	3,46%	5	5,88%	20	5,21%	9	4,27%	8	4,27%
Val(V)	43	7,19%	38	6,26%	85	5,88%	30	7,81%	13	6,16%	16	6,16%
Sum	598	100,00%	607	100,00%		100,00%	384	100,00%	211	100,00%	231	100,00%
BF	11,37		16,97		10,59		9,64		17,06		8,23	

The "Bradford factor" and the amount of arginine of Bovine gamma globulin (BGG) converges to the respective factor and amount of LmrA, so BGG was chosen for the usage as protein standard.

2.6 One dimensional SDS-PAGE

The SDS-poly acrylamid gel electrophoresis was performed according to Laemmli (Laemmli 1970). For running a gel with membrane protein fraction, the sample was preincubated with 1 % (m/vol) DDM to solute the membrane proteins. Gels were stained with Comassie® Brilliant Blue staining solution.

2.7 Western Blot

Western Blots were arranged as described elsewhere (Zapf *et al.* 2007) with some modifications. A semi-dry electroblot was performed at 0.8 mA/cm^2 for approximately1.5 h with a CAPS-methanol transfer buffer (Matsudaira 1987). Membrane was incubated in a ratio of 1:5000 with a primary anti His-Tag rabbit antibody, which was purchased from Biomol (Biomol GmbH, Hamburg, Germany). Secondary antibody (goat antirabbit-IgG-AP conjugate (Dako Deutschland GmbH, Hamburg, Germany) was used in a ratio of 1:5000 and incubated for 1 h, also. Staining was carried out using BCIP and NBT (Gerbu Biotechnik, München, Germany) in 0·1 m Tris-HCl, 0·1 m NaCl, 5 mm $MgCl_2$, pH 9·5 and stopped by addition of 10 mm EDTA.

2.8 Lipid extraction from *Lb. plantarum* TMW 1.460

The membrane lipids of *Lactobacillus plantarum* TMW 1.460 were extracted and purified from the cell mass as described in (Margolles *et al.* 1999) with some modifications. 10 L of modified MRS, pH 6.5 (per litre: 2 g meat extract, 10 g tryptic digest casein peptone, 4 g yeast extract, 1,8 g potassium dihydrogen phosphate, 5 g sodium acetate, 4 g diammonium

hydrogen citrate, 88 g glucose, 0.1 g magnesium sulfate, 0.05 g manganous sulphate, 0.5 µg each of cobalamin, folic acid, niacin, pantothenic acid, pyridoxal, and thiamin) were inoculated over night with 50 mL culture of *Lactobacillus plantarum* TMW 1.460 and incubated for three days at 30°C. The pH was adjusted every day to 6.0 with 5 M NaOH under non sterile conditions. The suspension was then centrifuged at 30°C and 2500 g for 90 min, washed twice with 50 mM potassium phosphate, pH 7.0, and centrifuged for 15 min and 4000 g at 30°C. After centrifugation, cells were frozen (-20°C) and thawed, resuspended in 20 mM potassium phosphate, pH 7.0, at a concentration of 1 g/mL (wet weight) and incubated at 37° C for 30 min with 4.2 mg/mL lysozyme and 0.01 mg/mL DNase. The following steps were carried out under N_2 atmosphere to avoid lipid oxidation. Chloroform and methanol were added at a volume ration of 1:2 and the suspension was stirred overnight at 4°C. Cell debris was removed by centrifugation at 2000 g for 10 min. The supernatant was added to chloroform and water (volume ratio 1:1:1) and the mixture was stirred for 3 h at RT. Phase separation was permitted over night at 4°C and the chloroform phase was evaporated in a rotary evaporator at 40°C and redissolved in chloroform to a concentration of 0.1 g/mL. One aliquot of the mixture was given to 10 aliquots of ice cold acetone containing 5 mM β-mercatoethanol, stirred over night at 4°C and centrifuged at 1000 g at RT. The supernatant, including neutral lipids and glycolipids, was dried in a rotary evaporator. The pellet, including the phospholipids, was dried under nitrogen flow. Both fractions were resuspended in diethyl ether containing 5 mM β-mercatoethanol to a concentration of 0.1 g/15 mL, stirred for 1 h and centrifuged at 1000 g at RT. Pellets were dried under a nitrogen flow and supernatants in a rotary evaporator

2.9 Thin layer chromatography (TLC)

A rapid thin layer chromatography method was used according to Vogels method to separate phospholipids and neutral lipids (Vogel *et al.* 1961). The described protocol was used with some modifications. As stationary phase silica gels plates (Kieselgel 60 WF) were purchased from Merck, Germany. The mobile phase was a mixture of hexan/ethyl acetate/acetic acid in a volume ratio of 59/10/1. Dried TLC-plates were sprayed uniformly with a solution of 2 % phosphomolybdic acid in ethanol and incubated for 5 min at 200° C in the drying oven. Organic substances reduced Mo(VI) to Mo(IV). resulting in spots.

2.10 Liposome preparation

Lipid and cholesterol stock solutions were prepared in chloroform. Laurdan was dissolved in chloroform at a concentration of 1 mmol/L, DMPC, DOPC, DMPC containing 10 mol%

cholesterol, the natural lipid extract and vesicles containing the 1:2:1 molar ratio of DOPC, DPPC and cholesterol were prepared together with the fluorophore Laurdan (if needed for measurements of membrane fluidity via the GP-values). After co-dissolving the respective lipids and fluorophore, the solvent chloroform was removed by a flow of nitrogen gas. Then the samples were dried under high vacuum pumping for several hours (usually over night) to completely remove the remaining solvent. The resulting dry film was then resuspended in 20 mM HEPES buffer, pH 7.2, vortexed and sonicated for 15 min in a bath-type sonicator (Bandelin SONOREX RK100SH). Large unilamellar vesicles (LUV) were produced by five freeze-thaw cycles (freezing in liquid nitrogen and slow-thawing in a warm water bath) followed by 9 passages through two stacked Nucleopore polycarbonate membranes of 200 nm pore size in a mini-extruder. The samples were kept at 65°C during the extrusion procedure. The final vesicle solution contained a 1:500 fluorophore to lipid mixture on a molecular basis for GP measurements, if needed.

For producing DNA loaded liposomes, the same protocol was followed with some modifications according to protocols published elsewhere (Lurquin *et al.* 2000; Pupo *et al.* 2005). DNA from calf thymus was swelled in 20 mM HEPES buffer, pH 7.2 and treated in bath tvpe sonicator for 60 min to get small fragments of the strands and to reduce the viscosity. This suspension with a concentration of 1 mg DNA per ml was used to swell the dry film of lipids from the vacuum drying step. Instead of 9 passages through the polycarbonate membranes only 5 passages were carried out, because the high viscosity causes bursting of the membrane very easily. Suspension with LUV was treated with DNAse for 30 min at 30° C to remove extraliposomal DNA in the solution.

2.11 Proteoliposome preparation

Proteoliposomes of LmrA with DMPC, DMPC+10 mol % cholesterol, natural lipid extract and raft mixture (DOPC:DPPC:Chol-1:2:1) were prepared by the detergent removal method (Rigaud *et al.* 1995). The required amount of the detergent DDM to destabilize different liposomes was determined by light scattering measurement. Liposomes were destabilized by the slow addition of the required amount of detergent DDM and kept stirring at room temperature for 10 min. For the reconstitution, the purified LmrA was mixed with DDM destabilized liposomes in 1:20 weight ratio and incubated for 30 min at room temperature under gentle stirring. The detergent was removed by 2 successive extractions with SM2 bio-beads. These polystyrene beads were thoroughly washed with methanol and water before using. A wet weight of 80 mg bio-beads/mL of liposomes was used to extract the detergent

for the first and 160 mg for the second time. First extraction was performed at room temperature for 2 h and the second at 4 ℃ for 16 h. Finally, proteoliposomes were collected by centrifugation (280000 g, 30 min, 20 ℃) and resuspended in 20 mM HEPES buffer pH 7.0 at a final lipid concentration of 4 mg/mL.

2.12 Pressure treatment

For all pressure treatments, three independent equipments were used and the temperature was under control of a tempering unit.

2.12.1 Pressure treatment while using the Perkin Elmer fluorescence spectrometer

For the pressure treatment while measuring flourecence intensity with the Perkin Elmer fluorescence spectrometer, high pressure equipment from Dunze GmbH (Hamburg, Germany) was used, which allowed to fit a sapphire window (SITEC Sieber Engineering AG, Zürich, Schweiz) in the sealing packet. The spectrometer was connected to the sapphire window with glass fibers. The pressure media was polyethylene glycol and the sample was protected by rubber plugs was directly connected to the sapphire window.

2.12.2 Pressure treatment while using the K2 flourometer

Pressure media was ethanol and the pressure was regulated with a hand operated spindle. The sample in a glass bottle was sealed with laboratory film and the bottle was stored in the pressure vessel behind sapphire windows, which is part of the K2 spectrometer.

2.12.3 Pressure treatment for determining the ATPase activity

Proteoliposomes or ATPase assay solution (with or without drugs, respectively) were inserted to a reaction tube and sealed with a silicone plug. The tubes were stored on ice till they were put in the reactor. The reactor of the high pressure equipment (Dunze GmbH, Hamburg) has had a capacity of 500ml and was under control of a tempering unit which kept the temperature stable at 30°C (+/- 0.1°C). Active fluid medium was water and pressure was increased with a pneumatic pressure pump and adjusted with a hand-operated spindle. Pressure was kept stable (±1MPa) and observed with a fitted mechanical barometer. After decompression the samples were put on ice immediately.

2.13 Fluorescence spectroscopy

The fluorescence spectroscopic measurements were performed using a Perkin Elmer LB50 luminescence fluorometer or a K2 multifrequency phase and modulation fluorometer with

photon counting mode equipment (ISS Inc., Champaign, Ill). The operational principle of the multifrequency fluorometer has been described in detail elsewhere (Spencer *et al.* 1970; Gratton *et al.* 1983)..The temperature was controlled to ±0.1°C by a circulating water bath.

2.13.1 Imidazole spectra

Imidazole spectra were taken from 300 to 500 nm with an excitation wave length of 280 nm. In all cases, the slit width was 2.5 nm.

2.13.2 Hoechst 33342 spectra and intensity

For intensity measurements an excitation wave length of 355 nm and a emission wave length of 457 was chosen as previously used (Margolles *et al.* 1999). Normally, the slit widths of 3 nm each were adjusted. To check the dependency of the fluorescence intensity of the dye and to avoid quenching effects, fluorescence was determined in dependency of the environment (lipid and aqueous phase), concentration and the pH

2.13.3 Ethidium bromide spectra and intensity

For the dye ethidium bromide for the excitation wave length was 500 nm and 580 nm for the emission with slit widths of 5 nm each.

2.13.4 Membrane fluidity

The phase state of the lipid membrane systems was determined by the Laurdan fluorescence technique. The emission spectrum of the environmentally sensitive fluorescence probe 6 dodecanoyl-2-dimethyl-aminonapthalene (Laurdan) was used to scrutinize the phase behavior of the lipid bilayer system. Laurdan is a naphthalene based amphiphilic molecule with fluorescence excitation and emission spectra, which are is very sensitive to the polarity and to the dipolar dynamics of the environment (Parasassi *et al.* 1986; Yu *et al.* 1996). The quantum yield of Laurdan is much higher in the membrane than in aqueous solution. When inserted into a membrane, Laurdan aligns its lauroyl tail with the lipid moiety and locates its naphthalene ring near the phospholipids glycerol backbone. Studies using phospholipid vesicles demonstrated that Laurdan is sensitive to the dynamics as well as the polarity of the surrounding membrane. Therefore, Laurdan can distinguish whether a membrane is in an ordered or fluid-like phase state (Parasassi *et al.* 1991). When Laurdan is embedded in a lipid membrane, it exhibits a 50 nm red shift of the emission spectrum as the membrane changes from the gel into the liquid-crystalline phase. The spectral changes of the emission spectrum

of Laurdan is generally quantified by the so-called generalized polarization function, which is defined as

$$GP = \frac{(IB - IR)}{(IB + IR)}$$

where IB and IR are the fluorescence intensities at 440 nm (characteristic for an ordered, gel phase state environment) and 490 nm (characteristic for a fluid, liquid-crystalline lipid state), respectively (Parasassi *et al.* 1990). Hence, GP values range from -1 to +1. In phase coexistence regions, the GP values exhibit values typical for fluid (liquid-disordered) and gel-type (liquid-ordered) domains. Hence the measured GP values of our system reflect the overall phase behavior and fluidity of the membrane as a function of temperature and pressure.

2.14 ATPase assay

The ATPase activity of the LmrA containing proteoliposomes was determined by measurement of free phosphate using the colorimetric assay of Lanzetta *et al.* (Lanzetta *et al.* 1979) with Triton-X 100 instead of Sterox as detergent. Proteoliposomes (1 mg/mL) were preincubated in 20 mM HEPES-KOH (pH 7.0) for 10 min on ice with/without drugs. The reaction was started with the addition of 1 mM Mg-ATP (stock solution containing 50 mM Na-ATP, 37.5 mM $MgCl_2$, 12.5 mM $MgSO_4$) and incubated at 30°C at the respective pressure. At regular time intervals, samples of 100 µL were transferred to a PE-cuvette containing 500 µL of malachite green molybdate reagent and mixed. After 5 min 250 µl 34 % citric acid was added and the mixture was incubated in the dark at 30 °C for 30 min and compared with a phosphorus standard at 650 nm. All experiments were performed in the presence of 0.1 mM orthovanadate in parallel and subtracted from the readings.

For measurements of the reversibility of the inhibition of the ATPase activity, samples were pressure treated without Mg-ATP, incubated for 10 min on ice immediately after decompression, and the ATPase activity was determined at ambient pressure and 30 °C afterwards.

3 Results

3.1 *horA* screening

In contrast to Ulmer (Ulmer *et al.* 2002), *horA* was not detected in *Lactobacillus plantarum* TMW 1.460, but *lmrA* was verified using the specific screening primer lmrA_screen_for, horA_screen_for and ScreenGen_rev. The primer lmrA_screen_for is specific to *lmrA* and horA_screen_for is specific to *horA* while the reverse primer ScreenGen_rev is located in the conserved region of the ABC in the Walker motif B and binds well in both genes. This results in approximately 550 bp amplification for a *lmrA* and an approximately 1000 bp amplification for a *horA* gene. The screening for a functional *horA*-gene in *Lactobacillus brevis* was further performed with 13 beer spoiling strains. Surprisingly, only half of the beer spoiling bacteria strains showed a specific PCR amplification. The strain TMW 1.6 showed a PCR-product with the expected size but also unspecific products and screening was not followed further on. The *horA* gene from TMW 1.465 was cloned into pBAD vector and sequenced. A deletion after bp 99 was detected, which leads to a stop codon after 36 amino acids and renders the protein nonfunctional. For optionally ongoing cloning steps, the other *horA* genes were subcloned into pST1-Blue vector and sequenced to differ functional genes from nonfunctional ones. Table 3.1 gives an overview to the existence and functionality of *horA* in the tested and sequenced strains and figure 3.1 an idea about the relationship to each other.

Table 3.1. Existence and functionality of the gene horA in beer spoiling bacteria strains of *Lactobacillus brevis*. Given is the number of the strain. A "-" indicates the lack of *horA*, "u" means the existence of a non-functional *horA* and "f" the existence of a functional gene. "nc" indicates, that a gene was detected using the specific primers but the sequence was not further checked.

Strain	1.1282	1.1283	1.1284	1.1326	1.310	1.311	1.313
horA	f	-	-	-	-	-	f
Strain	1.317	1.465	1.483	1.485	1.599	1.6	
horA	f	u	f	u	-	nc	

Figure 3.1. Relationship of the gene horA in beer spoiling bacteria strains of *Lactobacillus brevis* to each other. The sequence from the original published *horA* was taken from the paper from Sami (Sami *et al.* 1997) The cladogram was calculated using the sequence analysis internet tool "ClustalW2" from European Bioinformatics Institute.

An amino acid sequence alignment of the functional HorA is given in the appendix. The HorA from the strain TMW 1.1282 differs only in one amino acid from the published HorA from Sami (Sami *et al.* 1997). After 384 amino acids serine changed to a phenylalanine in the region of the Walker motif A. The other HorAs vary to this difference in up to five amino acid changes, three of them in the TMS one (TMW 1.317, 1.483 and 1.1313 changed a leucine with phenylalanine, amino acid 38), two (in TMW 1.317, 1.483 and 1.313 serine displaces phenylalanine after 90 amino acids) and five (1.1317, 1.483 and 1.1313 changed after 271 amino acids glutamic acid with glycine). Agin in the same strains, after 366 amino acids, aspartic acid replaces glycine in the cytoplasmatic domain of the transport protein. TMW 1.1317 completes its sequence with tryptophan instead of glycine like the other sequenced strains. *Lb. brevis* TMW 1.485 shows a deletion after 688 bp leading to an amino acid sequence break off after 223 amino acids in the loop between TMS four and five and renders the protein nonfunctional. For the following cloning steps HorA from TMW 1.1282 was chosen, the sequence of which was most similar to the one published by Sami (Sami *et al.* 1997).

3.2 Construction of an expression system for membrane MDR-transport proteins

For the expression of membrane the transport proteins LmrA and HorA, both genes were cloned into the *E. coli* expression vector pBAD*Myc*HisB. From previous works in the institute an additional construct of with *horA* with a C-terminal hexa-histidine Tag in pET3a existed. The three constructs were induced with different concentrations of arabinose or IPTG, respectively, and were incubated for varying times to determine the best expression conditions. To prevent forming of inclusion bodies, expression was tested also in the presence of 400 mM sorbitol. No suitable circumstances for a satisfying expression of the membrane

protein were detected (data not shown). Protein concentrations were either too low or toxicity led to a stop of growth of the culture. To check, whether the construct was able to express LmrA or HorA at all, expression was verified with a His-tag affinity western blot. One of the western blots is shown in Figure 3.2 where crude extracts of induced and non-induced *E. coli* BL21 cells or *E. coli* TOP TEN cells harboring pET3a-HisTag *horA*, pBAD*lmrA* or pBAD*horA*, respectively, were used.

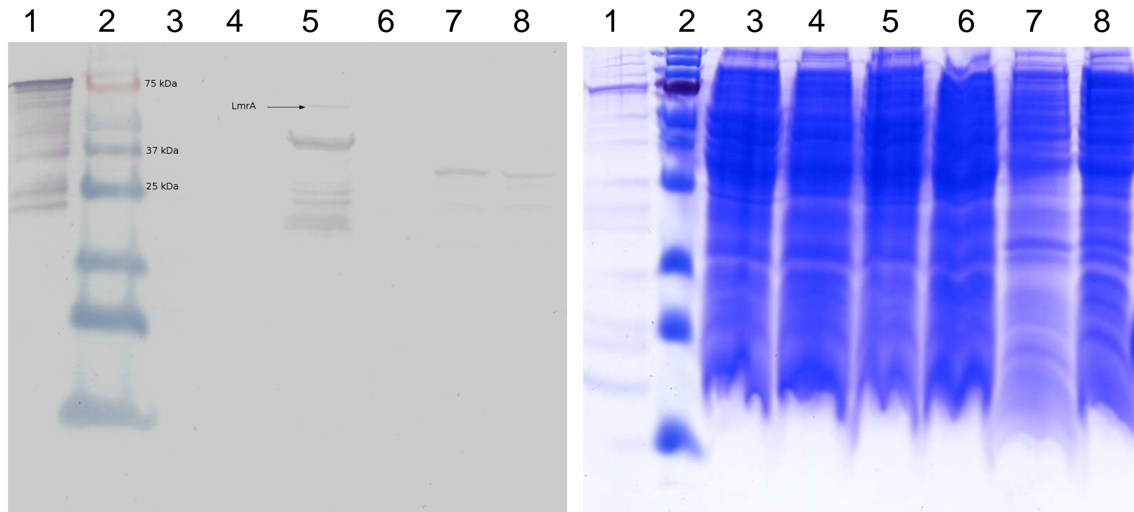


Figure 3.2. Western blot and SDS-page of crude extracts of cells of *E .coli* BL21 harbouring the expression vector pET3a-HisTag with *horA*, pBAD*lmrA* or pBAD*horA*. On the left hand side the western blot is shown and on the right hand side the associated SDS-page is given. In lane (1) a his-tagged protein (levansucrase with a molecular weight of approximately 93 kDa) is used as a control for the antibodies, respectively. Lane (2) is loaded with a prestained molecular marker in each case. In lane (3) and (4) crude extract from induced and non induced *E. coli* TOP TEN with pBAD*horA* is coated, respectively. *E. coli* TOP TEN with pBAD*lmrA* non induced is coated in lane (5) and non-induced in lane (6). Crude extract from IPTG induced cells of *E. coli* BL21 with pET3a-HisTag*horA* is loaded to lane (7) and to lane (8) crude extract from non-induced cells is coated. Expression was started with 1 mM arabinose or IPTG, respectively, if indicated and incubated for further 90 min.

The pET3a vector system shows basal expression even under non-induced conditions. The presence of glucose, which should inhibit the expression, has had no influence to this effect (data not shown). The detected band of the expressed protein was in the range of approximately 30 kDa, which is about half of the size expected for HorA. Constructs of pBAD and lmrA with the arabinose controlled promoter showed no basal activity like the pET3a vector. The western blot shows for induced cells a fine band at the expected size of approximately 60 kDa. But like in the case of HorA expression with pET3a strong bands with a smaller size appeared. Here a strong band of about 50 kDa and in addition bands in the

region of approximately 20 to 25 kDa appeared. Constructs with *horA* and pBAD produced no His-Tag antibody detectable band at all. Cloning experiments were redone using a Sal I restriction site at the c-terminal of the both genes resulting in a loss of the *myc*-epitope in the pBAD constructs. The elimination of the *myc*-epitope had no positive effects to the expression / detection results.

Although *Lactococcus lactis* MG1363 showed good transformability with pGKV259, a construct of pGKV259 using the blunt end restriction site Pme I could not be established. Satisfying amounts of LmrA and HorA were expressed using the pNZ8048 vector system for *Lc. lactis* NZ9000 (figures 3.3 & 3.4). This system is under control of the nisin induced promoter *nisA*. The vector pNH*lmrA* with an N-terminal His-Tag already existed (Margolles *et al.* 1999; van den Berg van Saparoea *et al.* 2005). The vector pNH282, a derivate of pNZ8048 containing the *horA* gene from *Lb. brevis* TMW 1.1282 with a N-terminal hexa-histidine tag was constructed via ligation of the PCR product using the primers horAHisTPscI_for and horAHindIII_rev. Though *horA* contains a Nco I binding site in the middle of the sequence and the pNZ8048 vector requires a Nco I cut ligation directly after the promoter and the following RBS, for the PCR amplification the 5' primer had a Psc I binding site. Nco I and Psc I have compatible overlapping sticky ends, which allow a uncomplicated ligation in the same frame without further modifications.

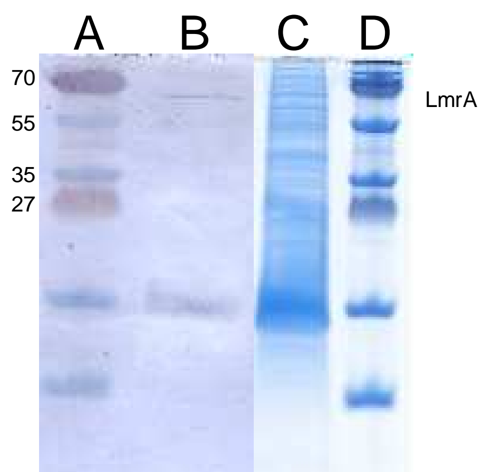


Figure 3.3. Western blot and SDS-page of membrane protein fraction of HorA expressing cells of *Lc. lactis* NZ9000 *ΔlmrA*. Blot and SDS-pager were performed as explained in materials and methods part. Lane (A) and (D) were loaded with a prestained protein standard. Lane (B) and (C) were loaded with solubilized membrane proteins from membrane vesicles. Lanes (A) and (B) show the reaction of the western blot and lanes (C) and (D) show the comassie blue stained SDS-page. The upper band of the blot shows the expected size for LmrA, the band strong band in the low mass region I a false positive reaction resulting from a huge protein accumulation, which can be seen in the SDS-page (lane C).

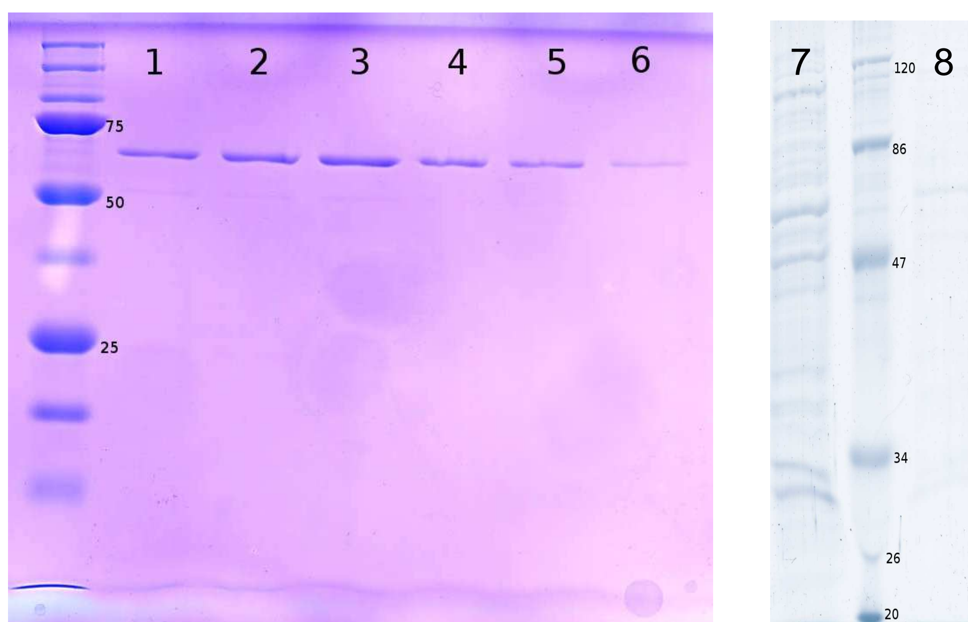


Figure 3.4. SDS-page of the fractions of the purification of LmrA/HorA from membrane vesicles from induced *Lc. lactis* NZ9000 ΔlmrA cells harbouring pNH*lmrA*.or pNH282, respectively. Next to the protein standard in the left frame, six numbered LmrA purification fractions (as described in materials and methods part) are shown. The right frame shows two lanes of HorA purification. Lane 7 gives a total membrane protein fraction of induced *Lc. lactis* NZ9000 ΔlmrA pNH282 cells (membrane vesicles). Lane 8 gives the band of the purified HorA.

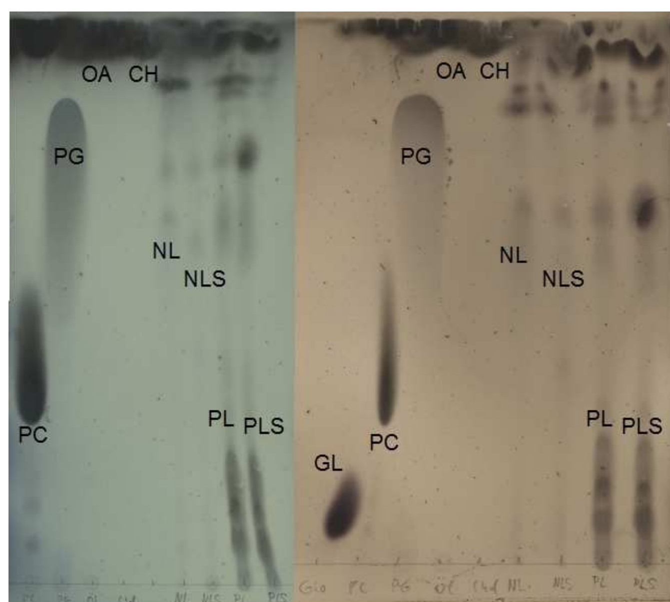
3.3 Lipid extraction from *Lb. plantarum* TMW 1.460

The biomass from a ten liter culture of *Lactobacillus plantarum* TMW 1.460 was approximately 150 g wet weight. A mass of 313 mg total lipids could be extracted. To precipitate the phospholipids from the neutral fraction, acetone insoluble material was removed (Roux *et al.* 1974; Gebhardt 1983; Ruiz Budria *et al.* 1989) and the purity of all lipid fractions was determined using tlc giving chromatograms from the neutral/glycolipid fraction and contaminated fractions of phospholipids (Figure 3.4). The neutral lipid fraction was chosen for the experiments, because tlc did not reveal any impurities within these, and the influence from other substances and contaminations, such as remaining hydrophobic proteins, could thus be excluded. Table 1 gives an overview of the ratio of lipid fractions and in Figure 3.5 the purity of the fractions is shown with tlc.

Table 3.1. Overview of the natural lipid fractions extracted from *Lactobacillus plantarum* TMW 1.460.

Fraction	Mass	Percentage	Source	Substance
total lipid	313 mg	100 %	chloroform/methanol/water extraction 1:2:1	lipid mixture
phosphorous fraction s	84 mg	approx. 27 %	acetone precipitation from total lipid, soluble in ether	phosphoglycerolipid + phosphatidylcholine and non lipids
phosphorous fraction n	23 mg	approx. 7 %	acetone precipitation from total lipid, insoluble in ether	phosphatidylcholine + non lipids, bigger part of phosphoglycerolipid
neutral fraction s	206 mg	approx. 66 %	acetone soluble fraction, soluble in ether	neutral lipids

s: soluble, n: insoluble.



Abbreviations in the picture are related to the applied probe.

Standards:
GL glucose
PC phosphatidylcholine
PG phosphoglcerolipid
OA hexane acid
CH cholesterol
Sample:
NL neutral lipid fraction
NLS neutral lipid fraction, non soluble in ether
PL phospholipid fraction
PLS phospholipid fraction, non soluble in ether

Figure 3.5 Thin layer chromatography of the natural lipid fractions explained in table 3.1. Tlc was performed as explained in materials and methods part. As indicated in the legend different class of lipids act as standards. On the right hand side, the tlc was redone with glucose representing a non lipid organic substance with polar abilities. Neutral fractions are predominately free of loaded contingents and show only weak spots at the level of phosphoglycerolipids. The phospholipid fractions contains a large amount of phosphoglycerolipids and as expected phosphatidylcholine. But there are also several spot in the level of glucose what might indicate non-lipid contaminations like degenerated polar lipid parts (choline) or hangover of membrane proteins.

3.4 Reconstitution of His-tagged LmrA/HorA

To test the self association of LmrA and HorA to a functional homodimer, the transport protein in liposomes consisting of different lipids were reconstituted. During the reconstitution, the concentration of imidazole, remaining from the LmrA purification, was

monitored via fluorescence spectroscopy as seen in figure 3.6. After the reconstitution, imidazole could not be detected any more. Figure 1 shows the reduction of flourecence intensity of imidazole of a BioBeads® treated solution. LUVs could be produced from DOPC, DMPC, DMPC + 10 % mol cholesterol and the model raft mixture. Natural lipids emerged as too compact for extrusion through a filter (filter was blocked and bursted while extrusion), hence multi lamellar vesicles (MLV) (procedure was stopped after the freeze-thaw steps) were used for reconstitution.

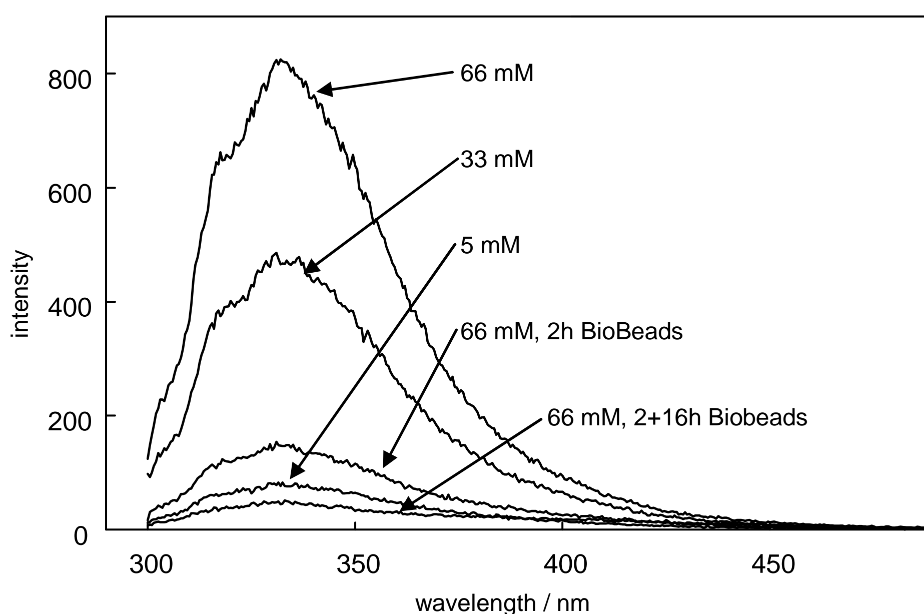


Figure 3.6 Decrease of imidazole fluorescence intensity during adsorbent treatment. Fluorescence intensity of DDM containing elution buffer B with imidazole was determined as explained in Materials and Methods. The different imidazole concentration is denoted with arrows. The time of adsorbent treatment also, where applicable ("2 h BioBeads" stands for the first and "2+16 h BioBeads" for both extraction steps as described in Materials and Methods).

3.5 Impact of reconstitution of vesicles on membrane fluidity

The influence of the reconstitution steps of proteoliposomes regarding the membrane fluidity was investigated using natural lipids and different amounts of LmrA. Liposomes and proteoliposomes containing LmrA with a ratio of 1:20, 1:50 and 1:100 were prepared and the GP values were determined. Figure 3.7 shows GP values of different vesicles depending on pressure and temperature.

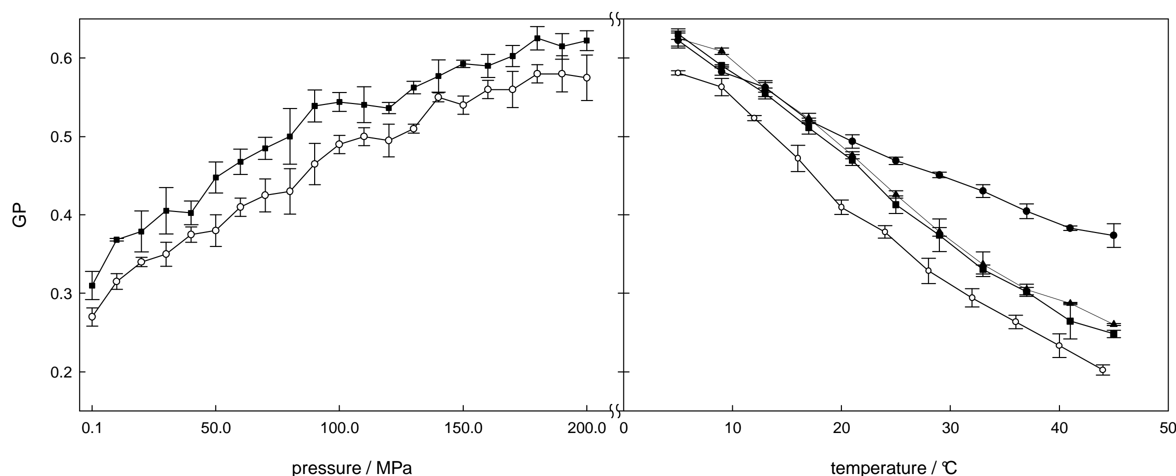


Figure 3.7 GP values of liposomes with and without LmrA in dependence of pressure (left window) or temperature (right window). GP values were determined as described in materials and methods section. Open circles (—○—) symbolizes liposomes from natural glycolipids from *Lb. plantarum* TMW 1.460 which was cultivated at 30° C. Filled triangles (—▲—) symbolizes proteoliposomes with a protein lipid ratio of 1:20 (w/w) and filled squares (—■—) give the values from proteoliposomes with a protein lipid ratio of 1:50 (w/w). Proteoliposomes with a ratio of 1:100 (w/w) are pictured with filled circles(—●—).Error bars illustrate the standard deviation of three measurements.

Table 3.3 Exact pressure dependant GP-values belonging to figure 3.7. Natural glycolipid extract from *Lb. plantarum* TMW 1.460 was used for vesicle preparationand were used for reconstitution of LmrA in a protein lipid ratio of 1:50, if required. GP measurements were arranged in dependence of pressure. Standard deviation results from the measurements each.

Pressure in MPa	Proteoliposomes 1:50		Liposomes	
	GP	Standard deviation	GP	Standard deviation
0,1	0,31	0,02	0,27	0,01
10	0,37	0,00	0,32	0,01
20	0,38	0,03	0,34	0,01
30	0,41	0,03	0,35	0,02
40	0,40	0,02	0,38	0,01
50	0,45	0,02	0,38	0,02
60	0,47	0,02	0,41	0,01
70	0,48	0,01	0,43	0,02
80	0,50	0,04	0,43	0,03
90	0,54	0,02	0,47	0,03
100	0,54	0,01	0,49	0,01
110	0,54	0,02	0,50	0,01
120	0,54	0,01	0,50	0,02
130	0,56	0,01	0,51	0,01
140	0,58	0,02	0,55	0,01
150	0,59	0,00	0,54	0,01
160	0,59	0,01	0,56	0,01
170	0,60	0,01	0,56	0,02
180	0,63	0,01	0,58	0,01
190	0,61	0,02	0,58	0,02
200	0,62	0,01	0,58	0,03

Table 3.4 Exact temperature dependant GP-values belonging to figure 3.7. Natural glycolipid extract from *Lb. plantarum* TMW 1.460 was used for vesicle preparation. Vesicles were used for reconstitution of LmrA in the ratio given in the table, if required. GP measurements were arranged in dependence of temperature. Standard deviation results from the measurements each.

Temperature in :°C	Liposomes		Proteoliposomes protein : lipid 1 : 20		Proteoliposomes protein : lipid 1 : 50		Proteoliposomes protein : lipid 1 : 100	
	GP value	Standard devitation	GP value	Standard devitation	GP value	Standard devitation	GP value	Standard devitation
5	0,58	0,00	0,63	0,01	0,62	0,01	0,62	0,01
9	0,56	0,01	0,59	0,00	0,61	0,00	0,59	0,01
13	0,52	0,00	0,56	0,01	0,56	0,01	0,56	0,00
17	0,47	0,02	0,51	0,01	0,52	0,01	0,53	0,02
21	0,41	0,01	0,47	0,01	0,48	0,00	0,48	0,00
25	0,38	0,01	0,41	0,01	0,43	0,00	0,46	0,01
29	0,33	0,02	0,37	0,02	0,38	0,01	0,43	0,00
33	0,29	0,01	0,33	0,01	0,34	0,02	0,43	0,01
37	0,26	0,01	0,30	0,01	0,30	0,01	0,42	0,00
41	0,23	0,02	0,26	0,02	0,29	0,00	0,42	0,01
45	0,20	0,01	0,25	0,00	0,26	0,00	0,43	0,02

No sharp phase transitions were observed, rather a continuous decrease of GP with increasing temperature is seen and vice versa in case of an increasing pressure. Reconstitution of LmrA leads to a steadily higher GP value, meaning an increase of the acyl chain order. The effect is strongest at temperatures higher than about 25ºC in the case of a protein lipid ratio of 1:100 (w/w) and minor pronounced for a ratio of 1:20 and 1:50. No difference can be observed for these two different ratios. Upon pressurization of the non-reconstituted natural membrane system, the overall conformational order of the membrane increases continuously in the whole pressure range covered from 0.1 to 200 MPa and reconstitution of LmrA leads to an overall increase in the conformational order of the acyl chains throughout the whole pressure range. The effect depends on the reconstituted protein as the simple destabilization of liposomes with DDM and the following removal of the detergent with BioBeads had no impact on the GP over the observed pressure range (data not shown).

3.6 Fluorescence measurement of transport activity

For all measurements with testing character, e.g for establishing a method to measure the activity of the protein under HHP influence, proteoliposomes with LmrA were used.

3.6.1 Transport of Hoechst 33342

As higher fluorescence activity in lipid environments is described and was used already to determine transport activity of membrane transport systems (Margolles *et al.* 1999; van Veen *et al.* 1999; van den Berg van Saparoea *et al.* 2005) the possibility of measurements of membrane transport under HHP was determined. To avoid quenching effects, the fluorescence activity of Hoechst 33324 was investigated in dependency of the concentration in a lipid environment and aqueous solution and the intensity in dependence to the pH were part if interest. The aqueous solution was modelled with 100 mM HEPES-KOH buffer, pH 7.0. A lipid phase was modelled with rapeseed oil, saturated with 100 mM HEPES-KOH buffer, pH 7.0. The relative fluorescence activity of Hoechst 33342 is shown in figure 3.8.

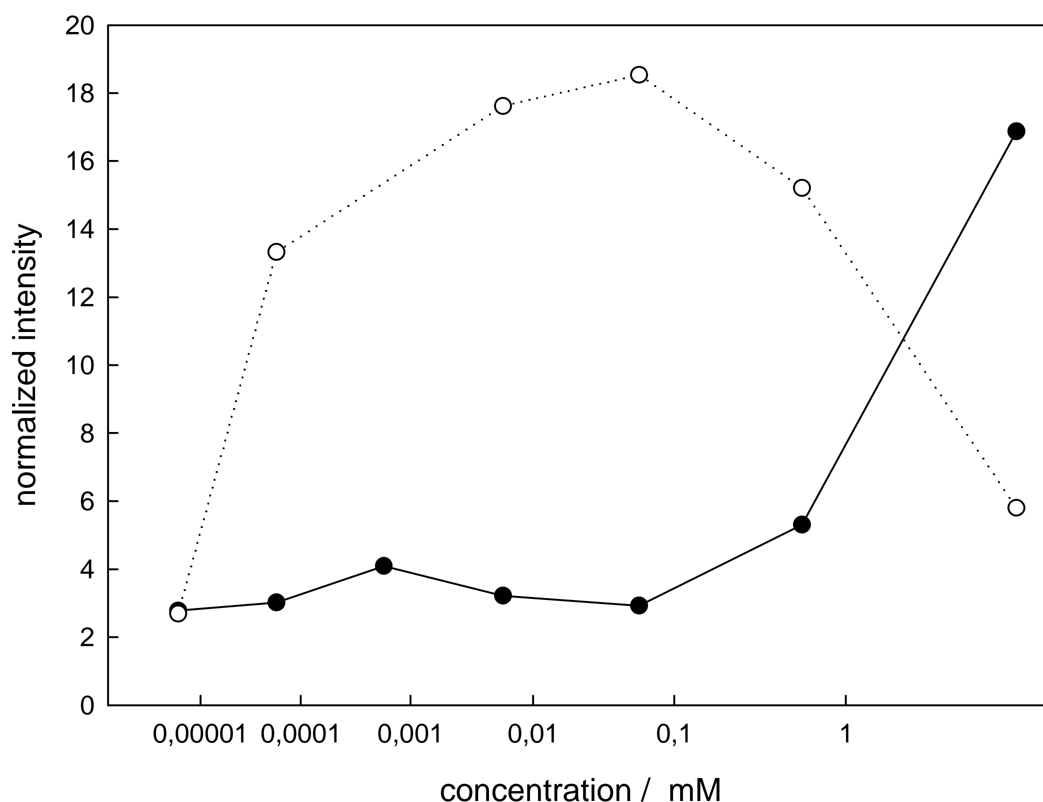


Figure 3.8 Increase of the fluorescence activity of Hoechst 33342 in dependence of the concentration. Aqueous phase was modelled with 100 mM HEPES-KOH, pH 7.0 and is schown with filled circles (—●—). Lipis phase was modelled with rapsseed oil saturated with 100 mM HEPES-KOH, pH 7.0. Lipis phase is shown with (··○··). Relative fluorescence activity was calculated with subtracting the basal intensity of the respective phase without the addition of the fluorophore Hoechst 33342.

As the active transport of Hoechst 33342 from the inner leaflet of the membrane to the outside should be determined, a concentration with the following possibilities should be used: no quenching effects should appear and the intensity in the membrane (lipid environment) must be measurable differently compared to the aqueous phase. Quenching effects appear in lipid environments from concentrations higher than 0.1 mM. For further measurements concentrations of 1,6 µM were not exceeded.

The dye offered in the presence of liposomes, 1 mg/ml strong dependency to the pH resulting in different intensities as shown in figure 3.9.

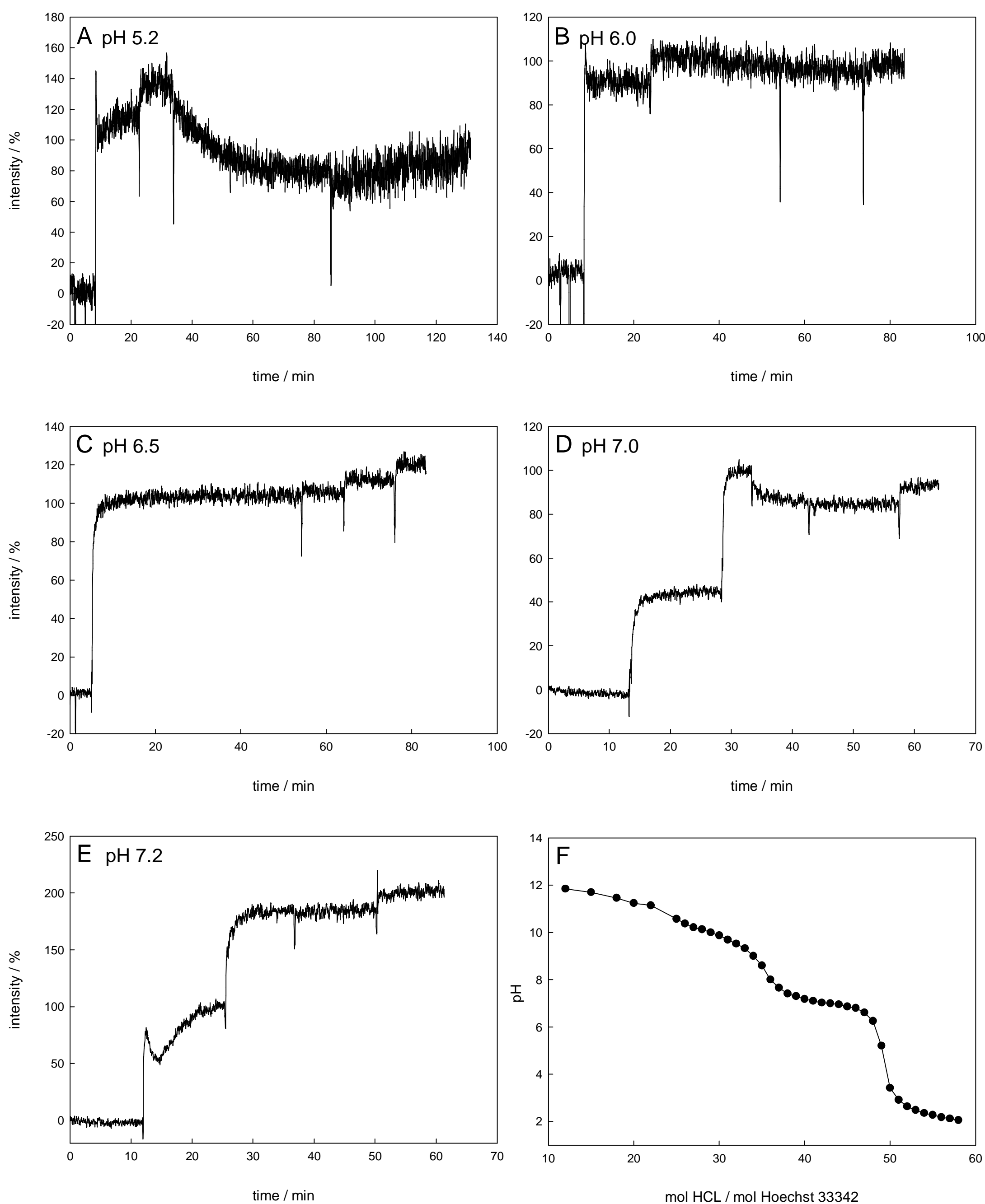


Figure 3.9 Relative fluorescence intensity of the dye Hoechst 33342 in the presence of liposomes (1 mg/ml) and in dependency to the pH of the environment. Intensity was measured under the conditions described in the material and methods part. Intensity was calculated with setting the basal activity as 0 % and the intensity after the addition of Hoechst 3342 was set to 100 %. End concentration of Hoechst 33342 and MG-ATP was 1 µM and 2 mM, respectively. In Frame (A) the intensity is followed at pH 5.2. After 8.3 min the dye was added, Mg-ATP followed after 21.6 min. KOH donation (resulting in a increase of the pH about 0.2) was carried out after 33.3 min. Valinomycin and nigericin (1 mM each) adjusted the pH after 66.6 min. For the further pH steps pH 6.0 in frame (B), pH 6.5in frame (C), pH 7.0 in frame (D) and pH 7.2 in frame (E) the order of the donation of substances was the same but were carried out at different time steps. Sole exception was at pH 7.0 when Hoechst 33342 donation was splitted into two steps. Time point of each donation is indicated in the graphs by strong intensity changes or sharp peaks in the graph resulting from the pipette dip in the cuvette. Frame (F) shows a titration curve of Hoechst 33342 against hydrochloric acid.

Addition of Mg-ATP at pH 5.2 after 21.6 min resulted in a jump up of the intensity. Increasing the outer pH slightly (ΔpH 0.2) led to a slow decrease of fluorescence intensity. At the higher pH of 6.0 an increase of the extra liposomal pH had no influence to the intensity. The intensity at an initial pH of 6.5 (figure 3.9 C) behaved differently, when every addition of any tested substance to the cuvette lead to a slight increase of the intensity. A decrease of intensity can be followed, when ATP is given to proteoliposomes at pH 7.0 (figure 3.9 D). Interestingly, at a slight higher pH of 7.2 the effect is drastically reversed with an increase of intensity about more then 50 % (figure 3.9 E). Experiments were redone in HEPES buffer system at pH 7.0 and Mg-ATP was exchanged with an ion solution (PO4- and K+) to simulate the same ion loading potential. No change in intensity appeared. Repetition of the experiment with ATP and without liposomes (100 mM HEPESbuffer, pH 7.0, dye, ATP, KOH and drugs) showed a similar graph as in the case of the measurements with liposomes and at pH 7.2 with an increase of intensity after addition of ATP (data not shown). This indicates that changes in the fluorescence intensity are caused by the Mg-ATP and its derivates and not by its charge. To check, if in case of a pH dependent fluorescence activity plays a role, Hoechst 33342 was titrated against hydrochloric acid to find out existing pK_a (figure 3.9 F).

3.6.2 Transport of ethidium bromide

To check the functionality of reconstituted membrane transporter proteins and the effect of HHP, the transporter LmrA was reconstituted in DNA loaded liposomes consisting of DMPC and DNA of calf thymus. Protein lipid ratio was 1:20 (w/w).

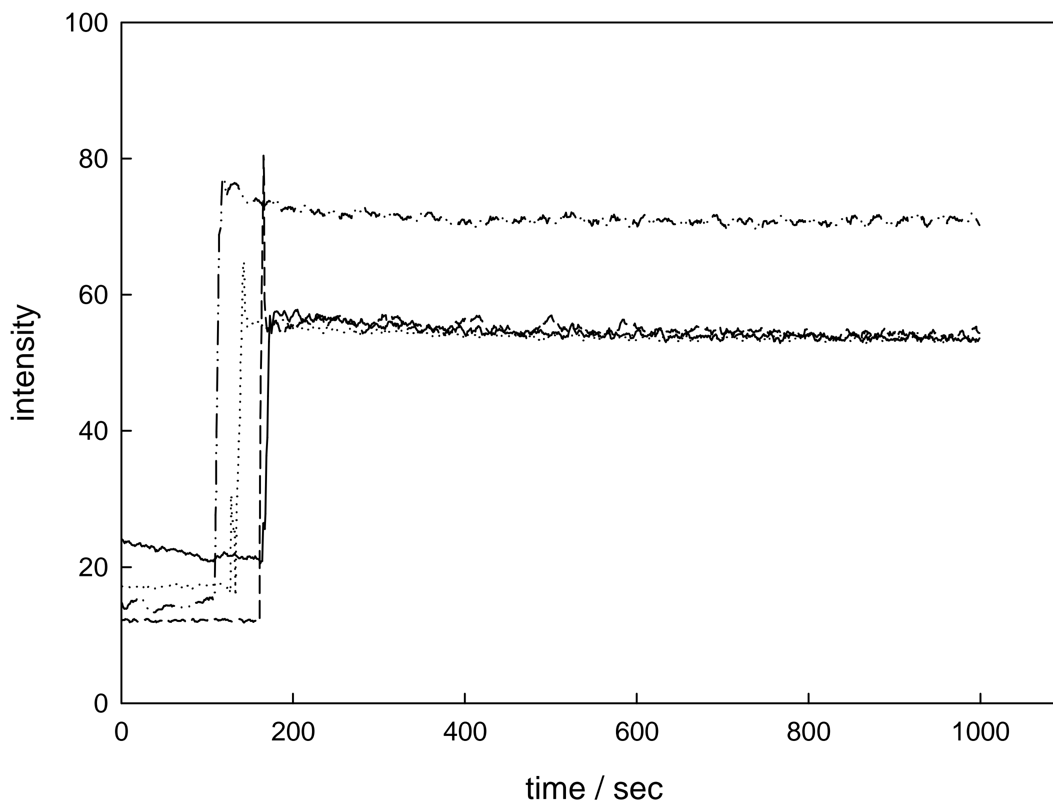


Figure 3.10 Increase of the fluorescence intensity of ethidium bromide in presence of proteoliposomes. Settings for the measurements are described in material and methods part. Proteoliposomes from DMPC and LmrA in a ratio of 20:1 (w/w) was used in a concentration of 1 mg/ml. Intensity increases immense after the donation ethidium bromide to a concentration of o.2 µM. When necessary, Mg-ATP had the concentration of 2 mM. Dashed line with points (— · · — ·) gives the intensity of proteoliposomes with Mg-ATP treated for 20 min at 30° C and 0.1 MPa. Same sample without Mg-ATP is pictured with a dashed line (– – – – – ·). The dotted line (..........) symbolizes a pressure treatment at 50 MPa and full line (————) stands for a pressure treatment at 20 MPa, measurements in the presence of Mg-ATP, both.

Both HHP treated samples showed slightly increased basal intensity. HHP treated samples show the same resulting intensity as non-energized proteoliposomes stored at ambient pressure and thereby no transport of ethidium bromide inside into the vesicle. The only active transport of Ethidium to the encapsulated DNA was observed for proteoliposomes treated at ambient pressure and measured in the presence of Mg-ATP.

3.7 ATPase activity of MDR transorters of the ABC family under HHP

All measurements of the ATPase activity were performed at least 3 times with proteoliposomes from independent reconstitutions (LmrA overexpressed in autonomous batches, also) in the presence of ethidium bromide as transport substrate and with added orthovanadate in parallel to get the ethidium-assisted, orthovanadate-sensitive ATPase activity of LmrA or HorA, respectively. Protein lipid ratio was 1:20 (w/w), always. ATPase activities in all vesicles were measured at ambient pressure and in steps up to 200 MPa (except for DMPC). Figure 3.11 visualizes the inactivation of the vanadate sensitive ATPase activity in DMPC vesicles with LmrA in the presence or absence of ethidium bromide. HHP inactivation makes no difference between basal ATPase activity or transport coupled activity. Both are inhibited to the same extent.

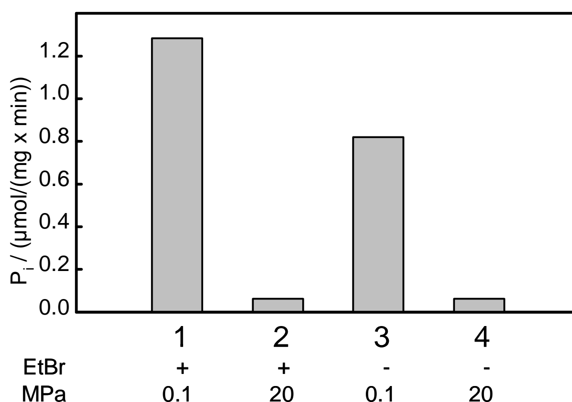


Figure 3.11 Dependency of ATPase activity on pressure. ATPase activity from proteoliposomes with DMPC and LmrA at 30° C. is shown in bar (1) for proteoliposomes at ambient pressure in the presence of ethidium bromide (EtBr), (2) with ethidium bromide at 20 MPa, (3) proteoliposomes at ambient pressure lacking ethidium bromide and (4) at 20 MPa and without ethidium bromide.

3.7.1 LmrA

As shown in fig. 3.12, nearly total inactivation was observed in DMPC vesicles when the pressure was increased to 20 MPa and higher at 80 MPa (fig. 3.12 E). Inactivation was tested for reversibility of the inhibition. For pressures of 40 MPa and 80 MPa, the remaining activity was approximately 12 % (0.12±0.07 µmol/(mg min) and 0.12±0.04 µmol/(mg min), respectively), only. Conversely, in pure DOPC vesicles (fig. 3.12 A) LmrA retained - at least in part - its activity upon increasing pressure. For this lipid and the remaining lipid systems, a continuous decrease of the activity was expected. Rather, the values follow a more or less

non-linear pressure dependence with local maxima of activity at 80 MPa (120 MPa for DMPC with cholesterol) and 200 MPa. In the case of DOPC, the activity drops to about 40% at approx. 40 and 120 MPa, but increases again at higher pressure, essentially reaching the initial ambient pressure-activity around 200 MPa. For proteoliposomes from natural lipids (fig. 3.12 B), the LmrA activity decreases markedly at low pressures (up to 40 MPa), and stays essentially constant at a level lower than 50% of its initial activity up to 200 MPa (fig. 2B, table 3.5). A different behavior is observed for the more-component proteoliposomes DMPC/ 10 mol % cholesterol and the model raft mixture (Figs. 3.12 C & D, and table 3.5). Their activity follows a non-monotonous behavior with an initial decrease up to 40 MPa and a marked increase of the activity again at and above 80 MPa, even exceeding the ambient pressure data by 100 % at 200 MPa.

The LmrA ATPase activity in the vesicles without cholesterol was found to be largely inhibited by high hydrostatic pressure, but partially recovers from 80 MPa upwards in the case of DOPC. No reversibility is observed and hence no full activity can be restored after high pressure treatment, as observed for the natural lipids at 160 MPa (-0.004±0.01 µmol/(mg min)) and DMPC (see above). Also, the high activity of LmrA in the DMPC/cholesterol vesicles at high pressures above 80 MPa (Table 2) was not retained after decompression of the system. An activity of only 0.34±0.24 µmol/(mg min) (41±29 %) and 0.41±0.19 µmol/(mg min) (49±23%) was recovered after pressurization to 160 and 200 MPa, respectively.

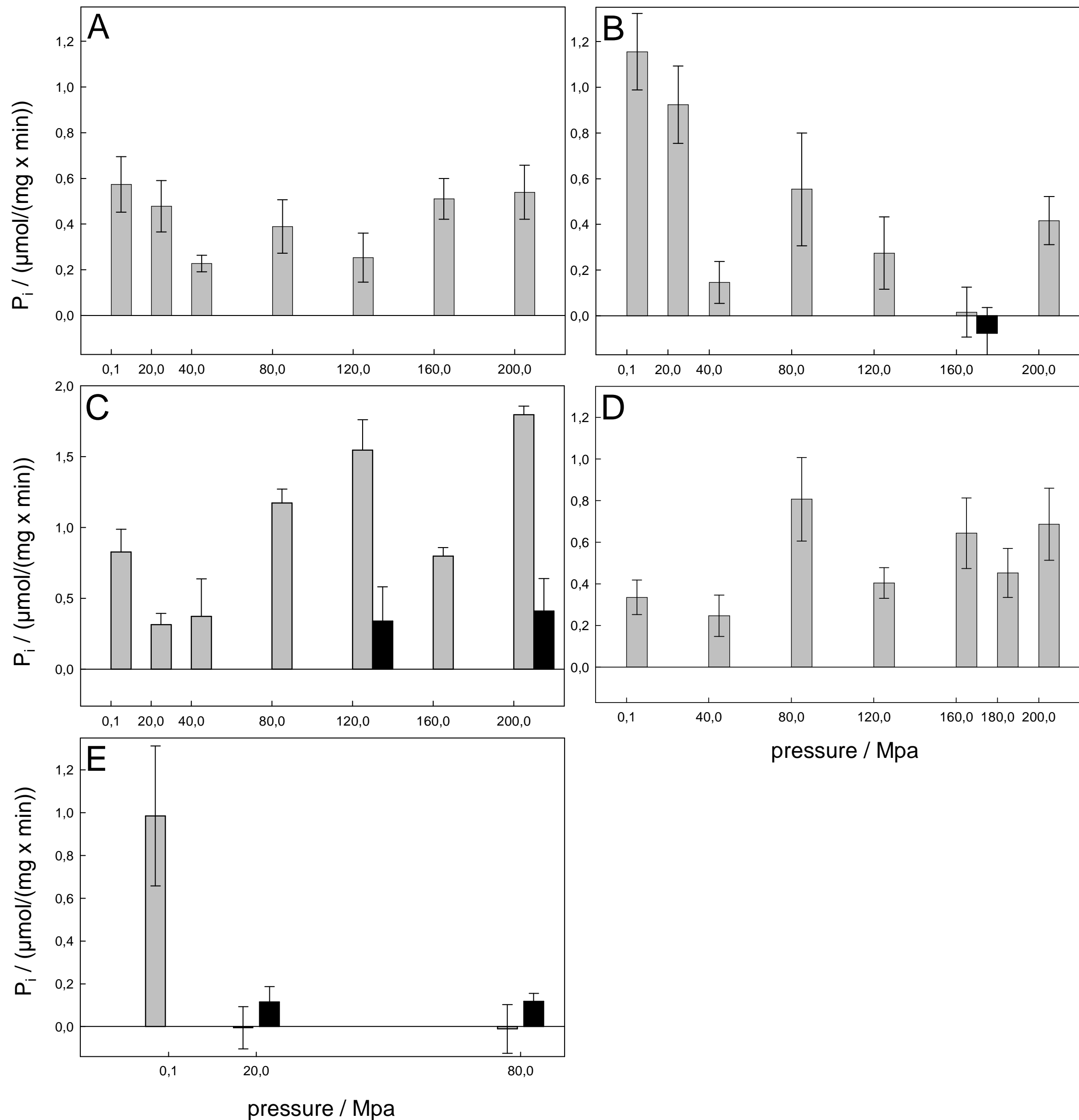


Figure 3.12 Pressure dependence of the ethidium-assisted ATPase activity of LmrA. The activity is measured by the release of phosphate (P_i) from ATP and given in micromole per minute and milligram of LmrA under the conditions explained in Materials and Methods. Grey bars represent the activity under high hydrostatic pressure; black bars symbolize the activity at ambient pressure after the high pressure treatment. Error bars illustrate the standard deviation for the particular experiments (the number of experiments, n, is given in table 3.5). A: proteoliposomes made from DOPC, B: natural lipids from Lb. plantarum, C: DMPC with 10 mol% of cholesterol, D model raft mixture, E: pure DMPC as membrane lipid.

Table 3.5: Ethidium-assisted, orthovanadate-sensitive ATPase activity of proteoliposomes at 30 °C in dependency of lipid environment and pressure. The activity is determined by calculating the release of phosphate from ATP in micromole per minute and milligram of LmrA under the conditions explained in materials and methods part. Percentage values of the activity are related to the ATPase activity under ambient pressure conditions. Standard deviations are given and the number of experiments, *n*, is given in brackets.

Lipid Vesicle	Activity at 0.1 MPa		Activity at 20 MPa		Activity at 40 MPa		Activity at 80 MPa		Activity at 120 MPa		Activity at 160 MPa		Activity at 180 MPa		Activity at 200 MPa	
	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%
DOPC	0,57 ±0,12 (3)	100 ±21	0,48 ±0,11 (3)	83 ±20	0,23 ±0,04 (3)	40 ±6	0,39 ±0,12 (4)	68 ±20	0,25 ±0,11 (3)	44 ±19	0,51 ±0,09 (3)	89 ±16			0,54 ±0,12 (3)	94 ±21
DMPC	0,99 ±0,33 (3)	100 ±33	-0,01 ±0,10 (3)	-1 ±10			-0,01 ±0,11 (3)	-1 ±12								
Natural lipids	1,16 ±0,17 (3)	100 ±14	0,92 ±0,17 (3)	80 ±15	0,15 ±0,09 (3)	13 ±8	0,55 ±0,25 (3)	48 ±21	0,27 ±0,16 (3)	24 14	0,02 ±0,11 (7)	1 ±9			0,42 ±0,11 (4)	36 ±9
Model raft mixture	0,34 ±0,08 (3)	100 ±25			0,25 ±0,10 (3)	74 ±30	0,81 ±0,20 (3)	241 ±60	0,40 ±0,07 (3)	121 ±22	0,64 ±0,17 (3)	192 ±51	0,45 ±0,12 (3)	135 ±35	0,69 ±0,17 (3)	205 ±52
DMPC+10 % Cholesterol	0,83 ±0,16 (3)	100 ±19	0,31 ±0,08 (3)	38 ±10	0,37 ±0,26 (3)	45 ±32	1,17 ±0,10 (3)	142 ±12	1,55 ±0,21 (3)	187 ±26	0,80 ±0,06 (3)	97 ±7			1,79 ±0,06 (3)	217 ±7

3.7.2 HorA

In the following graphs, black bars represent the activity of HorA in the respective lipid environment. For a better possibility of comparison with LmrA, the activity of LmrA at the respective pressure is given with the grey bars. Figure 3.13 shows the activity of HorA in a pure DMPC lipid environment. Like in the case of LmrA, nearly total inactivation occurred at a treatment of 80 MPa. Notably, the HorA activity seems to be less sensitive to the pressure in this lipid system as LmrA as the activity drops down at 20 MPa to a value of the activity of LmrA at ambient pressure.

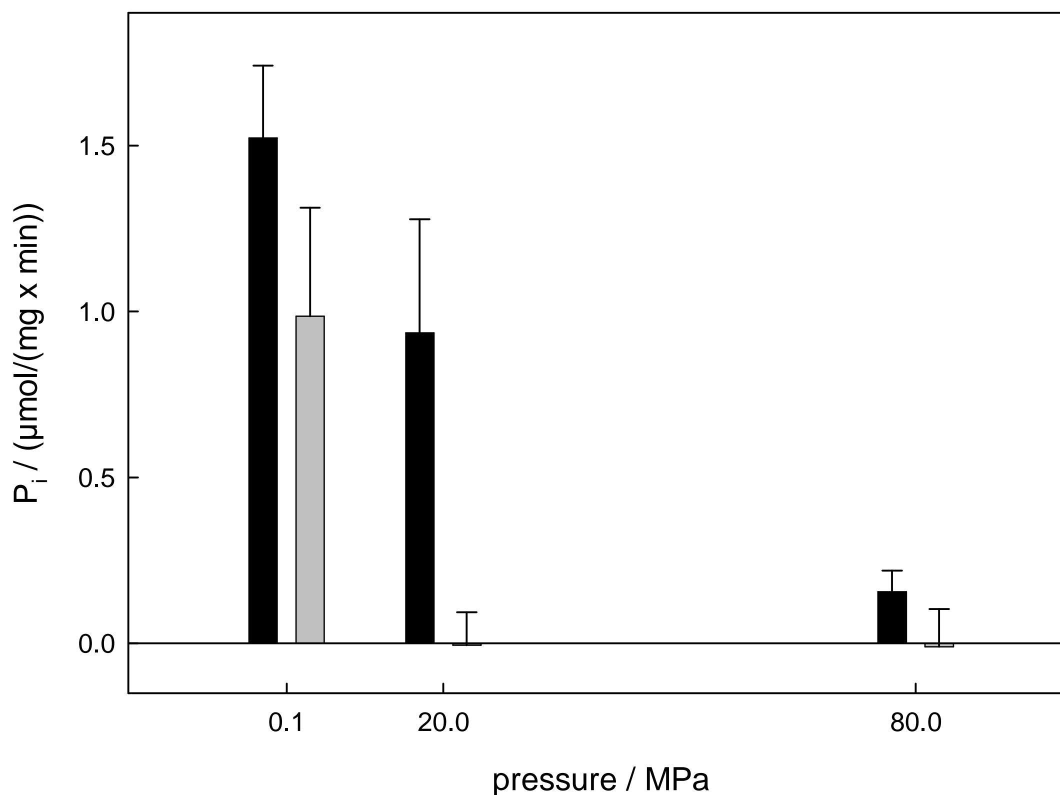


Figure 3.13 Pressure dependence of the ethidium-assisted ATPase activity of HorA in LUV of DMPC. The activity is measured by the release of phosphate (P_i) from ATP and given in micromole per minute and milligram of LmrA under the conditions explained in materials and methods. Black bars represent the activity under high hydrostatic pressure (grey bars symbolize the activity of LmrA as already shown under 3.7.1). Error bars illustrate the standard deviation of three particular measurements.

Conversely, in pure DOPC vesicles (fig. 3.14) HorA retained its activity upon increasing pressure and even several fold outreaches the initial value. The activity rose to about 240 % and 520 % at approx. 40 and 80 MPa, but decreases again at higher pressure, reaching nearly the initial ambient pressure-activity around 120 MPa and further increases again up to 400 – 500 % in the remaining pressure range observed. For this lipid and regarding the observations with LmrA, a stable activity to a less or greater extent was expected. The values follow more or less non-linear pressure dependence with local maxima of activity at 80 MPa. Throughout the observed pressure range, the activity stays at higher levels than at ambient pressure. At 0.1 MPa and at 20 MPa, activity of LmrA and HorA correlates to each other, although HorA slightly increases and LmrA slightly decreases. But both are in the range of approximately

0.5 µmol/(mg x min) (see tables 3.5 & 3.6). At increasing pressures they act completely different and activities diverge.

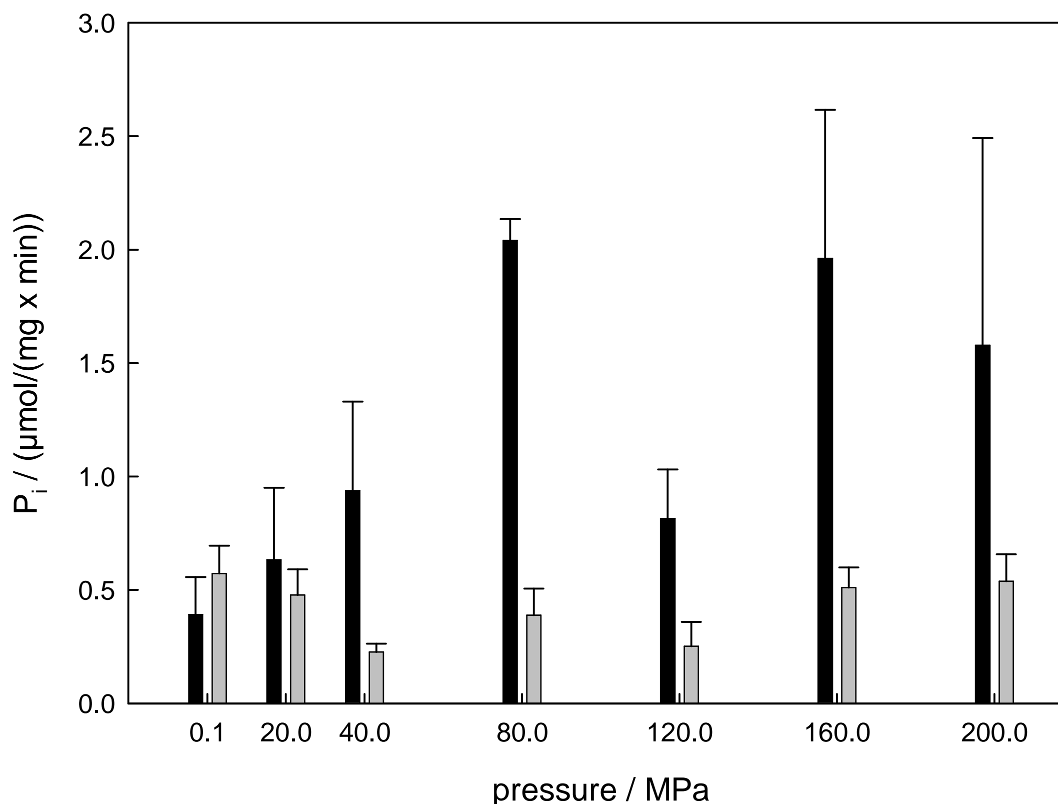


Figure 3.14 Pressure dependence of the ethidium-assisted ATPase activity of HorA in LUV of DOPC. Activity is measured by the release of phosphate (P_i) from ATP and given in micromole per minute and milligram of LmrA. Black bars represent the activity under high hydrostatic pressure (grey bars symbolize the activity of LmrA as already shown under chapter 3.7.1). Error bars illustrate the standard deviation of three particular measurements.

Similar effects as in systems with LmrA were expected with HorA reconstituted in the same liposomes. But the convergence was only partially. For cholesterol containing systems, differences were only minor or not observable as supposed. For DOPC and natural lipids, the activities at ambient pressures were similar, but with increasing pressure, the values from HorA overtop the initial activities, always. Figure 3.15 gives the activity of proteoliposomes of natural lipids.

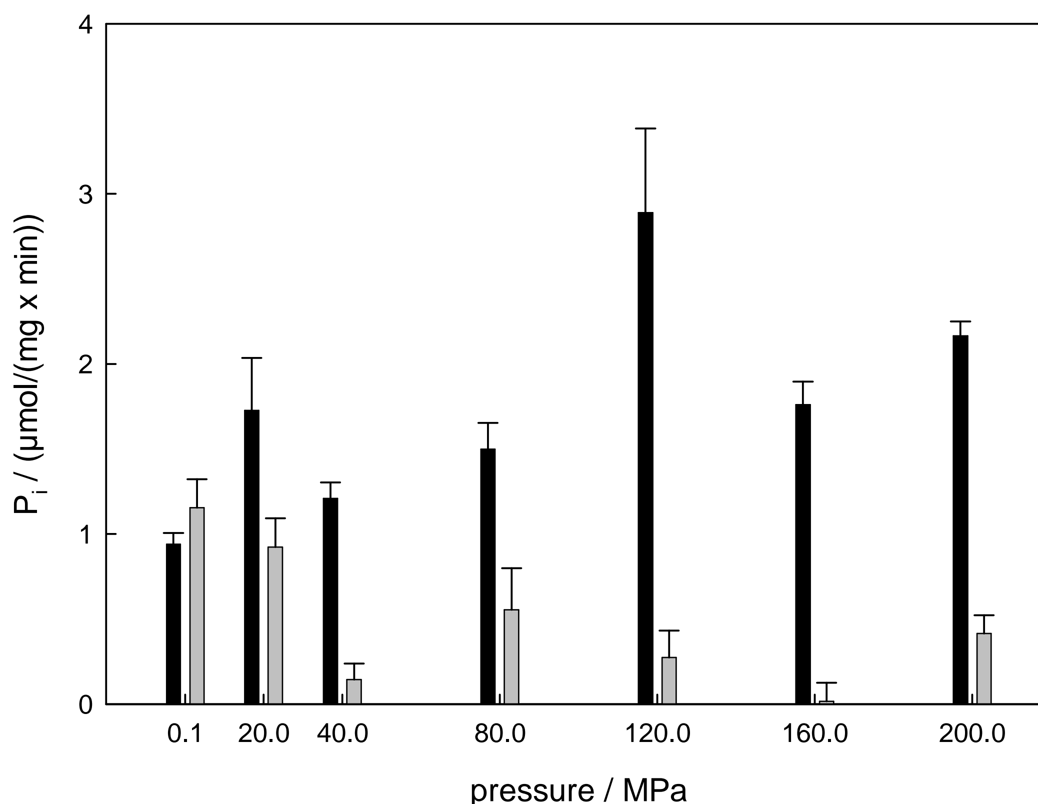


Figure 3.15 Pressure dependence of the ethidium-assisted ATPase activity of HorA in LUV of natural lipid fractions from *Lb. plantarum* TMW1.460. Activity is measured by the release of phosphate (P_i) from ATP and given in micromole per minute and milligram of LmrA. Black bars represent the activity under high hydrostatic pressure (grey bars symbolize the activity of LmrA as already shown under chapter 3.7.1). Error bars illustrate the standard deviation of three particular measurements.

HorA ATPase activiy stays essentially constant at a level higher than its initial activity up to 200 MPa. Activity tends to result in higher values with increasing pressure with the exeption of two local maxima at 20 and 120 MPa. Totally convergent to LmrA is the change of the HorA activity over pressure in the model raft mixture lipid system. Activity follows a non-monotonous behavior with a decrease at 40 MPa and a marked increase of the activity again at and above 80 MPa, even exceeding the ambient pressure data by 120 % at 200 MPa. Similar to the other more compenent (more then two) lipid system, the acticity tends to higher values over the pressure range, except at 40 MPa. But with an immense maximum at 20 MPa with nearly three fold activity as compared to ambient pressure. LmrA was not tested for this pressure step.

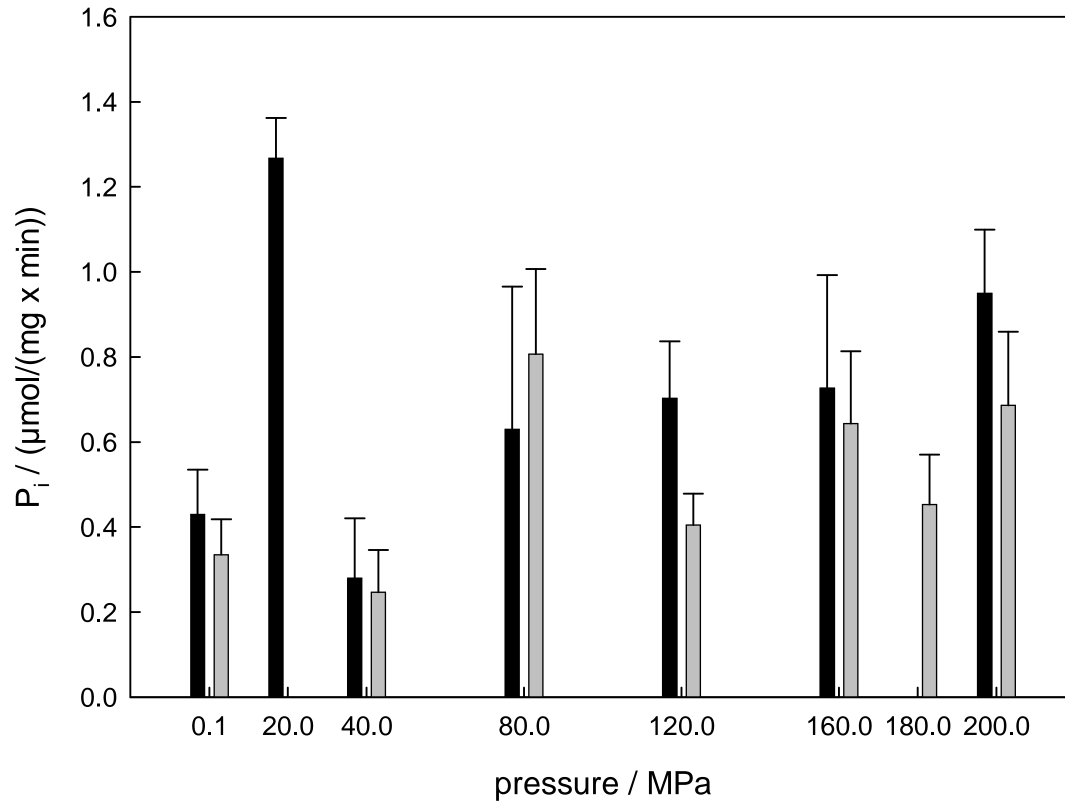


Figure 3.16 Pressure dependence of the ethidium-assisted ATPase activity of HorA in LUV of model raft mixture. Activity is measured by the release of phosphate (P_i) from ATP and given in micromole per minute and milligram of LmrA. Black bars represent the activity under high hydrostatic pressure (grey bars symbolize the activity of LmrA as already shown under chapter 3.7.1). Error bars illustrate the standard deviation of three particular measurements.

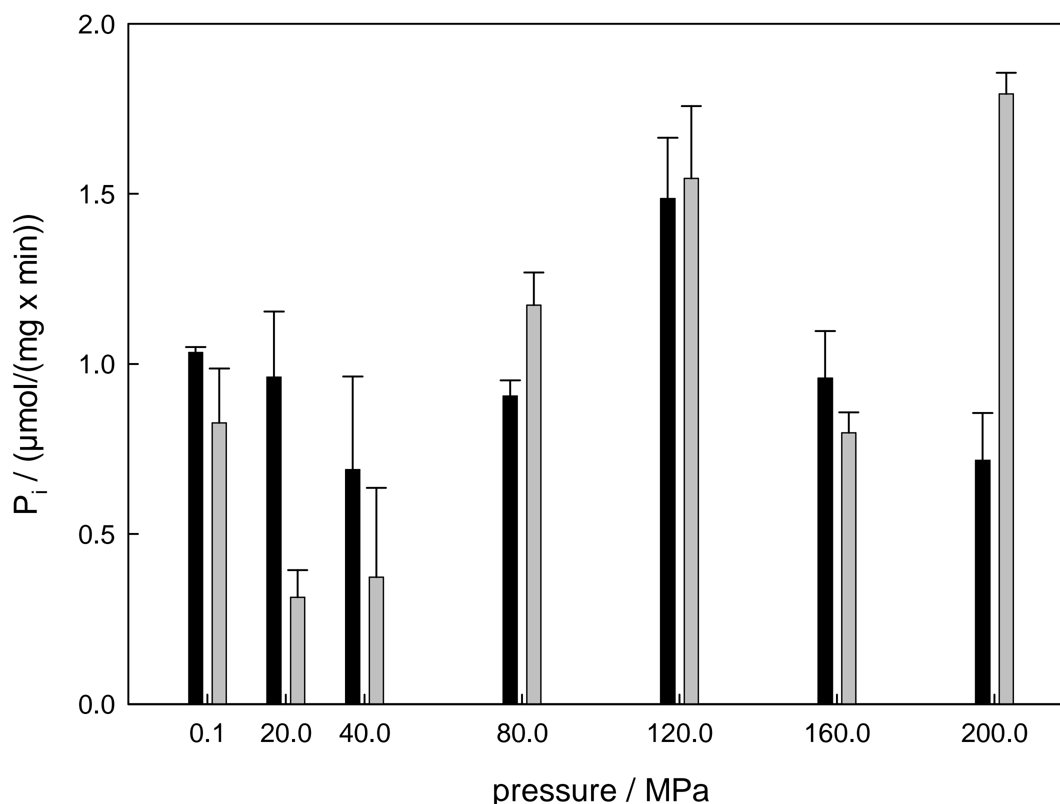


Figure 3.17 Pressure dependence of the ethidium-assisted ATPase activity of HorA in LUV of DMPC and 10 mol% cholesterol. Activity is measured by the release of phosphate (P_i) from ATP and given in micromole per minute and milligram of LmrA. Black bars represent the activity under high hydrostatic pressure (grey bars symbolize the activity of LmrA as already shown under chapter 3.7.1). Error bars illustrate the standard deviation of three particular measurements.

The activity in the second cholesterol containing system DMPC with 10 mol% cholesterol follows predominantly the levels which were previously shown from LmrA with a huge local maximum at 120 MPa. Levels in both systems are similar except, that HorA shows no reduced activity at 20 MPa and stayed nearly unimpaired at this pressure and no huge increase at 200 MPa is observed with only 69 % of the initial value.

The HorA ATPase activity in the vesicles containing only saturated acyl chains as in the case of DMPC was found to be largely inhibited by high hydrostatic pressure. Even at 20 MPa, where activity under pressure was nearly to the initial activity, it drops down after decompression to 0.28±0.04 µmol/(mg min) (18±2%). Notably the inactivation at 80 MPa under pressure was reversibly 0.74±0.1 µmol/(mg min) (48±7%).

Table 3.6: Ethidium-assisted, orthovanadate-sensitive ATPase activity of HorA containing proteoliposomes at 30 °C in dependency of lipid environment and pressure. The activity is determined by calculating the release of phosphate from ATP in micromole per minute and milligram of LmrA under the conditions explained in materials and methods part. Percentage values of the activity are related to the ATPase activity under ambient pressure conditions. Standard deviations are given and the number of experiments, *n*, is given in brackets.

Lipid Vesicle	Activity at 0.1 MPa		Activity at 20 MPa		Activity at 40 MPa		Activity at 80 MPa		Activity at 120 MPa		Activity at 160 MPa		Activity at 200 MPa	
	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%	µmol/ (mg min)	%
DOPC	0,39 ±0,17 (3)	100 ±42	0,63 ±0,32 (3)	162 ±83	0,94 ±0,39 (3)	240 ±100	2,04 ±0,09 (3)	522 ±24	0,82 ±0,21 (3)	208 ±55	1,96 ±0,65 (3)	501 ±167	1,58 ±0,91 (3)	404 ±233
DMPC	1,52 ±0,22 (3)	100 ±14	0,94 ±0,34 (3)	61 ±23			0,16 ±0,06 (3)	10 ±4						
Natural lipids	,94 ±0,06 (3)	100 ±7	1,73 ±0,31 (3)	183 ±33	1,21 ±0,09 (3)	128 ±10	1,5 ±0,15 (3)	159 ±16	2,89 ±0,5 (3)	307 ±53	1,76 ±0,14 (3)	187 ±14	2,16 ±0,09 (3)	230 ±9
Model raft mixture	0,43 ±0,11 (3)	100 ±24	1,27 ±0,09 (3)	295 ±22	0,28 ±0,14 (3)	65 ±33	0,63 ±0,34 (3)	147 ±78	0,70 ±0,13 (3)	164 ±31	0,73 ±0,27 (3)	169 ±62	0,95 ±0,15 (3)	221 ±35
DMPC+10 % Cholesterol	1,03 ±0,02 (3)	100 ±2	0,96 ±0,19 (3)	93 ±19	0,69 ±0,27 (3)	67 ±27	0,91 ±0,05 (3)	88 ±4	1,49 ±0,18 (3)	144 ±17	0,96 ±0,14 (3)	93 ±13	0,72 ±0,14 (3)	69 ±14

4 Discussion

At the beginning of this study, we set out whether the protein or the lipid environment influences the transport functions in the first glance. Actually we substantiated that the lipid environment influences the transport protein LmrA and its activity in an active and direct way transmitted by the structural attributes of the membrane. But the influence differs considerably when the transport system is replaced with a related homodimeric transporter and thereby may play a minor role for other kinds of membrane transport systems and can not be adapted completely to all membrane proteins. Thus, the basic ability to dimerize to a functional dimer is given by the protein structure, while the membrane structure can modulate this within the given frame.

4.1 Screening for *horA*

Although, HorA is described to play a crucial role in the hops resistance mechanisms of *Lactobacillus* and other species in beer spoiling processes, (Sami *et al.* 1997; Sakamoto *et al.* 2001; Suzuki *et al.* 2002; Suzuki *et al.* 2005; Haakensen *et al.* 2008) we were not able to detect *horA* in all of the beer spoiling bacteria tested. In contrast to previous works, *Lb. plantarum* TMW 1.460 did not show *horA* with the used method. Even as the sequence of *horA* from TMW 1.460 is not published, a discrepancy in the sequence to the originally published *horA* from *Lb. brevis,* leading to an insecurity of the *horA* screening primer, can not be excluded. Retrospectively it could not debarred, whether the formerly used strain TMW 1.460 has been displaced from a contamination or may have lost a plasmid encoded horA gene upon unavoidable sub culturing steps. Looking on all tested beer spoiling *Lb. brevis* strains, only 50 % (six out of twelve) carried a *horA* gene. It was more surprising, that only 2/3 of the found genes are in a functional state (four of six). *Lb. brevis* TMW 1.465 shows a stop codon in after 36 amino acids (deletion after 99 bp) and *Lb. brevis* TMW 1.485 shows a deletion after 688 bp leading to a amino acid sequence break off after 223 amino acids and renders the protein nonfunctional. Notably, the both deletions are both in the TMD, but are at completely different segments of the gene. While the deletion in TMW 1.465 took place in the sequence of the first TMS, the protein translation of the HorA protein from *Lb. brevis* TMW 1.485 stops in the loop after the fourth TMS and before the fifth TMS. Although these two strains lack HorA transport, they represent strong beer spoiling ability (Patrick Preissler, TMW, personal communication, unpublished). This supports the observation of Behr, that HorA transport is not obligatory for the hop resistance of bacteria (Behr *et al.* 2006). For the cloning steps and the measurements depending on the membrane

phase, HorA from TMW 1.1282 was chosen. Its sequence is close to the originally published one. Allthough it was not aim to check a special HorA from a certain strain, but a functional system with the ability of transporting ethidium bromide was needed.

4.2 Construction of an expression system for membrane proteins

From previous works, a clone existed carrying the pET3a-HisT vector with a *horA* gene. This construct lost the possibility of induction and expression every time after some over night cultures. It is already described, that usual E. coli based expression systems reach their limit with the espression of putative toxic multidrug resistance membrane proteins (Mokhonova *et al.* 2005). Western blot revealed that the expression system standing under control of the lac-promoter was not completely deactivated in the absence of lactose or IPTG, as it was used in our case. Hypothesized, that HorA is a putative toxic protein for *E. coli*, it seemed to lead to instability of the construct. Also new transformations of *E. coli* with the plasmid were not stable. Therefore, another expression system was used to clone *horA* and *lmrA*. The *E. coli* expression vector pBAD/Myc-His (B) stands under the control of the *araB* arabinose promoter as per description in the recommendations of the supplier (Invitrogen). Lacking arabinose and in the presence of glucose, this promoter is nearly totally repressed, as verified with the western blot. But the induction with arabinose led to the expression of only low amount of protein with the expected size, most of the his-tagged protein shows a band in the SDS-PAGE of about 40 kDa. It can not be excluded that the protein is completely denatured or partly runs faster because internal crosslinks (or the C-terminal hexa histidine tag) prevent complete unfolding in SDS and thus cause the more compact protein to run faster in SDS gels or that it binds more SDS (cause of its hydrophobic character) as it is described for other membrane proteins (Chen *et al.* 1996; Heinrich *et al.* 2000; Drew *et al.* 2006). Also, the gels exhibit several bands of different sizes. The amount of recovereable protein was also very low and could not be improved with different expression conditions like varying concentration of arabinose or different expresion times. To exclude, that the myc-epitope has a negative impact on folding the protein or on the purification via His-Tag trapping with Ni-affinity chromatography, the cloning steps were repeated by using primers with Sal I binding sites for the 3'-end of the sequence leading to a loss of the myc-epitope in the resulting construct. No improvement of the protein yield indicated absence of a negative influence of the epitope. In all our experiments, c-terminal His-tags were used so far in gram negative expression systems. Membrane proteins and especially MDR transporters are often toxic to the

expression host and lead to inclusion bodies when expressed in common *E. coli* expression systems (Mokhonova *et al.* 2005). The NICE system is described as excellent for the expression of prokaryotic and eukaryotic membrane proteins (Mierau *et al.* 2005) in gram positive bacteria. Under the control if the nisin promoter *nisA* two constructs with *lmrA* and *horA* were used for further experiments. Both proteins carried an N-terminal hexa histidine tag and could be purified in adequate amounts for the following experiments. Western blot analysis of the crude cell extracts and the membrane fractions showed a significant band at the expected protein size. Also, purification fractions delivered pure protein with the expected size. For the investigated MDR transporter, expression operates best in their "origin" host, a gram positive lactic acid bacteria and does work and stays functional with a hexa-histidine tag at the n-terminal of the protein.

4.3 Fluorescence measurements of transport substrates

To reach the aim of the study, it was decided to determine the activity of reconstituted membrane transporter systems und the direct influence of HHP. For that a system should have been established, that allows determination of either the direct transport of substrate or the intermediate consequence of the transport. Hoechst 33342 and ethidium bromide are well known transport substrates of both transport systems used in this study (Margolles *et al.* 1999; Ulmer *et al.* 2000; Sakamoto *et al.* 2001; Ulmer *et al.* 2002; Reuter *et al.* 2003; Balakrishnan *et al.* 2004; van den Berg van Saparoea *et al.* 2005; Venter *et al.* 2005). While Hoechst 33342 is hypothesized to have an increased fluorescence activity in a lipid environment, measurements of this dye and its intensity will directly correlate with the transport from the lipid environment (membrane) to the aqueous environment. On the other hand, ethidium is a well known dye for staining DNA. It is a cationic molecule that offers no direct changes in fluorescence intensity while diffusing through the membrane, but after binding to DNA after it has passed this barrier. Active transport of this dye to or away from DNA leads to changing fluorescence intensities. The measurement of these changes is thereby an intermediate consequence of the active transport.

4.3.1 Hoechst 33342

The dye Hoechst 33342 is a dye on the base of bis-benzimides with the ability to bind to DNA (Petit *et al.* 1993). Unlike the dye Hoechst 33258 is possesses an ethyl group that renders a more lipophilic character. The dye discloses a strong dependence to the pH of the environment. A titration against hydrochloric acid showed the existence of at least one strong pKa in the area of pH 7.0. According to other working groups, we reasoned the fluorescence intensity changes simply correspond to the pH changes, which complicates the interpretation of connection to transport (Aleman *et al.* 2005). Our results are evidence for the pH dependency of the fluorescence activity of the dye. Hoechst fluorescent dyes show affinity to adenosine rich regions (Comings 1975; Otto *et al.* 1985; Portugal *et al.* 1988; Petit *et al.* 1993). A further result of our measurements is that intensity changes can occur form the simple addition of ATP at an adequate pH (as in our case 7.2). Even such a simple system (compared with biological, whole bacteria containing systems) obtains many ions, which complicate the pH stability of a system under HHP because of potential equilibrium shifts. It is a simple thermodynamic effect, that the pressure influences the pH of even buffered systems. Although HEPES buffer is described as an excellent buffer for the stability under HHP, it shows a shift of ΔpH/100 MPa=-0.07 (Bernhardt *et al.* 1987). This little pH shift may have immense influence to the chemical nature and potential of molecular interactions and so interpretations of fluorescence intensity of Hoechst 33342 has to be taken with care (Aleman *et al.* 2005). As a conclusion, using this dye seemed not to be practicable for our project.

4.3.2 Ethidium bromide

Ethidium is a cationic aromatic molecule and finds application in staining DNA since more than 40 years (Waring 1965). After binding to DNA, the fluorescence intensity multiplies. Ethidium bromide is a transport substrate for HorA and LmrA (Margolles *et al.* 1999; Sakamoto *et al.* 2001; Ulmer *et al.* 2002; Balakrishnan *et al.* 2004). We have used proteoliposomes which were loaded with DNA (Lurquin *et al.* 2000; Pupo *et al.* 2005). The measurement of ethidium bromide in DNA loaded liposomes displays therefore a consequence of the active transport as a shift of the concentration of the dye in direction to the encapsulated DNA is necessary. In our measurements, the intensity with untreated proteoliposomes rise stronger in the presence of ATP than without ATP. Ethidium is transported directly to the interior of the vesicle. All HHP treated proteoliposomes exhibited the intensity level of untreated vesicles without ATP after the donation of ethidium bromide. The membrane transport is irreversibly inhibited. Notably, all high pressure treated vesicles

show a slightly increased basal intensity (before adding ethidium bromide). Maybe conformational changes of the proteoliposomes, residual DNA or DNAase outside the vesicles have altered, or membrane embedded DNA has been released due to the membrane phase transition of the DMPC vesicles during the pressurization. However, the slightly increased intensities did not affect the level after the donation of the fluorophore. The executed experiments show an active transport to the inner leaflet of the membrane and therefore to the encapsulated DNA. However, this just indicated, if the enzyme was active or not. Further experiments with the aim of reducing the transport process to measure the rise of the intensity over the time, as it would be necessary to determine a comparable turnover, lead to no better results. For this, the amount of ATP or ethidium donation was altered (data not shown). When ATP and the transport substrate were not the limiting factor for this method, we hypothesized, that the amount of encapsulated DNA was too low. It seemed so, that no matter, how fast or how much the dye would be transported to the DNA, the same was immediately saturated with ethidium und the complex has reached its maximum of fluorescence intensity. Equally, both fluorescence methods (with Hoechst 33342 and ethidium bromide) have one additional disadvantage: the reaction is always started with the addition of a substrate and the measurements need to start previously for comparing with the respective basal intensity. Hence, these methods can only act for determining the circumstances of the protein after a pressure treatment. Only “ex situ”, no “in situ” measurements. But after decompression, the membrane phase reconstitutes completely. Only irreversible damages to the protein would be captured. Interactions from lipids and membrane under HHP and resulting dissociation of the homodimer can not be detected.

4.4 Influence of the membrane protein content to membrane phase condition

Due to the complex nature of the natural lipid extract, no sharp phase transitions were observed, rather a continuous decrease of GP with increasing temperature is seen. Reconstitution of LmrA deeply increases the acyl chain order of the membrane. The effect is strongest at temperatures higher than about 25ºC in the case of a protein lipid ratio of 1:100 (w/w). For higher amounts of LmrA as like in case of a ratio of 1:20 and 1:50, the ordering effect is quite stable over the observed temperature range and lesser pronounced. No difference can be observed for these two different ratios. This may indicate, that there exists a significant concentration or at least an area of concentration of LmrA, where the influence of protein to lipid changes. As LmrA normally functions as a homodimer, low amounts of LmrA

may not form dimers and thereby influences as monomers the acyl chain order in a stronger way. Upon pressurization of the non-reconstituted natural membrane system, the overall conformational order of the membrane increases continuously in the whole pressure range covered from 0.1 to 200 MPa and reconstitution of LmrA leads to an overall increase in the conformational order of the acyl chains throughout the whole pressure range. The ordering effect depends on the reconstituted protein as the simple destabilization of liposomes with DDM and the following removal of the detergent with BioBeads leads to no impact to the GP over the observed pressure range.

4.5 ATPase activity under HHP

As it was not practicable to determine the transport of drugs like ethidium cations in proteoliposomes, for which functional crosstalk between the LmrA monomers (partially embedded in the membrane) is essential, it was decided to determine the accompanying ATPase activity (Bolhuis *et al.* 1996; van Veen *et al.* 2000; Balakrishnan *et al.* 2004). Transport escorting ATPase was secured by using the transport substrate ethidium bromide as an "inducer" of the ATP consuming process. ATPase activity was markedly increased in the presence of the transport substrate ethidium bromide as shown previously, and the transport was found to be directly connected with the ATP turnover and independent from other proteins (Balakrishnan *et al.* 2004). If an inactivation occurs for example as a result of a membrane phase transition, the basal activity is inhibited as well as the ethidium induced activity. On the other hand, orthovanadate is a well known inhibitor of the activity of LmrA, HorA and other ABC-transporters (Urbatsch *et al.* 1995; van Veen *et al.* 1996; Margolles *et al.* 1999; Sakamoto *et al.* 2001; Balakrishnan *et al.* 2004). Binding and inhibition of a vanadate-enzyme complex was studied under high hydrostatic pressure conditions (Ronzani *et al.* 1991). Orthovanadate induced inhibitory effects are nearly pressure insensitive at low pressure regions up to 30 MPa and are not affected at higher pressures. Therefore, the method used in these experiments is suitable to reveal the influence of changes in the lipid membrane on the activity of the enzyme while minimizing any artifacts.

Regarding all lipid model systems (except pure DMPC), no linear course of the activity values with pressure was detected. A more or less multiphasic behavior was observed, with maxima at 0.1 MPa and 200 MPa and local extrema in the range of 80 MPa to 120 MPa. This indicates that different effects may influence the activity of the enzyme.

Membrane phase transitions are known to affect membrane transport systems (Ulmer *et al.* 2000; Ulmer *et al.* 2002; Kilimann *et al.* 2005). Regarding the decrease of the activity of LmrA in the low pressure range up to 40 MPa, the loss of activity goes along with an increase of *GP*-values (i.e., the overall chain order parameter) of all lipid systems as seen previously .(Periasamy *et al.* 2008). A similar behavior has been observed for other homodimeric membrane proteins in living systems under moderate pressure conditions (up to 50 MPa, unpublished data). Proteoliposomes from DMPC reach the pressure-induced gel phase after a rather sharp transition around 20-30 MPa at 30°C. The LmrA activity is lost rapidly after only a small pressurization step of 20 MPa and this loss was found to be not reversible. Hence, when this lipid system comprised of fatty acids with solely saturated C14 acyl chains reaches the gel phase, total inactivation of LmrA is induced. Notably, a significant hydrophobic mismatch between DMPC and LmrA could be revealed by the AFM- and *GP*-data, which might also disfavor optimal LmrA function.

Periasamy *et al*. observed that DMPC proteoliposomes containing 10 mol% cholesterol exhibit a distinct area of coexistence of gel/fluid phases (Periasamy *et al.* 2008). Thus, no sharp fluid-to-gel phase transition is detected upon pressurization. Interestingly, LmrA is not inactivated in this model membrane system and retains its activity even up to 200 MPa, where the average *GP*-value of ~0.55 indicates gel-like tight chain packing. This suggests that only a sharp phase transition from a fluid-like to an ordered gel phase without having the possibility for lipid sorting and reorganization of the coexisting domain structure (as is feasible in DMPC/cholesterol mixtures), impairs the LmrA ATPase activity.

Due to the very low main transition temperature of DOPC (about -20 ^{o}C), DOPC proteoliposomes remain in the liquid-disordered state under all pressure conditions studied. The activity of the incorporated LmrA first decreases and then increases again upon pressurization, finally reaching the initial values at pressures of 200 MPa. Notably, for DOPC and LmrA no significant hydrophobic mismatch was found.

Heterogeneous lipid membranes with the existence of so called “liquid-ordered” domains should be represented by the model raft mixture DOPC/DPPC/cholesterol (1:2:1). Atomic force microscopy (AFM) data of proteoliposomes from the model raft mixture taken at ambient pressure show an equal distribution of the protein in the liquid-ordered and liquid-disordered domains of the membrane (Periasamy *et al.* 2008). Hence, for LmrA there seems to be no preference for a particular phase at ambient pressure. With increasing pressure in these kinds of lipid systems, different activity levels of LmrA should result from changes in

the lateral organization, redistribution of coexisting phases and changes in phase state of the lipid system. Similar to the DMPC/cholesterol system, the LmrA activity increases significantly at high pressures (above 40 MPa), and remains high even at 200 MPa, where the average *GP* value determined indicates existence of a densely packed membrane.

Interestingly, the pressure dependence of the LmrA activity in a neutral lipid environment from a natural source remains rather constant (in accordance with an almost pressure-insensitive *GP*-value). Also for this system, the drastic increase in *GP* upon incorporation of the protein into the neutral natural lipid mixture indicates drastic hydrophobic mismatch. This might cause its low pressure-resistance, as observed in DMPC bilayers.

Notably, the AFM images of proteoliposomes of LmrA reconstituted in DMPC, DMPC + cholesterol and the model raft mixture always present regions without or with less protein. This may indicate an affinity of LmrA towards LmrA-enriched regions and a minor affinity towards particular lipid molecules and membrane phases, thus fostering homodimer formation which is required for its function. Periasamy *et al*. (2008) also reports on a thicker membrane in these protein-enriched regions. The thickness of fluid-like membranes or domains increases with increasing hydrostatic pressure as shown previously (Kato *et al.* 2002; Winter 2002; Winter *et al.* 2005). If the thickness of the membrane affects the affinity of LmrA to particular membrane regions, this may lead to an increased distribution of LmrA in the whole membrane, thus reducing the chance of building homodimers required for activity. A membrane with a thicker lipid bilayer but still with a fluid like character, which can be achieved by moderate pressurization, may thus lead to a decrease in activity as it is observed for low pressures up to 40 MPa. In accordance with the *GP*-data, upon further increase of pressure, no marked changes in bilayer thickness can be expected for these more-component model membranes.

Assuming that LmrA is a simple kind of an ATPase and the equation

$$ATP + n\ Et_{in} \leftrightarrow [ATP\text{-}E\text{-}n Et] \leftrightarrow ADP + P_i + n\ Et_{out}$$

is valid (with *n*Et as the amount of ethidium being transported with the turnover of one ATP and [ATP-E-*n*Et] standing for the substrate enzyme complex), this system could have similar thermodynamic features like CF_oF_1ATPase, for which an increasing activity under high hydrostatic pressure up to 80 MPa was determined (Souza *et al.* 2004). For the CF_oF_1ATPase,

a negative activation volume $\Delta V^{\#}$ of -24 mL/mol was calculated (Souza *et al.* 2004), meaning, that the ATPase activity increases by increasing pressure. In our case, a different scenario is observed. Only for some of the systems, an increase of pressure leads to an increase of the activity, but only at medium to high pressures. The different behavior may essentially be due to the different structural properties of the proteins (homodimeric transport proteins and oligomeric CF_oF_1ATPase) and the dimerization reaction needed for LmrA and HorA function in the membranous context.

Raft building, budding and fission effects in vesicles in the absence and presence of pressure have been intensively discussed in recent years (Farge *et al.* 1992; Sackmann *et al.* 1995; Tsafrir *et al.* 2003; Staneva *et al.* 2004; Li *et al.* 2005; Perrier-Cornet *et al.* 2005b; Nicolini *et al.* 2006a). For heterogeneous membranes, such as cholesterol-containing model raft mixtures, an increase of pressure of a few 10 MPa may be sufficient to induce budding and fission of lipid daughter vesicles to avoid a drastic pressure-induced increase of the line tension between domains. Raft building and budding can result in locally ordered lipid structures, which provide an environment for a membrane bound protein more suitable for acquiring activity and improving its resistance towards lateral pressure or other external stress factors. Such factors might play a role in interpreting the different behavior of the systems DMPC/cholesterol and DOPC/DPPC/cholesterol, which exhibit irregular pressure dependencies of the ATPase activity and an increased ATPase activity (2-fold) even at 200 MPa.

HorA and LmrA show 53 % homology in their amino acid sequence. HorA is seven amino acids shorter. An alignment of both sequences can help to identify TMS with nearly the same length and size. As the ABC and therefore the cytoplasmic domain reveals a strong homology, the ATPase abilities and the pressure effects to this cytoplasamic domain should be the similar. Regional distinctions were mainly detected in the TMD and there especially in the TMS one, two and six. Differences in the activity under HHP are therefore mainly influenced by a different impact of the membrane phase to the TMD.

HorA dissociation exhibits a stronger resistance against the HHP treatment in DMPC vesicles as LmrA. Indeed, the ATPase activity is strongly repressed at 80 MPa as in for LmrA, but the inactivation is fully reversible. As expected, the HorA ATPase activity in lipid systems containing cholesterol range in the same level and with nearly similar behaviour. Additionally in DMPC and cholesterol containing liposomes, an inhibition at 20 MPa is not perceivable and no two fold activity (as in the case of LmrA) is found at 200 MPa. In this lipid system,

HorA is quite stable and not affected by phase transitions except for an increase at 120 MPa as it was observed for LmrA, too. Equally in the model raft mixture, the activity rises up to approximately the two fold at 200 MPa after a slight decrease at 40 MPa. Notably is an approximately three fold activity at 20 MPa but comparative values for LmrA are missing. In the case of natural lipids and liposomes made of DOPC, HorA tends to raised activities over the pressure and reaches the highest values. Due to the sequence, structure a functionality of HorA and its consensuses to LmrA, a lower hydrophobic mismatch I hypothesized for DOPC vesicles.

Taken together, these data clearly indicate that highest LmrA activities, even exceeding the one at ambient pressure, may be achieved for lipid matrices with small hydrophobic mismatch and the ability of lipid sorting to guarantee optimal adjustment of the lipid layers surrounding the protein, also under high pressure conditions. Remarkably, a high overall order parameter and dense packing of the lipid membrane in these systems does foster efficient protein function. Such efficient packing with optimal lipid adjustment to prevent (also pressure-induced) hydrophobic mismatch might be a particular prerequisite for the homodimer formation and hence function of LmrA. Apparently this is not transferable to other ABC transporter systems. Indeed the relative protein HorA is also inhibited from the sharp phase to phase transition in a system showing a high hydrophobic mismatch, but for the other lipid systems tested, the activity of HorA increases to a greater or lesser extent. The protein also attains higher activities in lipid systems with a low hydrophobic mismatch. HorA seems thereby more unaffected from the possibility of lipid sorting and principles of the reaction volume may get more impact.

5 Summary

In this work we could show, that HHP is a powerful tool to study the effect of membrane structure on the activity of membrane proteins. Depending on the membrane lipid composition, the lipid bilayer thickness and the ability to form coexisting lipid phases influencing lipid/protein interactions, the ATPase activity of LmrA and HorA is modulated apart from the influence of additional substances or temperature changes and in a pressure range, where *Lb. plantarum* is not inhibited already but membrane transport is influenced from the membrane phase (Molina-Gutierrez *et al.* 2002; Ulmer *et al.* 2002; Kilimann *et al.* 2005; Perrier-Cornet *et al.* 2005a). Lipid sorting may play an important role, but more in a way of raft building. Budding and fission has not yet been reported in vesicles of that size (LUV) and is not a possibility in the natural cell, where the membrane is reinforced from a rigid cell wall.

Secondary, and apart from the focus of this study, it was shown, that the membrane transporter HorA, which was hypothesized to play a crucial role in the resistance of beer spoiling bacteria, is unsuitable as a single factor for classifying and determining the possibility of a bacteria towards the ability of spoiling beer. Less of half of the tested beer spoiling strains possesses a functional horA gene without any mutation leading to a non-functional protein.

Usual *E. coli* expression systems evidenced as unsuitable for the expression of ABC membrane transporter from lactic acid bacteria, as the proteins offered either toxicity to the host or the protein could not be purified in satisfying amounts.

The fluorescence dye Hoechst 33342 emerged as a very difficult fluorophore to interpret. Intensity can depend on the simple presence of ATP in the solution and is strongly affected from the pH and thereby from unavoidable small changes upon pressure changes. Transport of ethidium bromide on the other hand turned out to work for the determination of the funtionaltiy of the membrane transporter, if it was reconstituted into DNA loaded liposomes and ethidium is a transport substrate, of course. But the activity can not be distinguished in an exacter manner than active or non-active.

To conclude, it was proven, that the effect of high hydrostatic pressure on the membrane bound protein LmrA and HorA is largely transmitted from changes in the lipid environment. Regarding the activity of the protein, this can have “negative” effects, as it is the case for pure DMPC vesicles, or “positive” effects, when cholesterol is added or heterogeneous model raft

mixtures are present. A sharp pressure-induced fluid-to-gel phase transition without extended two-phase region and the possibility for lipid sorting, such as for DMPC bilayers, has a drastic inhibitory effect. Otherwise, an overall fluid-like, liquid-disordered membrane phase over the whole pressure-range covered, with suitable hydrophobic matching, such as for DOPC, prevents the membrane protein from high pressure inactivation even up to 200 MPa. Membrane protein interactions were not completely transferable to allied protein. HorA is less affected and the role of lipid sorting plays thereby a minor role for this protein. Hence, a series of membrane physical-chemical effects seems to influence the lipid-protein interaction, activity and pressure stability of membrane proteins. Moreover, when comparing the few data existing in the literature on pressure effects on membrane proteins so far, it seems also to be clear that the specific nature of the membrane protein (e.g., oligomeric assembly, a required dimerization reaction) plays a significant role in its membrane attribute dependent activity (altered with pressure treatment) and stability as well. But for both systems it can be remarked, that for the dimerization and therefore for active membrane transport, a low hydrophobic mismatch, which can result from a membrane phase transition, is playing the crucial role and thereby for the membrane transport of homodimeric membrane transporters.

6 Zusammenfassung

Mit dieser Arbeit wurde gezeigt, dass das thermodynamische „Werkzeug“ hoher hydrostatischer Druck ein hervorragendes Mittel darstellt, den Einfluss der Membranstruktur auf die Aktivität von Membranproteinen zu ermitteln. Dabei wurde die ATPase Aktivität von LmrA und HorA in Abhängigkeit von der Lipidzusammensetzung, dem Durchmesser der Lipiddoppelschicht und der Möglichkeit, Koexistenzbereiche von verschiedenen Fluiditätsphasen, welche die Lipid/Protein-Wechselwirkungen beeinflussen, zu entwickeln, moduliert. Unter dem Ausschluss von Konzentrationsänderungen und Temperaturschwankungen wurde hierzu ein Druckbereich gewählt, in welchem *Lb. plantarum* zwar noch nicht abgetötet wird, der Membrantransport wohl aber bereits beeinflusst ist (Molina-Gutierrez *et al.* 2002; Ulmer *et al.* 2002; Kilimann *et al.* 2005; Perrier-Cornet *et al.* 2005a). Gezielte Anordnung von Membranlipiden kann eine wichtige Rolle spielen, nicht jedoch in Form vom „Knospung und Spaltung“, die noch nicht bei „kleineren“ Vesikeln dieser Größe (LUV) beobachtet worden ist weder noch bei vegetativen Milchsäurebakterien, deren Membran von einer festen Zellwand gestützt ist und einen derartigen Effekt nicht zuläßt.

Nebenbei konnte gezeigt werden, dass der Membrantransporter HorA, der als enorm wichtig mit der Hopfenresistenz von bierverderbenden Michsäurebakterien erachtet wird, alleine stehend absolut ungeeignet für die Kategorisierung als Bierverderber ist. Nicht einmal die Hälfte der untersuchten Bierverderbern hatte ein funktionales *horA* Gen, das keine Mutation aufweist, die zu einem Kettenabbruch und damit zu einem funktionslosen Protein führt.

Herkömmliche Expressionssysteme für *E. coli* stellten ungeeignete Systeme für die Expression von Membrantransportern der ABC-Familie aus Milchsäurebakterien dar. Entweder zeigte das Protein Toxizität gegenüber dem Wirt oder es konnte nicht in zufriedenstellender Qualität aufgereinigt werden.

Der Fluoreszenzfarbstoff Hoechst 33342 erwies sich als ungeeigneter Farbstoff. Seine Fluoreszenzintensität zeigte sich leicht von mehreren Parametern beeinflussbar und ist somit schwer interpretierbar. Es konnte gezeigt werden, dass charakteristische Intensitätsverläufe der Fluoreszenz rein durch die Zugabe von Mg-ATP erzeugt werden kann, ohne die Anwesenheit von Membranen geschweige denn Proteoliposomen mit Membrantransportern. Ebenso zeigt Hoechst 33342 eine enorme pH-Abhängigkeit in seiner Fluoreszenzeigenschaft. Ein weiterer negativer Aspekt ist, dass ein absolut stabiler pH-Wert unter verschiedenen Hochdruckbedingungen nicht realisierbar ist. Der Transport von Ethidiumbromid ist hingegen

geeignet, die Funtionalität von rekonstituierten Membrantransportern nachzuweisen, wenn diese Ethidium als Transportsubstrat akzeptieren und in DNA beladene Liposomen eingebaut werden. Diese Methode eignet sich jedoch nur bedingt, um die Transportaktivität näher zu charakterisieren und lässt nur eine Aussage bezüglich aktiv oder inaktiv zu.

Letztendlich wurde erwiesen, dass der Effekt durch hohe hydrostatische Drücke auf die Membrantransportproteine LmrA und HorA zum großen Teil durch Veränderungen in der Lipidphase vermittelt werden. Im Bezug auf die Aktivität des Proteins kann dies negative Auswirkungen haben, wie z. B. der Fall in reinen DMPC Vesikel, oder positive Auswirkungen, falls Cholesterin anwesend ist oder heterogene „Model Raft“ Mischungen zur Anwendung kommen. Ein schneller, druckinduzierter Phasenwechsel von einer Flüssigphase hin zur Gelphase ohne größeren Bereich der Koexistenz beider Phasen nebeneinander und ohne die Möglichkeit der Selbstordung der Lipide, wie es bei Lipiddoppelschichten aus DMPC gegeben ist, hat enorme hemmende Auswirkungen. Andererseits, eine durchgehende flüssig-geordnete Membranphase über den gesamten Druckbereich hinweg, verbunden mit einer entsprechenden hydrophoben Verträglichkeit der TMD mit der Lipiddoppelschicht wie im Falle von DOPC, schützt das Protein LmrA vor einer hochdruckinduzierten Inaktivierung sogar bis Drücke von 200 MPa. Die beobachteten Wechselbeziehungen sind nicht vollständig auf andere verwandte Membranproteine übertragbar. Für HorA spielt die Möglichkeit der Lipidsortierung eine weniger tragende Rolle als für LmrA. Demzufolge ist es eine Reihe von chemisch-physikalischen Effekten, die die Lipid-Protein Wechselwirkungen mitbestimmen und die Druckstabilität von Membranproteinen beeinflussen. Vergleicht man des Weiteren die noch spärliche Literatur zur Druckauswirkung auf Membranproteine, wird weiter verdeutlicht, das die artspezifische Eigenschaft des Proteins (z.B. ein oligmerer Aufbau und/oder eine benötigte Dimerisierung) eine bedeutende Rolle in der (druckgesteuerten) Membranzustand abhängigen Aktivität und Stabilität spielt. Für die beiden untersuchten Proteine jedoch kann übereinstimmend festgestellt werden, dass für ihre Dimerisierung und somit für den aktiven Transport eine adäquate hydrophobe Übereinstimmung mit der Lipiddoppelschicht vorliegen muss, die auch eine Folge des Membranphasenübergangs sein kann, und somit folglich eine ausschlaggebende Rolle beim Membrantransport von homodimeren Transportsystemen spielt.

7 References

Aleman, C., A. M. Namba and J. Casanovas (2005). "Acid-base and electronic structure-dependent properties of Hoechst 33342." J Biomol Struct Dyn **23**(1): 29-36.

Allain, J. M. and M. Ben Amar (2006). "Budding and fission of a multiphase vesicle." Eur Phys J E Soft Matter **20**(4): 409-20.

Balakrishnan, L., H. Venter, R. A. Shilling and H. W. van Veen (2004). "Reversible transport by the ATP-binding cassette multidrug export pump LmrA: ATP synthesis at the expense of downhill ethidium uptake." J Biol Chem **279**(12): 11273-80.

Barman, H., M. Walch, S. Latinovic-Golic, C. Dumrese, M. Dolder, P. Groscurth and U. Ziegler (2006). "Cholesterol in negatively charged lipid bilayers modulates the effect of the antimicrobial protein granulysin." J Membr Biol **212**(1): 29-39.

Barwe, S. P., S. Kim, S. A. Rajasekaran, J. U. Bowie and A. K. Rajasekaran (2007). "Janus model of the Na,K-ATPase beta-subunit transmembrane domain: distinct faces mediate alpha/beta assembly and beta-beta homo-oligomerization." J Mol Biol **365**(3): 706-14.

Behr, J., M. G. Ganzle and R. F. Vogel (2006). "Characterization of a highly hop-resistant Lactobacillus brevis strain lacking hop transport." Appl Environ Microbiol **72**(10): 6483-92.

Bernhardt, G., A. Distèche, R. Jaenicke, B. Koch, H.-D. Lüdemann and K.-O. Stetter (1987). "Effect of carbon dioxide and hydrostatic pressure on the pH of culture media and the growth of methanogens at elevated temperature." Applied Microbiology and Biotechnology **28**(2): 176-181.

Bolhuis, H., D. Molenaar, G. Poelarends, H. W. van Veen, B. Poolman, A. J. Driessen and W. N. Konings (1994). "Proton motive force-driven and ATP-dependent drug extrusion systems in multidrug-resistant Lactococcus lactis." J Bacteriol **176**(22): 6957-64.

Bolhuis, H., G. Poelarends, H. W. van Veen, B. Poolman, A. J. Driessen and W. N. Konings (1995). "The Lactococcal lmrP gene encodes a proton motive force-dependent drug transporter." J Biol Chem **270**(44): 26092-8.

Bolhuis, H., H. W. van Veen, D. Molenaar, B. Poolman, A. J. Driessen and W. N. Konings (1996). "Multidrug resistance in Lactococcus lactis: evidence for ATP-dependent drug extrusion from the inner leaflet of the cytoplasmic membrane." Embo J **15**(16): 4239-45.

Boonyaratanakornkit, B. B., C. B. Park and D. S. Clark (2002). "Pressure effects on intra- and intermolecular interactions within proteins." Biochim Biophys Acta **1595**(1-2): 235-49.

Boyer, P. D. (1993). "The binding change mechanism for ATP synthase--some probabilities and possibilities." Biochim Biophys Acta **1140**(3): 215-50.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-54.

Cevc, G. (1993). Phospholipids handbook. New York, Marcel Dekker Inc.

Chen, G. Q. and J. E. Gouaux (1996). "Overexpression of bacterio-opsin in Escherichia coli as a water-soluble fusion to maltose binding protein: efficient regeneration of the fusion protein and selective cleavage with trypsin." Protein Sci **5**(3): 456-67.

Comings, D. E. (1975). "Mechanisms of chromosome banding. VIII. Hoechst 33258-DNA interaction." Chromosoma **52**(3): 229-43.

Cordeiro, Y., J. Kraineva, M. C. Suarez, A. G. Tempesta, J. W. Kelly, J. L. Silva, R. Winter and D. Foguel (2006). "Fourier transform infrared spectroscopy provides a fingerprint for the tetramer and for the aggregates of transthyretin." Biophys J **91**(3): 957-67.

Drew, D., M. Lerch, E. Kunji, D. J. Slotboom and J. W. de Gier (2006). "Optimization of membrane protein overexpression and purification using GFP fusions." Nat Methods **3**(4): 303-13.

Ellison, R. T., 3rd (1994). "The effects of lactoferrin on gram-negative bacteria." Adv Exp Med Biol **357**: 71-90.

Ellison, R. T., 3rd, F. M. LaForce, T. J. Giehl, D. S. Boose and B. E. Dunn (1990). "Lactoferrin and transferrin damage of the gram-negative outer membrane is modulated by Ca2+ and Mg2+." J Gen Microbiol **136**(7): 1437-46.

Engelman, D. M., Y. Chen, C. N. Chin, A. R. Curran, A. M. Dixon, A. D. Dupuy, A. S. Lee, U. Lehnert, E. E. Matthews, Y. K. Reshetnyak, A. Senes and J. L. Popot (2003). "Membrane protein folding: beyond the two stage model." FEBS Lett **555**(1): 122-5.

Farge, E. and P. F. Devaux (1992). "Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids." Biophys J **61**(2): 347-57.

Federici, L., B. Woebking, S. Velamakanni, R. A. Shilling, B. Luisi and H. W. van Veen (2007). "New structure model for the ATP-binding cassette multidrug transporter LmrA." Biochem Pharmacol **74**(5): 672-678.

Froud, R. J., C. R. Earl, J. M. East and A. G. Lee (1986a). "Effects of lipid fatty acyl chain structure on the activity of the (Ca2+ + Mg2+)-ATPase." Biochim Biophys Acta **860**(2): 354-60.

Froud, R. J., J. M. East, O. T. Jones and A. G. Lee (1986b). "Effects of lipids and long-chain alkyl derivatives on the activity of (Ca2+-Mg2+)-ATPase." Biochemistry **25**(23): 7544-52.

Gebhardt, D. O. (1983). "The acetone precipitation step is needed in determining the lecithin/ sphingomyelin (LS) ratio of amniotic fluid." Clin Chem **29**(1): 214.

Gratton, E. and M. Limkeman (1983). "A continuously variable frequency cross-correlation phase fluorometer with picosecond resolution." Biophys J **44**(3): 315-24.

Grimard, V., C. Vigano, A. Margolles, R. Wattiez, H. W. van Veen, W. N. Konings, J. M. Ruysschaert and E. Goormaghtigh (2001). "Structure and dynamics of the membrane-embedded domain of LmrA investigated by coupling polarized ATR-FTIR spectroscopy and (1)H/(2)H exchange." Biochemistry **40**(39): 11876-86.

Gross, M. and R. Jaenicke (1994). "Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes." Eur J Biochem **221**(2): 617-30.

Haakensen, M. and B. Ziola (2008). "Identification of novel horA-harbouring bacteria capable of spoiling beer." Can J Microbiol **54**(4): 321-5.

Heinrich, S. U., W. Mothes, J. Brunner and T. A. Rapoport (2000). "The Sec61p Complex Mediates the Integration of a Membrane Protein by Allowing Lipid Partitioning of the Transmembrane Domain." Cell **102**(2): 233-244.

Herberhold, H., S. Marchal, R. Lange, C. H. Scheyhing, R. F. Vogel and R. Winter (2003). "Characterization of the pressure-induced intermediate and unfolded state of red-shifted green fluorescent protein--a static and kinetic FTIR, UV/VIS and fluorescence spectroscopy study." J Mol Biol **330**(5): 1153-64.

Herberhold, H. and R. Winter (2002). "Temperature- and pressure-induced unfolding and refolding of ubiquitin: a static and kinetic Fourier transform infrared spectroscopy study." Biochemistry **41**(7): 2396-401.

Heremans, K. and L. Smeller (1998). "Protein structure and dynamics at high pressure." Biochim Biophys Acta **1386**(2): 353-70.

Higgins, C. F. (1992). "ABC transporters: from microorganisms to man." Annu Rev Cell Biol **8**: 67-113.

Holo, H. and I. F. Nes (1989). "High-Frequency Transformation, by Electroporation, of Lactococcus lactis subsp. cremoris Grown with Glycine in Osmotically Stabilized Media." Appl Environ Microbiol **55**(12): 3119-3123.

Hoover, D. G., C. Metrick, A. M. Papineau, D. F. Farkas and D. Knorr (1989). "Biological effects of high hydrostatic pressure on microorganisms." Food Technol. **43**: 99-107.

Hormann, S., C. Scheyhing, J. Behr, M. Pavlovic, M. Ehrmann and R. F. Vogel (2006). "Comparative proteome approach to characterize the high-pressure stress response of Lactobacillus sanfranciscensis DSM 20451(T)." Proteomics **6**(6): 1878-85.

Kangur, L., K. Timpmann, x and A. Freiberg (2008). "Stability of Integral Membrane Proteins under High Hydrostatic Pressure: The LH2 and LH3 Antenna Pigment−Protein Complexes from Photosynthetic Bacteria." J. Phys. Chem. B **112**(26): 7948-7955.

Kato, M., R. Hayashi, T. Tsuda and K. Taniguchi (2002). "High pressure-induced changes of biological membrane. Study on the membrane-bound Na(+)/K(+)-ATPase as a model system." Eur J Biochem **269**(1): 110-8.

Kilimann, K. V., C. Hartmann, A. Delgado, R. F. Vogel and M. G. Ganzle (2005). "A fuzzy logic-based model for the multistage high-pressure inactivation of Lactococcus lactis ssp. cremoris MG 1363." Int J Food Microbiol **98**(1): 89-105.

Kingsley, D. H., D. G. Hoover, E. Papafragkou and G. P. Richards (2002). "Inactivation of hepatitis A virus and a calicivirus by high hydrostatic pressure." J Food Prot **65**(10): 1605-9.

Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. Fiers, W. Stiekema, R. M. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos and R. J. Siezen (2003). "Complete genome sequence of Lactobacillus plantarum WCFS1." Proc Natl Acad Sci U S A **100**(4): 1990-5.

Kleinschmidt, J. H. (2003). "Membrane Proteins - Introduction." Cellular and Molecular Life Sciences **60**(8): 1527 - 1528.

Knorr, D., B. I. Ade-Omowaye and V. Heinz (2002). "Nutritional improvement of plant foods by non-thermal processing." Proc Nutr Soc **61**(2): 311-8.

Kornblatt, M. J., R. Lange and C. Balny (2004). "Use of hydrostatic pressure to produce 'native' monomers of yeast enolase." Eur J Biochem **271**(19): 3897-904.

Koseki, S., Y. Mizuno and K. Yamamoto (2008). "Use of mild-heat treatment following high-pressure processing to prevent recovery of pressure-injured Listeria monocytogenes in milk." Food Microbiol **25**(2): 288-93.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-685.

Lanzetta, P. A., L. J. Alvarez, P. S. Reinach and O. A. Candia (1979). "An improved assay for nanomole amounts of inorganic phosphate." Anal Biochem **100**(1): 95-7.

Lewington, J., S. D. Greenwawy and B. J. Spillane (1987). "Rapid small scale preparation of bacterial genomic DNA, suitable for cloning and hybridization analysis." Letters in Applied Microbiology **5**(3): 51-53.

Li, L., X. Liang, M. Lin, F. Qiu and Y. Yang (2005). "Budding dynamics of multicomponent tubular vesicles." J Am Chem Soc **127**(51): 17996-7.

Lipowsky, R. and E. Sackmann (1995). Structure and dynamics of membranes. Amsterdam ; New York, Elsevier Science.

Lopez-Pedemonte, T. J., A. X. Roig-Sagues, A. J. Trujillo, M. Capellas and B. Guamis (2003). "Inactivation of spores of Bacillus cereus in cheese by high hydrostatic pressure with the addition of nisin or lysozyme." J Dairy Sci **86**(10): 3075-81.

Lurquin, P. F. and K. Athanasiou (2000). "Electric field-mediated DNA encapsulation into large liposomes." Biochem Biophys Res Commun **267**(3): 838-41.

Marchal, S., E. Shehi, M. C. Harricane, P. Fusi, F. Heitz, P. Tortora and R. Lange (2003). "Structural instability and fibrillar aggregation of non-expanded human ataxin-3 revealed under high pressure and temperature." J Biol Chem **278**(34): 31554-63.

Marchal, S., J. Torrent, P. Masson, J. M. Kornblatt, P. Tortora, P. Fusi, R. Lange and C. Balny (2005). "The powerful high pressure tool for protein conformational studies." Braz J Med Biol Res **38**(8): 1175-83.

Margolles, A., M. Putman, H. W. van Veen and W. N. Konings (1999). "The purified and functionally reconstituted multidrug transporter LmrA of Lactococcus lactis mediates the transbilayer movement of specific fluorescent phospholipids." Biochemistry **38**(49): 16298-306.

Masschalck, B., R. Van Houdt and C. W. Michiels (2001). "High pressure increases bactericidal activity and spectrum of lactoferrin, lactoferricin and nisin." Int J Food Microbiol **64**(3): 325-32.

Matsudaira, P. (1987). "Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes." J Biol Chem **262**(21): 10035-8.

McKeegan, K. S., M. I. Borges-Walmsley and A. R. Walmsley (2003). "The structure and function of drug pumps: an update." Trends Microbiol **11**(1): 21-9.

Mierau, I. and M. Kleerebezem (2005). "10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis." Appl Microbiol Biotechnol **68**(6): 705-17.

Mokhonova, E. I., V. V. Mokhonov, H. Akama and T. Nakae (2005). "Forceful large-scale expression of "problematic" membrane proteins." Biochem Biophys Res Commun **327**(3): 650-5.

Molina-Garcia, A. D. and P. D. Sanz (2002). "Anisakis simplex larva killed by high-hydrostatic-pressure processing." J Food Prot **65**(2): 383-8.

Molina-Gutierrez, A., V. Stippl, A. Delgado, M. G. Ganzle and R. F. Vogel (2002). "In situ determination of the intracellular pH of Lactococcus lactis and Lactobacillus plantarum during pressure treatment." Appl Environ Microbiol **68**(9): 4399-406.

Molina-Hoppner, A., W. Doster, R. F. Vogel and M. G. Ganzle (2004). "Protective effect of sucrose and sodium chloride for Lactococcus lactis during sublethal and lethal high-pressure treatments." Appl Environ Microbiol **70**(4): 2013-20.

Mouritsen, O. G. and M. Bloom (1984). "Mattress model of lipid-protein interactions in membranes." Biophys J **46**(2): 141-53.

Munoz, M., B. de Ancos, C. Sanchez-Moreno and M. P. Cano (2007). "Effects of high pressure and mild heat on endogenous microflora and on the inactivation and sublethal injury of Escherichia coli inoculated into fruit juices and vegetable soup." J Food Prot **70**(7): 1587-93.

Murdock, C. A., J. Cleveland, K. R. Matthews and M. L. Chikindas (2007). "The synergistic effect of nisin and lactoferrin on the inhibition of Listeria monocytogenes and Escherichia coli O157:H7." Lett Appl Microbiol **44**(3): 255-61.

Nicolini, C., A. Celli, E. Gratton and R. Winter (2006a). "Pressure tuning of the morphology of heterogeneous lipid vesicles: a two-photon-excitation fluorescence microscopy study." Biophys J **91**(8): 2936-42.

Nicolini, C., J. Kraineva, M. Khurana, N. Periasamy, S. S. Funari and R. Winter (2006b). "Temperature and pressure effects on structural and conformational properties of POPC/SM/cholesterol model raft mixtures--a FT-IR, SAXS, DSC, PPC and Laurdan fluorescence spectroscopy study." Biochim Biophys Acta **1758**(2): 248-58.

Otto, F. and K. C. Tsou (1985). "A comparative study of DAPI, DIPI, and Hoechst 33258 and 33342 as chromosomal DNA stains." Stain Technol **60**(1): 7-11.

Parasassi, T., F. Conti and E. Gratton (1986). "Time-resolved fluorescence emission spectra of Laurdan in phospholipid vesicles by multifrequency phase and modulation fluorometry." Cell Mol Biol **32**(1): 103-8.

Parasassi, T., G. De Stasio, A. d'Ubaldo and E. Gratton (1990). "Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence." Biophys J **57**(6): 1179-86.

Parasassi, T., G. De Stasio, G. Ravagnan, R. M. Rusch and E. Gratton (1991). "Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan fluorescence." Biophys J **60**(1): 179-89.

Paredes-Sabja, D., M. Gonzalez, M. R. Sarker and J. A. Torres (2007). "Combined effects of hydrostatic pressure, temperature, and pH on the inactivation of spores of Clostridium perfringens type A and Clostridium sporogenes in buffer solutions." J Food Sci **72**(6): M202-6.

Periasamy, N., H. Teichert, K. Weise, R. F. Vogel and R. Winter (2008). "Effects of temperature and pressure on the lateral organization of model membranes with functionally reconstituted multidrug transporter LmrA." Biochim Biophys Acta.

Periasamy, N. and R. Winter (2006). "The effects of temperature, pressure and peptide incorporation on ternary model raft mixtures--a Laurdan fluorescence spectroscopy study." Biochim Biophys Acta **1764**(3): 398-404.

Perrier-Cornet, J.-M., S. Tapin, S. Gaeta and P. Gervais (2005a). "High-pressure inactivation of Saccharomyces cerevisiae and Lactobacillus plantarum at subzero temperatures." Journal of Biotechnology **115**(4): 405-412.

Perrier-Cornet, J. M., K. Baddouj and P. Gervais (2005b). "Pressure-induced shape change of phospholipid vesicles: implication of compression and phase transition." J Membr Biol **204**(3): 101-7.

Petit, J. M., M. Denis-Gay and M. H. Ratinaud (1993). "Assessment of fluorochromes for cellular structure and function studies by flow cytometry." Biol Cell **78**(1-2): 1-13.

Pleban, K., A. Macchiarulo, G. Costantino, R. Pellicciari, P. Chiba and G. F. Ecker (2004). "Homology model of the multidrug transporter LmrA from Lactococcus lactis." Bioorg Med Chem Lett **14**(23): 5823-6.

Poelarends, G. J. and W. N. Konings (2002a). "The transmembrane domains of the ABC multidrug transporter LmrA form a cytoplasmic exposed, aqueous chamber within the membrane." J Biol Chem **277**(45): 42891-8.

Poelarends, G. J., P. Mazurkiewicz and W. N. Konings (2002b). "Multidrug transporters and antibiotic resistance in Lactococcus lactis." Biochim Biophys Acta **1555**(1-3): 1-7.

Popot, J. L. and D. M. Engelman (2000). "Helical membrane protein folding, stability, and evolution." Annu Rev Biochem **69**: 881-922.

Portugal, J. and M. J. Waring (1988). "Assignment of DNA binding sites for 4',6-diamidine-2-phenylindole and bisbenzimide (Hoechst 33258). A comparative footprinting study." Biochim Biophys Acta **949**(2): 158-68.

Powalska, E., S. Janosch, E. Kinne-Saffran, R. K. Kinne, C. F. Fontes, J. A. Mignaco and R. Winter (2007). "Fluorescence spectroscopic studies of pressure effects on Na+,K(+)-ATPase reconstituted into phospholipid bilayers and model raft mixtures." Biochemistry **46**(6): 1672-83.

Pupo, E., A. Padron, E. Santana, J. Sotolongo, D. Quintana, S. Duenas, C. Duarte, M. C. de la Rosa and E. Hardy (2005). "Preparation of plasmid DNA-containing liposomes using a high-pressure homogenization--extrusion technique." J Control Release **104**(2): 379-96.

Reuter, G., T. Janvilisri, H. Venter, S. Shahi, L. Balakrishnan and H. W. van Veen (2003). "The ATP binding cassette multidrug transporter LmrA and lipid transporter MsbA have overlapping substrate specificities." J Biol Chem **278**(37): 35193-8.

Richter, M. L., R. Hein and B. Huchzermeyer (2000). "Important subunit interactions in the chloroplast ATP synthase." Biochim Biophys Acta **1458**(2-3): 326-42.

Rigaud, J. L., B. Pitard and D. Levy (1995). "Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins." Biochim Biophys Acta **1231**(3): 223-46.

Ronzani, N., L. Stephan and W. Hasselbach (1991). "Pressure effects on the binding of vanadate to the sarcoplasmic reticulum calcium-transport enzyme." Eur J Biochem **201**(1): 265-71.

Roux, J. F., J. Nakamura and M. Frosolono (1974). "Fatty acid composition and concentration of lecithin in the acetone fraction of amniotic fluid phospholipids." Am J Obstet Gynecol **119**(6): 838-43.

Ruiz Budria, J., J. M. Abbad Bara, E. Fabre Gonzalez, M. T. Higueras Sanz and J. L. Serrano Ostariz (1989). "Phospholipid surface tension: the diagnostic utility of amniotic fluid and its lipid extract, an analysis of the value of precipitation with cold acetone." Clin Chem **35**(5): 800-3.

Sachs, G., K. Munson, V. N. Balaji, D. Aures-Fischer, S. J. Hersey and K. Hall (1989). "Functional domains of the gastric HK ATPase." J Bioenerg Biomembr **21**(5): 573-88.

Sackmann, E. and T. Feder (1995). "Budding, fission and domain formation in mixed lipid vesicles induced by lateral phase separation and macromolecular condensation." Mol Membr Biol **12**(1): 21-8.

Sakamoto, K. and W. N. Konings (2003). "Beer spoilage bacteria and hop resistance." Int J Food Microbiol **89**(2-3): 105-24.

Sakamoto, K., A. Margolles, H. W. van Veen and W. N. Konings (2001). "Hop resistance in the beer spoilage bacterium Lactobacillus brevis is mediated by the ATP-binding cassette multidrug transporter HorA." J Bacteriol **183**(18): 5371-5.

Sami, M., H. Yamashita, T. Hirono, H. Kadokura, K. Kitamoto, K. Yoda and M. Yamasaki (1997). "Hop-Resistant Lactobacillus brevis Contains a Novel Plasmid Harboring a Multidrug Resistance-Like Gene." JOURNAL OF FERMENTATION AND BIOENGINEERING **84**(1): 1-6.

San Martin, M. F., G. V. Barbosa-Canovas and B. G. Swanson (2002). "Food processing by high hydrostatic pressure." Crit Rev Food Sci Nutr **42**(6): 627-45.

Scheyhing, C. H., S. Hormann, M. A. Ehrmann and R. F. Vogel (2004). "Barotolerance is inducible by preincubation under hydrostatic pressure, cold-, osmotic- and acid-stress conditions in Lactobacillus sanfranciscensis DSM 20451T." Lett Appl Microbiol **39**(3): 284-9.

Sedmak, J. J. and S. E. Grossberg (1977). "A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250." Anal Biochem **79**(1-2): 544-52.

Smeller, L. (2002). "Pressure-temperature phase diagrams of biomolecules." Biochim Biophys Acta **1595**(1-2): 11-29.

Souza, M. O., T. B. Creczynski-Pasa, H. M. Scofano, P. Graber and J. A. Mignaco (2004). "High hydrostatic pressure perturbs the interactions between CF(0)F(1) subunits and induces a dual effect on activity." Int J Biochem Cell Biol **36**(5): 920-30.

Spencer, R. D. and G. Weber (1970). "Influence of Brownian rotations and energy transfer upon the measurements of fluorescence lifetimes." J. Chem. Phys. **52**: 1654-1663.

Staneva, G., M. I. Angelova and K. Koumanov (2004). "Phospholipase A2 promotes raft budding and fission from giant liposomes." Chem Phys Lipids **129**(1): 53-62.

Suzuki, K., K. Iijima, K. Ozaki and H. Yamashita (2005). "Isolation of a hop-sensitive variant of Lactobacillus lindneri and identification of genetic markers for beer spoilage ability of lactic acid bacteria." Appl Environ Microbiol **71**(9): 5089-97.

Suzuki, K., M. Sami, K. Iijima, K. Ozaki and H. Yamashita (2006). "Characterization of horA and its flanking regions of Pediococcus damnosus ABBC478 and development of more specific and sensitive horA PCR method." Lett Appl Microbiol **42**(4): 392-9.

Suzuki, K., M. Sami, H. Kadokura, H. Nakajima and K. Kitamoto (2002). "Biochemical characterization of horA-independent hop resistance mechanism in Lactobacillus brevis." Int J Food Microbiol **76**(3): 223-30.

Tardieu, A., V. Luzzati and F. C. Reman (1973). "Structure and polymorphism of the hydrocarbon chains of lipids: a study of lecithin-water phases." J Mol Biol **75**(4): 711-33.

Tassou, C. C., E. Z. Panagou, F. J. Samaras, P. Galiatsatou and C. G. Mallidis (2008). "Temperature-assisted high hydrostatic pressure inactivation of Staphylococcus aureus in a ham model system: evaluation in selective and nonselective medium." J Appl Microbiol **104**(6): 1764-73.

Tieking, M., M. A. Ehrmann, R. F. Vogel and M. G. Ganzle (2005). "Molecular and functional characterization of a levansucrase from the sourdough isolate Lactobacillus sanfranciscensis TMW 1.392." Appl Microbiol Biotechnol **66**(6): 655-63.

Tsafrir, I., Y. Caspi, M. A. Guedeau-Boudeville, T. Arzi and J. Stavans (2003). "Budding and tubulation in highly oblate vesicles by anchored amphiphilic molecules." Phys Rev Lett **91**(13): 138102.

Ulmer, H. M., M. G. Ganzle and R. F. Vogel (2000). "Effects of high pressure on survival and metabolic activity of Lactobacillus plantarum TMW1.460." Appl Environ Microbiol **66**(9): 3966-73.

Ulmer, H. M., H. Herberhold, S. Fahsel, M. G. Ganzle, R. Winter and R. F. Vogel (2002). "Effects of pressure-induced membrane phase transitions on inactivation of HorA, an ATP-dependent multidrug resistance transporter, in Lactobacillus plantarum." Appl Environ Microbiol **68**(3): 1088-95.

Urbatsch, I. L., B. Sankaran, J. Weber and A. E. Senior (1995). "P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site." J Biol Chem **270**(33): 19383-90.

van den Berg van Saparoea, H. B., J. Lubelski, R. van Merkerk, P. S. Mazurkiewicz and A. J. Driessen (2005). "Proton motive force-dependent Hoechst 33342 transport by the ABC transporter LmrA of Lactococcus lactis." Biochemistry **44**(51): 16931-8.

van Veen, H. W., C. F. Higgins and W. N. Konings (2001a). "Molecular basis of multidrug transport by ATP-binding cassette transporters: a proposed two-cylinder engine model." J Mol Microbiol Biotechnol **3**(2): 185-92.

van Veen, H. W., C. F. Higgins and W. N. Konings (2001b). "Multidrug transport by ATP binding cassette transporters: a proposed two-cylinder engine mechanism." Res Microbiol **152**(3-4): 365-74.

van Veen, H. W., A. Margolles, M. Muller, C. F. Higgins and W. N. Konings (2000). "The homodimeric ATP-binding cassette transporter LmrA mediates multidrug transport by an alternating two-site (two-cylinder engine) mechanism." Embo J **19**(11): 2503-14.

van Veen, H. W., M. Putman, A. Margolles, K. Sakamoto and W. N. Konings (1999). "Structure-function analysis of multidrug transporters in Lactococcus lactis." Biochim Biophys Acta **1461**(2): 201-6.

van Veen, H. W., K. Venema, H. Bolhuis, I. Oussenko, J. Kok, B. Poolman, A. J. Driessen and W. N. Konings (1996). "Multidrug resistance mediated by a bacterial homolog of the human multidrug transporter MDR1." Proc Natl Acad Sci U S A **93**(20): 10668-72.

Venter, H., S. Shahi, L. Balakrishnan, S. Velamakanni, A. Bapna, B. Woebking and H. W. van Veen (2005). "Similarities between ATP-dependent and ion-coupled multidrug transporters." Biochem Soc Trans **33**(Pt 5): 1008-11.

Venter, H., R. A. Shilling, S. Velamakanni, L. Balakrishnan and H. W. Van Veen (2003). "An ABC transporter with a secondary-active multidrug translocator domain." Nature **426**(6968): 866-70.

Vigano, C., A. Margolles, H. W. van Veen, W. N. Konings and J. M. Ruysschaert (2000). "Secondary and tertiary structure changes of reconstituted LmrA induced by nucleotide binding or hydrolysis. A fourier transform attenuated total reflection infrared spectroscopy and tryptophan fluorescence quenching analysis." J Biol Chem **275**(15): 10962-7.

Vogel, W. C., W. M. Doizaki and L. Zieve (1961). "Rapid thin-layer chromatographic separation of phospholipids and neutral lipids of serum " J. Lipid Res **3**(1): 138-140.

Waring, M. J. (1965). "Complex formation between ethidium bromide and nucleic acids." J Mol Biol **13**(1): 269-82.

Winter, R. (2002). "Synchrotron X-ray and neutron small-angle scattering of lyotropic lipid mesophases, model biomembranes and proteins in solution at high pressure." Biochim Biophys Acta **1595**(1-2): 160-84.

Winter, R. and W. Dzwolak (2005). "Exploring the temperature-pressure configurational landscape of biomolecules: from lipid membranes to proteins." Philos Transact A Math Phys Eng Sci **363**(1827): 537-62; discussion 562-3.

Wuytack, E. Y., S. Boven and C. W. Michiels (1998). "Comparative study of pressure-induced germination of Bacillus subtilis spores at low and high pressures." Appl Environ Microbiol **64**(9): 3220-4.

Yamauchi, K., M. Tomita, T. J. Giehl and R. T. Ellison, 3rd (1993). "Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment." Infect Immun **61**(2): 719-28.

Yu, W., P. T. So, T. French and E. Gratton (1996). "Fluorescence generalized polarization of cell membranes: a two-photon scanning microscopy approach." Biophys J **70**(2): 626-36.

Zapf, M. W., S. Theisen, S. Rohde, F. Rabenstein, R. F. Vogel and L. Niessen (2007). "Characterization of AfpA, an alkaline foam protein from cultures of Fusarium culmorum and its identification in infected malt." J Appl Microbiol **103**(1): 36-52.

8 Appendix

List of publications that resulted from this dissertation:

Papers

Holger Teichert, Nagarajan Periasamy, Roland Winter and Rudi F. Vogel, Influence of membrane lipid composition on the activity of functionally reconstituted LmrA under high hydrostatic pressure, High Pressure Research, 2009, in press

Nagarajan Periasamy, **Holger Teichert**, Katrin Weise, Rudi F. Vogel and Roland Winter, Effects of temperature and pressure on the lateral organization of model and natural membrane lipids with functionally reconstituted multidrug transporter LmrA, Biochimica et Biophysica Acta, 2008, Oct. 17th, article in press

Rudi F. Vogel, Kai Linke, **Holger Teichert**, Matthias A. Ehrmann, High pressure modulated transport and signalling functions of membrane proteins in models an *in vivo*, Journal of Physics:Conference Series, Joint 21st AIRAPT And 45th EHPRG International Conference On High Pressure Science And Technology, paper 0250, in press

Poster Presentations

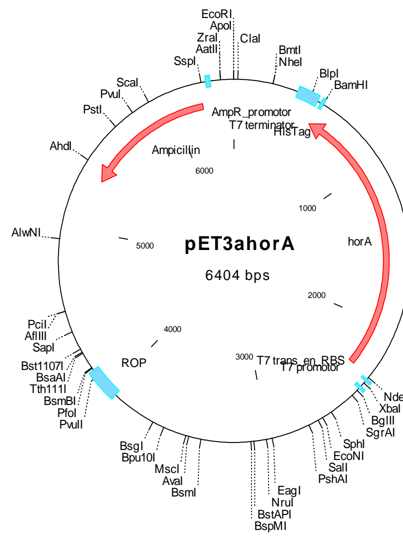
Nagarajan Periasamy, **Holger Teichert**, Rudi F. Vogel, Roland Winter, Funtional reconstituted multidrug transporter LmrA in model and natural membrane lipids and lipid bilayer-protein interaction – The effects of temperature and pressure, 44th EHPRG International Conference, Prague, September 4 – 8, 2006

Rudi Vogel, **Holger Teichert**,. Kai Linke, Matthias Ehrmann, High pressure modulated transport and signalling functions of membrane proteins in models an *in vivo*, VAAM meeting 9 – 11.03.2008 in Frankfurt, Germany

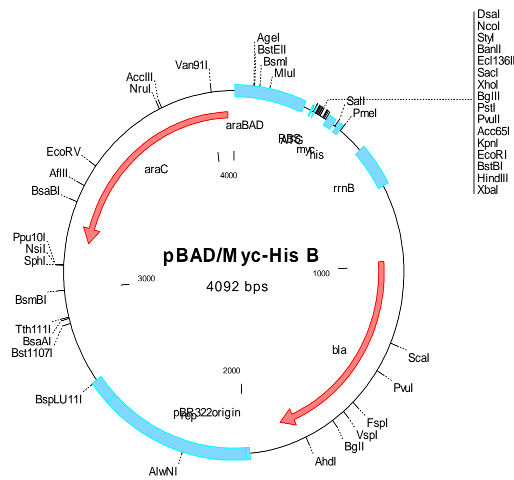
Vector maps of the plasmids constructed or used in this work:

pSTblue1 vector is used for subcloning of *horA* in *E. coli* and for sequencing the gene.

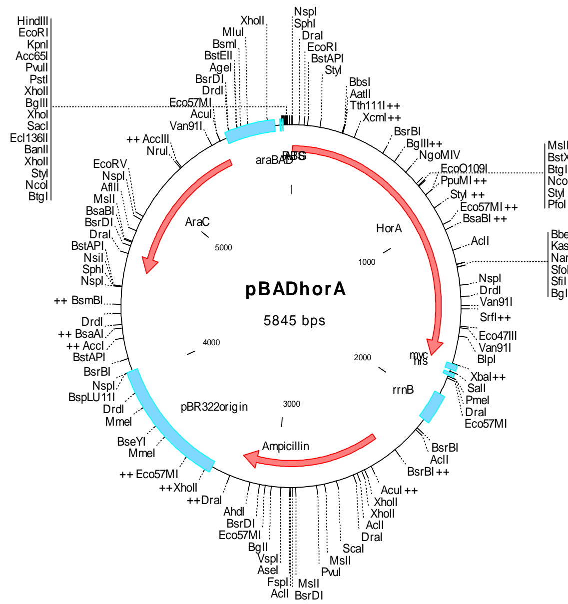
pSTblue1 vector with horA from TMW 1.1282 as it was used for sequencing.



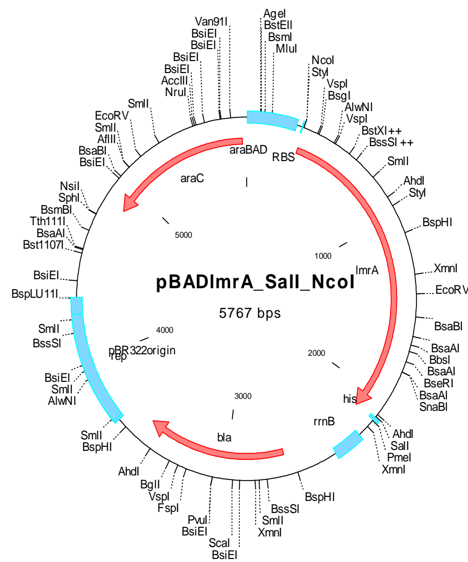
E. coli expression vector pET3a with *horA* and c-terminal His-tag.



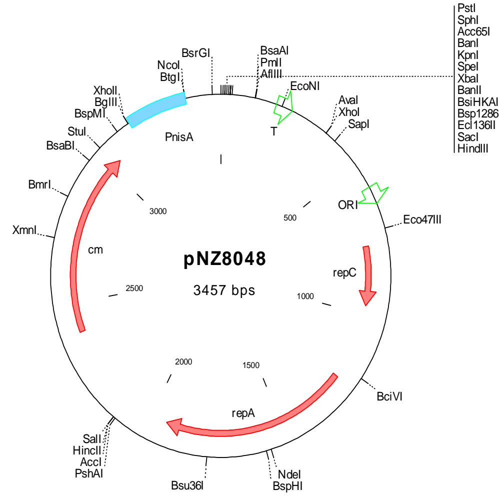
E. coli expression vector pBAD*Myc*HisB



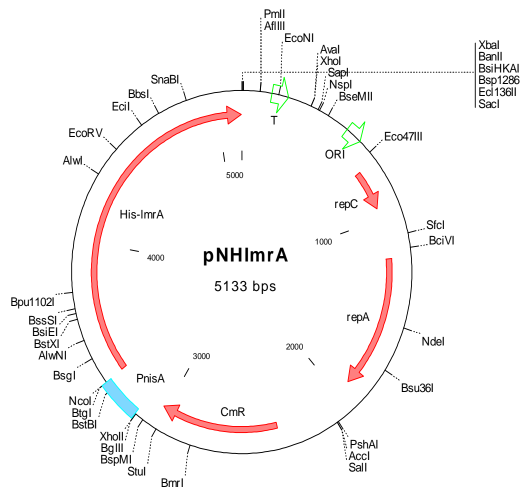
E. coli expression vector pBAD*Myc*HisB with insert *horA* and *myc* epitope.



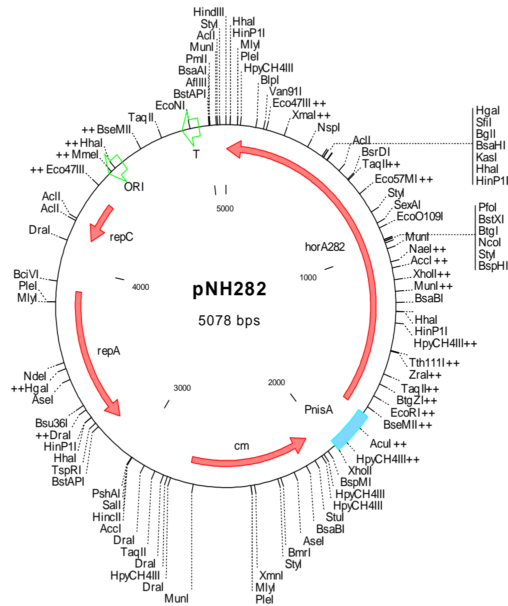
E. coli expression vector pBAD*Myc*HisB with insert *lmrA* and witout the *myc* epitope. Epitope was resticted by using Sal I restriction of the vector.



Vector pNZ8048. An expression vector for *Lc. lactis* NZ9000 and derivates. For the host the existence of *niR* and *nisK* genes are necessary for expression.



Lactococcus lactis NZ9000 expression vector pNH*lmrA*. A derivate from pNZ8048.



Lactococcus lactis NZ9000 expression vector pNH282. A derivate of pNZ8048 with *horA* gene from *Lb. brevis* TMW 1.1282. The Nco I restriction site is lost through the ligation with Psc I restricted insert.

Alignment of the cloned *horA* genes found in beer spoiling *Lactobacillus brevis* stains. Given is the TMW number. The sequence from the originally published *horA* was taken from the literature (Sami *et al.* 1997). The N-terminal hexa histidine tag was part of the primer and was added for optionally ongoing cloning steps.

```
1.317           ATGTTACATCATCATCATCATCAT-CAAGCTCAGTCCAAGAACAATACCAAGTTTAACTT 59
1.483           ATGTTACATCATCATCATCATCAT-CAAGCTCAGTCCAAGAACAATACCAAGTTTAACTT 59
1.313           ATGTTACATCATCATCATCATCAT-CAAGCTCAGTCCAAGAACAATACCAAGTTTAACTT 59
1.485           ATGTTACATCATCATCATCATCAT-CAAGCTCAGTCCAAGAACAATACCAAGTTTAACTT 59
1.1282          ATGTTACATCATCATCATCATCAT-CAAGCTCAGTCCAAGAACAATACCAAGTTTAACTT 59
horA            ----------------------ATGCAAGCTCAGTCCAAGAACAATACCAAGTTTAACTT 38
                                      ** ***********************************

1.317           TAAAACATTTATGGGCCTAATCAACCGAATTCACCCCCGTTACTGGCAACTGCTGTTTGG 119
1.483           TAAAACATTTATGGGCCTAATCAACCGAATTCACCCCCGTTACTGGCAACTGCTGTTTGG 119
1.313           TAAAACATTTATGGGCCTAATCAACCGAATTCACCCCCGTTACTGGCAACTGCTGTTTGG 119
1.485           TAAAACATTTATGGGCCTAATCAACCGAATTCACCCCCGTTACTGGCAACTGCTGCTTGG 119
1.1282          TAAAACATTTATGGGCCTAATCAACCGAATTCACCCCCGTTACTGGCAACTGCTGCTTGG 119
horA            TAAAACATTTATGGGCCTAATCAACCGAATTCACCCCCGTTACTGGCAACTGCTGCTTGG 98
                ******************************************************* ****

1.317           TTTTTTTCTAGGAGTTGTCGCAACGGCGATGCAATTGATGGTTCCCGGCATCGCCAAGGG 179
1.483           TTTTTTTCTAGGAGTTGTCGCAACGGCGATGCAATTGATGGTTCCCGGCATCGCCAAGGG 179
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1.485           CTTTTTTCTAGGAGTTGTCGCAACGGCGATGCAATTGATGGTTCCCGGCATCGCCAAGGG 179
1.1282          CTTTTTTCTAGGAGTTGTCGCAACGGCGATGCAATTGATGGTTCCCGGCATCGCCAAGGG 179
horA            CTTTTTTCTAGGAGTTGTCGCAACGGCGATGCAATTGATGGTTCCCGGCATCGCCAAGGG 158
                 ***********************************************************

1.317           GATCATCAACTCAATCGGTCATTCAATGGATGTCGGCCTAATCGTTGCCGTCATTTTACT 239
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1.313           GATCATCAACTCAATCGGTCATTCAATGGATGTCGGCCTAATCGTTGCCGTCATTTTACT 239
1.485           GATCATCAACTCAATCGGTCATTCAATGGATGTCGGCCTAATCGTTGCCGTCATTTTACT 239
1.1282          GATCATCAACTCAATCGGTCATTCAATGGATGTCGGCCTAATCGTTGCCGTCATTTTACT 239
horA            GATCATCAACTCAATCGGTCATTCAATGGATGTCGGCCTAATCGTTGCCGTCATTTTACT 218
                ************************************************************

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1.483           ATTCGTTTTCAGTACCATTATTGGAGCCTCTTCCGGCAGTATTTTAGGCTTCTTCGGTGA 299
1.313           ATTCGTTTTCAGTACCATTATTGGAGCCTCTTCCGGCAGTATTTTAGGCTTCTTCGGTGA 299
1.485           ATTCGTTTTCAGTACCATTATTGGAGCCTTTTCCGGCAGTATTTTAGGCTTCTTCGGTGA 299
1.1282          ATTCGTTTTCAGTACCATTATTGGAGCCTTTTCCGGCAGTATTTTAGGCTTCTTCGGTGA 299
horA            ATTCGTTTCCAGTACCATTATTGGAGCCTTTTCCGGCAGTATTTTAGGCTTCTTCGGTGA 278
                ******** ******************** ******************************

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1.483           AGACGTCGTCTATAAGCTGCGAACAACACTTTGGGATAAAATCTTAACCCTGCCGGTGGG 359
1.313           AGACGTCGTCTATAAGCTGCGAACAACACTTTGGGATAAAATCTTAACCCTGCCGGTGGG 359
1.485           AGACGTCGTCTATAAGCTGCGAACAACACTTTGGGATAAAATCTTAACCCTGCCGGTGGG 359
1.1282          AGACGTCGTCTATAAGCTGCGAACAACACTTTGGGATAAAATCTTAACCCTGCCGGTGGG 359
horA            AGACGTCGTCTATAAGCTGCGAACAACACTTTGGGATAAAATCTTAACCCTGCCGGTGGG 338
                ************************************************************

1.317           TTATTTTGACCAAACCAAATCTGGCGAAATAACGTCCAGGTTGGTCAATGATTCCACACA 419
1.483           TTATTTTGACCAAACCAAATCTGGCGAAATAACGTCCAGGTTGGTCAATGATTCCACACA 419
1.313           TTATTTTGACCAAACCAAATCTGGCGAAATAACGTCCAGGTTGGTCAATGATTCCACACA 419
1.485           TTATTTTGACCAAACCAAATCTGGCGAAATAACGTCCAGGTTGGTCAATGATTCCACACA 419
1.1282          TTATTTTGACCAAACCAAATCTGGCGAAATAACGTCCAGGTTGGTCAATGATTCCACACA 419
horA            TTATTTTGACCAAACCAAATCTGGCGAAATAACGTCCAGGTTGGTCAATGATTCCACACA 398
                ************************************************************
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1.317           GGTCAAGGAACTGTTGGCCAATTCGGTTCCCAAAACCGCAACTTCGATTCTGCAACTGGT 479
1.483           GGTCAAGGAACTGTTGGCCAATTCGGTTCCCAAAACCGCAACTTCGATTCTGCAACTGGT 479
1.313           GGTCAAGGAACTGTTGGCCAATTCGGTTCCCAAAACCGCAACTTCGATTCTGCAACTGGT 479
1.485           GGTCAAGGAACTGTTGGCCAATTCGGTTCCCAAAACCGCAACTTCGATTCTGCAACTGGT 479
1.1282          GGTCAAGGAACTGTTGGCCAATTCGGTTCCCAAAACCGCAACTTCGATTCTGCAACTGGT 479
horA            GGTCAAGGAACTGTTGGCCAATTCGGTTCCCAAAACCGCAACTTCGATTCTGCAACTGGT 458
                ************************************************************

1.317           TGGCGCATTGGTCTTAATGCTCATCATGGACTGGCGGATGACTATCATTATGTTTATCGC 539
1.483           TGGCGCATTGGTCTTAATGCTCATCATGGACTGGCGGATGACTATCATTATGTTTATCGC 539
1.313           TGGCGCATTGGTCTTAATGCTCATCATGGACTGGCGGATGACTATCATTATGTTTATCGC 539
1.485           TGGCGCATTGGTCTTAATGCTCATCATGGACTGGCGGATGACTATCATTATGTTTATCGC 539
1.1282          TGGCGCATTGGTCTTAATGCTCATCATGGACTGGCGGATGACTATCATTATGTTTATCGC 539
horA            TGGCGCATTGGTCTTAATGCTCATCATGGACTGGCGGATGACTATCATTATGTTTATCGC 518
                ************************************************************

1.317           CGTTCCGCTCGTCTTGATCTGCCTGCTGCCAATTGTCCGCCAATCCCACAAAGTTGCCAG 599
1.483           CGTTCCGCTCGTCTTGATCTGCCTGCTGCCAATTGTCCGCCAATCCCACAAAGTTGCCAG 599
1.313           CGTTCCGCTCGTCTTGATCTGCCTGCTGCCAATTGTCCGCCAATCCCACAAAGTTGCCAG 599
1.485           CGTTCCGCTCGTCTTGATCTGCCTGCTGCCAATTGTCCGCCAATCCCACAAAGTTGCCAG 599
1.1282          CGTTCCGCTCGTCTTGATCTGCCTGCTGCCAATTGTCCGCCAATCCCACAAAGTTGCCAG 599
horA            CGTTCCGCTCGTCTTGATCTGCCTGCTGCCAATTGTCCGCCAATCCCACAAAGTTGCCAG 578
                ************************************************************

1.317           AGCGAGACAGGACGCACTGGCAGATCTCAATGGTAAAGCCGGTGAAATGCTGGGCGAAGT 659
1.483           AGCGAGACAGGACGCACTGGCAGATCTCAATGGTAAAGCCGGTGAAATGCTGGGCGAAGT 659
1.313           AGCGAGACAGGACGCACTGGCAGATCTCAATGGTAAAGCCGGTGAAATGCTGGGCGAAGT 659
1.485           AGCGAGACAGGACGCACTGGCAGATCTCAATGGTAAAGCCGGTGAAATGCTGGGCGAAGT 659
1.1282          AGCGAGACAGGACGCACTGGCAGATCTCAATGGTAAAGCCGGTGAAATGCTGGGCGAAGT 659
horA            AGCGAGACAGGACGCACTGGCAGATCTCAATGGTAAAGCCGGTGAAATGCTGGGCGAAGT 638
                ************************************************************

1.317           CCGTCTAGTCAAATCGTCTACCGCAGAAAACTTAGAACGAACAGCCGGCGATAAACGGAT 719
1.483           CCGTCTAGTCAAATCGTCTACCGCAGAAAACTTAGAACGAACAGCCGGCGATAAACGGAT 719
1.313           CCGTCTAGTCAAATCGTCTACCGCAGAAAACTTAGAACGAACAGCCGGCGATAAACGGAT 719
1.485           CCGTCTAGTCAAATCGTCTACCGCAGAAA-CTTAGAACGAACAGCCGGCGATAAACGGAT 718
1.1282          CCGTCTAGTCAAATCGTCTACCGCAGAAAACTTAGAACGAACAGCCGGCGATAAACGGAT 719
horA            CCGTCTAGTCAAATCGTCTACCGCAGAAAACTTAGAACGAACAGCCGGCGATAAACGGAT 698
                ***************************** ******************************

1.317           GTATCGCCTTTATCGCATCGGGTTAAAAGAAGCGATCTATGATTCAATTGCCGGACCTGT 779
1.483           GTATCGCCTTTATCGCATCGGGTTAAAAGAAGCGATCTATGATTCAATTGCCGGACCTGT 779
1.313           GTATCGCCTTTATCGCATCGGGTTAAAAGAAGCGATCTATGATTCAATTGCCGGACCTGT 779
1.485           GTATCGCCTTTATCGCATCGGGTTAAAAGAAGCGATCTATGATTCAATTGCCGGACCTGT 778
1.1282          GTATCGCCTTTATCGCATCGGGTTAAAAGAAGCGATCTATGATTCAATTGCCGGACCTGT 779
horA            GTATCGCCTTTATCGCATCGGGTTAAAAGAAGCGATCTATGATTCAATTGCCGGACCTGT 758
                ************************************************************

1.317           AATGGGCATGGTCATGATGGCCATGGTCCTGGGAATTCTGGGCTATGGTGCGATCCGGGT 839
1.483           AATGGGCATGGTCATGATGGCCATGGTCCTGGGAATTCTGGGCTATGGTGCGATCCGGGT 839
1.313           AATGGGCATGGTCATGATGGCCATGGTCCTGGGAATTCTGGGCTATGGTGCGATCCGGGT 839
1.485           AATGGGCATGGTCATGATGGCCATGGTCCTGGAAATTCTGGGCTATGGTGCGATCCGGGT 838
1.1282          AATGGGCATGGTCATGATGGCCATGGTCCTGGAAATTCTGGGCTATGGTGCGATCCGGGT 839
horA            AATGGGCATGGTCATGATGGCCATGGTCCTGGAAATTCTGGGCTATGGTGCGATCCGGGT 818
                ******************************** ***************************

1.317           TCGGGAAGGTGCCATTGATATTGGGACCTTATTTTCATTTCTGATGTACCTGGTTCAAAT 899
1.483           TCGGGAAGGTGCCATTGATATTGGGACCTTATTTTCATTTCTGATGTACCTGGTTCAAAT 899
1.313           TCGGGAAGGTGCCATTGATATTGGGACCTTATTTTCATTTCTGATGTACCTGGTTCAAAT 899
1.485           TCGGGAAGGTGCCATTGATATTGGGACCTTATTTTCATTTCTGATGTACCTGGTTCAAAT 898
1.1282          TCGGGAAGGTGCCATTGATATTGGGACCTTATTTTCATTTCTGATGTACCTGGTTCAAAT 899
horA            TCGGGAAGGTGCCATTGATATTGGGACCTTATTTTCATTTCTGATGTACCTGGTTCAAAT 878
                ************************************************************
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1.317           GATTAGTCCATTTGCGGTTCTCGGCCAATTCATGTCTGATGTTGCCAAGGCAAGTGGCTC 959
1.483           GATTAGTCCATTTGCGGTTCTCGGCCAATTCATGTCTGATGTTGCCAAGGCAAGTGGCTC 959
1.313           GATTAGTCCATTTGCGGTTCTCGGCCAATTCATGTCTGATGTTGCCAAGGCAAGTGGCTC 959
1.485           GATTAGTCCATTTGCGGTTCTCGGCCAATTCATGTCTGATGTTGCCAAGGCAAGTGGCTC 958
1.1282          GATTAGTCCATTTGCGGTTCTCGGCCAATTCATGTCTGATGTTGCCAAGGCAAGTGGCTC 959
horA            GATTAGTCCATTTGCGGTTCTCGGCCAATTCATGTCTGATGTTGCCAAGGCAAGTGGCTC 938
                ************************************************************

1.317           AACCACTCGAATCCAGGCATTATTGCAAACTCATGAAGAAGATCGTCTGACTGGAACGGA 1019
1.483           AACCACTCGAATCCAGGCATTATTGCAAACTCATGAAGAAGATCGTCTGACTGGAACGGA 1019
1.313           AACCACTCGAATCCAGGCATTATTGCAAACTCATGAAGAAGATCGTCTGACTGGAACGGA 1019
1.485           AACCACTCGAATCCAGGCATTATTGCAAACTCATGAAGAAGATCGTCTGACTGGAACGGA 1018
1.1282          AACCACTCGAATCCAGGCATTATTGCAAACTCATGAAGAAGATCGTCTGACTGGAACGGA 1019
horA            AACCACTCGAATCCAGGCATTATTGCAAACTCATGAAGAAGATCGTCTGACTGGAACGGA 998
                ************************************************************

1.317           TTTGGATATTGGCGATCAAACACTTCAGATGAACCACGTCAGTTTTTCTTATGATCAGCA 1079
1.483           TTTGGATATTGGCGATCAAACACTTCAGATGAACCACGTCAGTTTTTCTTATGATCAGCA 1079
1.313           TTTGGATATTGGCGATCAAACACTTCAGATGAACCACGTCAGTTTTTCTTATGATCAGCA 1079
1.485           TTTGGATATTGGCGATCAAACACTTCAGATGAACCACGTCAGTTTTTCTTATGATCAGCA 1078
1.1282          TTTGGATATTGGCGATCAAACACTTCAGATGAACCACGTCAGTTTTTCTTATGATCAGCA 1079
horA            TTTGGATATTGGCGATCAAACACTTCAGATGAACCACGTCAGTTTTTCTTATGATCAGCA 1058
                ************************************************************

1.317           TCACCCCATTTTATCCGACGTGTCGTTTACGGCAGAACCCAATTCGGTCATTGCCTTTGC 1139
1.483           TCACCCCATTTTATCCGACGTGTCGTTTACGGCAGAACCCAATTCGGTCATTGCCTTTGC 1139
1.313           TCACCCCATTTTATCCGACGTGTCGTTTACGGCAGAACCCAATTCGGTCATTGCCTTTGC 1139
1.485           TCACCCCATTTTATCCGGCGTGTCGTTTACGGCAGAACCCAATTCGGTCATTGCCTTTGC 1138
1.1282          TCACCCCATTTTATCCGGCGTGTCGTTTACGGCAGAACCCAATTCGGTCATTGCCTTTGC 1139
horA            TCACCCCATTTTATCCGGCGTGTCGTTTACGGCAGAACCCAATTCGGTCATTGCCTTTGC 1118
                ***************** ******************************************

1.317           CGGACCATCCGGCGGTGGCAAATCAACCATTTTCAGCTTAATTGAACGTTTTTATGAACC 1199
1.483           CGGACCATCCGGCGGTGGCAAATCAACCATTTTCAGCTTAATTGAACGTTTTTATGAACC 1199
1.313           CGGACCATCCGGCGGTGGCAAATCAACCATTTTCAGCTTAATTGAACGTTTTTATGAACC 1199
1.485           CGGACCATCCGGCGGTGGCAAATCAACCATTTTCAGCTTAATTGAACGTTTTTATGAACC 1198
1.1282          CGGACCATCCGGCGGTGGCAAATCAACCATTTTCAGCTTAATTGAACGTTTTTATGAACC 1199
horA            CGGACCATCCGGCGGTGGCAAATCAACCATTTCTAGCTTAATTGAACGTTTTTATGAACC 1178
                ********************************  **************************

1.317           TAACGAGGGCAGCATCACGATTGGCAATACCAATATTACTGATATTCAACTTGCCGATTG 1259
1.483           TAACGAGGGCAGCATCACGATTGGCAATACCAATATTACTGATATTCAACTTGCCGATTG 1259
1.313           TAACGAGGGCAGCATCACGATTGGCAATACCAATATTACTGATATTCAACTTGCCGATTG 1259
1.485           TAACGAGGGCAGCATCACGATTGGCAATACCAATATTACTGATATTCAACTTGCCGATTG 1258
1.1282          TAACGAGGGCAGCATCACGATTGGCAATACCAATATTACTGATATTCAACTTGCCGATTG 1259
horA            TAACGAGGGCAGCATCACGATTGGCAATACCAATATTACTGATATTCAACTTGCCGATTG 1238
                ************************************************************

1.317           GCGCCAGCAAATCGGCCTGGTCGGCCAAGACGCTGCGATCATGTCTGGAACGATTCGTTA 1319
1.483           GCGCCAGCAAATCGGCCTGGTCGGCCAAGACGCTGCGATCATGTCTGGAACGATTCGTTA 1319
1.313           GCGCCAGCAAATCGGCCTGGTCGGCCAAGACGCTGCGATCATGTCTGGAACGATTCGTTA 1319
1.485           GCGCCAGCAAATCGGCCTGGTCGGCCAAGACGCTGCGATCATGTCTGGAACGATTCGTTA 1318
1.1282          GCGCCAACAAATCGGCCTGGTCGGCCAAGACGCTGCGATCATGTCTGGAACGATTCGTTA 1319
horA            GCGCCAGCAAATCGGCCTGGTCGGCCAAGACGCTGCGATCATGTCTGGAACGATTCGTTA 1298
                ****** *****************************************************

1.317           CAATTTAACCTATGGTTTGCCGGGGCATTTTTCCGATGAACAGCTTTGGCATGTCTTGGA 1379
1.483           CAATTTAACCTATGGTTTGCCGGGGCATTTTTCCGATGAACAGCTTTGGCATGTCTTGGA 1379
1.313           CAATTTAACCTATGGTTTGCCGGGGCATTTTTCCGATGAACAGCTTTGGCATGTCTTGGA 1379
1.485           CAATTTAACCTATGGTTTGCCGGGGCATTTTTCCGATGAACAGCTTTGGCATGTCTTGGA 1378
1.1282          CAATTTAACCTATGGTTTGCCGGGGCATTTTTCCGATGAACAGCTTTGGCATGTCTTGGA 1379
horA            CAATTTAACCTATGGTTTGCCGGGGCATTTTTCCGATGAACAGCTTTGGCATGTCTTGGA 1358
                ************************************************************
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1.317           AATGGCTTACGCAACGCAATTTGTCCAGAAGATGCCTCGGGGCTTGGACACGGAAGTCGG 1439
1.483           AATGGCTTACGCAACGCAATTTGTCCAGAAGATGCCTCGGGGCTTGGACACGGAAGTCGG 1439
1.313           AATGGCTTACGCAACGCAATTTGTCCAGAAGATGCCTCGGGGCTTGGACACGGAAGTCGG 1439
1.485           AATGGCTTACGCAACGCAATTTGTCCAGAAGATGCCTCGGGGCTTGGACACGGAAGTCGG 1438
1.1282          AATGGCTTACGCAACGCAATTTGTCCAGAAGATGCCTCGGGGCTTGGACACGGAAGTCGG 1439
horA            AATGGCTTACGCAACGCAATTTGTCCAGAAGATGCCTCGGGGCTTGGACACGGAAGTCGG 1418
                ************************************************************

1.317           TGAGCGTGGAGTCAAGGTATCTGGGGGCCAACGCCAACGATTGGCGATTGCCCGGGCCTT 1499
1.483           TGAGCGTGGAGTCAAGGTATCTGGGGGCCAACGCCAACGATTGGCGATTGCCCGGGCCTT 1499
1.313           TGAGCGTGGAGTCAAGGTATCTGGGGGCCAACGCCAACGATTGGCGATTGCCCGGGCCTT 1499
1.485           TGAGCGTGGAGTCAAGGTATCGGGGGGCCAACGCCAACGATTGGCGATTGCCCGGGCCTT 1498
1.1282          TGAGCGTGGAGTCAAGGTATCGGGGGGCCAACGCCAACGATTGGCGATTGCCCGGGCCTT 1499
horA            TGAGCGTGGAGTCAAGGTATCGGGGGGCCAACGCCAACGATTGGCGATTGCCCGGGCCTT 1478
                ********************* **************************************

1.317           CCTGCGTAATCCAAAAATCTTAATGTTGGATGAAGCAACGGCGAGCCTGGATTCCGAGTC 1559
1.483           CCTGCGTAATCCAAAAATCTTAATGTTGGATGAAGCAACGGCGAGCCTGGATTCCGAGTC 1559
1.313           CCTGCGTAATCCAAAAATCTTAATGTTGGATGAAGCAACGGCGAGCCTGGATTCCGAGTC 1559
1.485           CCTGCGTAATCCAAAAATCTTAATGTTGGATGAAGCAACGGCGAGCCTGGATTCCGAGTC 1558
1.1282          CCTGCGTAATCCAAAAATATTAATGTTGGATGAAGCAACGGCGAGCCTGGATTCCGAGTC 1559
horA            CCTGCGTAATCCAAAAATATTAATGTTGGATGAAGCAACGGCGAGCCTGGATTCCGAGTC 1538
                ****************** *****************************************

1.317           CGAAATGATGGTCCAAAAAGCGCTGGACCAGTTGATGGCCAATCGAACAACATTGGTGAT 1619
1.483           CGAAATGATGGTCCAAAAAGCGCTGGACCAGTTGATGGCCAATCGAACAACATTGGTGAT 1619
1.313           CGAAATGATGGTCCAAAAAGCGCTGGACCAGTTGATGGCCAATCGAACAACATTGGTGAT 1619
1.485           CGAAATGATGGTCCAAAAAGCGCTGGACCAGTTGATGGCCAATCGAACAACATTGGTGAT 1618
1.1282          CGAAATGATGGTCCAAAAAGCGCTGGACCAGTTGATGGCCAATCGAACAACATTGGTGAT 1619
horA            CGAAATGATGGTCCAAAAAGCGCTGGACCAGTTGATGGCCAATCGAACAACATTGGTGAT 1598
                ************************************************************

1.317           CGCCCACAGGCTAAGCACAATTACCAACGCCGACGAAATTTATTTCATAGAAAACGGCAG 1679
1.483           CGCCCACAGGCTAAGCACAATTACCAACGCCGACGAAATTTATTTCATAGAAAACGGCAG 1679
1.313           CGCCCACAGGCTAAGCACAATTACCAACGCCGACGAAATTTATTTCATAGAAAACGGCAG 1679
1.485           CGCCCACAGGCTAAGCACAATTACCAACGCCGACGAAATTTATTTCATAGAAAACGGCAG 1678
1.1282          CGCCCACAGGCTAAGCACAATTACCAACGCCGACGAAATTTATTTCATAGAAAACGGCAG 1679
horA            CGCCCACAGGCTAAGCACAATTACCAACGCCGACGAAATTTATTTCATAGAAAACGGCAG 1658
                ************************************************************

1.317           GGTAACGGGCCAGGGAACCCACCAACAGTTAGTGAAAACGACTCCTTTGTATAGGGAGTA 1739
1.483           GGTAACGGGCCAGGGAACCCACCAACAGTTAGTGAAAACGACTCCTTTGTATAGGGAGTA 1739
1.313           GGTAACGGGCCAGGGAACCCACCAACAGTTAGTGAAAACGACTCCTTTGTATAGGGAGTA 1739
1.485           GGTAACGGGCCAGGGAACCCACCAACAGTTAGTGAAAACGACTCCTTTGTATAGGGAGTA 1738
1.1282          GGTAACGGGCCAGGGAACCCACCAACAGTTAGTGAAAACGACTCCTTTGTATAGGGAGTA 1739
horA            GGTAACGGGCCAGGGAACCCACCAACAGTTAGTGAAAACGACTCCTTTGTATAGGGAGTA 1718
                ************************************************************

1.317           TGTGAAAAATCAGAGCGCGACGAGCAACTGGTGA 1773
1.483           TGTGAAAAATCAGAGCGCGACGAGCAACGGGTGA 1773
1.313           TGTGAAAAATCAGAGCGCGACGAGCAACGGGTGA 1773
1.485           TGTGAAAAATCAGAGCGCGACGAGCAACGGGTGA 1772
1.1282          TGTGAAAAATCAGAGCGCGACGAGCAACGGGTGA 1773
horA            TGTGAAAAATCAGAGCGCGACGAGCAACGGGTGA 1752
                **************************** *****
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Partial alignment of the *horA* gene found in beer spoiling *Lactobacillus brevis* TMW 1.465. Given are the first 500 bp, each. The sequence from the originally published *horA* was taken from the literature (Sami *et al.* 1997).

```
1.465           ATGCAAGCTCAGTCCAAGAACAATACCAAGTTTAACTTTAAAACATTTATGGGCCTAATC 60
horA            ATGCAAGCTCAGTCCAAGAACAATACCAAGTTTAACTTTAAAACATTTATGGGCCTAATC 60
                ************************************************************

1.465           AACCGAATTCACCCCCGTTACTGGCAACTGCTGTTTGG-TTTTTTCTAGGAGTTGTCGCA 119
horA            AACCGAATTCACCCCCGTTACTGGCAACTGCTGCTTGGCTTTTTTCTAGGAGTTGTCGCA 120
                ********************************* **** *********************

1.465           ACGGCGATGCAATTGATGGTTCCCGGCATCGCCAAGGGGATCATCAACTCAATCGGTCAT 179
horA            ACGGCGATGCAATTGATGGTTCCCGGCATCGCCAAGGGGATCATCAACTCAATCGGTCAT 180
                ************************************************************

1.465           TCAATGGATGTCGGCCTAATCGTTGCCGTCATTTTACTATTCGTTTTCAGTACCATTATT 239
horA            TCAATGGATGTCGGCCTAATCGTTGCCGTCATTTTACTATTCGTTTCCAGTACCATTATT 240
                ********************************************** *************

1.465           GGAGCCTCTTCCGGCAGTATTTTAGGCTTCTTCGGTGAAGACGTCGTCTATAAGCTGCGA 299
horA            GGAGCCTTTTCCGGCAGTATTTTAGGCTTCTTCGGTGAAGACGTCGTCTATAAGCTGCGA 300
                ******* ****************************************************

1.465           ACAACACTTTGGGATAAAATCTTAACCCTGCCGGTGGGTTATTTTGACCAAACCAAGTCT 359
horA            ACAACACTTTGGGATAAAATCTTAACCCTGCCGGTGGGTTATTTTGACCAAACCAAATCT 360
                ******************************************************** ***

1.465           GGCGAAATAACGTCCAGGTTGGTCAATGATTCCACACAGGTCAAGGAACTGTTGGCCAAT 419
horA            GGCGAAATAACGTCCAGGTTGGTCAATGATTCCACACAGGTCAAGGAACTGTTGGCCAAT 420
                ************************************************************

1.465           TCGGTTCCCAAAACCGCAACTTCGATTCTGCAACTGGTTGGCGCATTGGTCTTAATGCTC 479
horA            TCGGTTCCCAAAACCGCAACTTCGATTCTGCAACTGGTTGGCGCATTGGTCTTAATGCTC 480
                ************************************************************

1.465           ATCATGGACTGGCGGATGACTATCATTATGTTTATCGCCGTTCCGCTCGTCTTGATCTGC 539
horA            ATCATGGACTGGCGGATGACTATCATTATGTTTATCGCCGTTCCGCTCGTCTTGATCTGC 540
                ************************************************************

1.465           CTGCTGCCAATTGTCCGCCAATCCCACAAAGTTGCCAGAGCGAGACAGGACGCACTGGCA 599
horA            CTGCTGCCAATTGTCCGCCAATCCCACAAAGTTGCCAGAGCGAGACAGGACGCACTGGCA 600
                ************************************************************
```

Amino acid sequence of the HorA fragment of TMW 1.485 from the start codon till abort of the translation.

Met Q A Q S K N N T K F N F K T F **Met** G L I N R I H P R Y W Q L L L G F F L G V V A T A **Met** Q L **Met** V P G I A K G I I N S I G H S **Met** D V G L I V A V I L L F V F S T I I G A F S G S I L G F F G E D V V Y K L R T T L W D K I L T L P V G Y F D Q T K S G E I T S R L V N D S T Q V K E L L A N S V P K T A T S I L Q L V G A L V L **Met** L I **Met** D W R **Met** T I I **Met** F I A V P L V L I C L L P I V R Q S H K V A R A R Q D A L A D L N G K A G E **Met** L G E V R L V K S S T A E T

Amino acid sequence of the HorA fragment of TMW 1.465 from the start codon till abort of the translation

Met Q A Q S K N N T K F N F K T F **Met** G L I N R I H P R Y W Q L L F G F F

Alignment of HorA from *Lb. brevis* TMW 1.1282 and LmrA from *Lactococcus lactis* MG 1363 to show varying and according domains.

```
1.1282          --------MQAQSKNNTKFNFKTFMGLINRIHPRYWQLLLGFFLGVVATAMQLMVPGIAK 52
LmrA            MERGPQMANRIEGKAVDKTSIKHFVKLIRAAKPRYLFFVIGIVAGIIGTLIQLQVPKMVQ 60
                         : :.*   * .:* *: **.  :***  :::*:. *::.* :** ** :.:

1.1282          GIINSIGHSMDVGLIVAVILLFVFSTIIGAFSGSILGFFGEDVVYKLRTTLWDKILTLPV 112
LmrA            PLINSFGHGVNGGKVALVIALYIGSAAVSAIAAIVLGIFGESVVKNLRTRVWDKMIHLPV 120
                 :***:**.:: * :. ** *:: *: :.*::. :**:***.** :*** :***:: ***

1.1282          GYFDQTKSGEITSRLVNDSTQVKELLANSVPKTATSILQLVGALVLMLIMDWRMTIIMFI 172
LmrA            KYFDEVKTGEMSSRLANDTTQVKNLIANSIPQAFTSILLLVGSIIFMLQMQWRLTLAMII 180
                 ***:.*:**::***.**:****:*:***:*:: **** ***::::** *:**:*: *:*

1.1282          AVPLVLICLLPIVRQSHKVARARQDALADLNGKAGEMLGEVRLVKSSTAENLERTAGDKR 232
LmrA            AVPIVMLIMFPIMTFGQKIGWTRQDSLANFQGIASESLSEIRLVKSSNAEKQASKKAEND 240
                ***:*:: ::**:  .:*:. :***:**:::* *.* *.*:******.**:   . .::

1.1282          MYRLYRIGLKEAIYDSIAGPVMGMVMMAMVLEILGYGAIRVREGAIDIGTLFSFLMYLVQ 292
LmrA            VNALYKIGVKEAVFDGLMSPVMMLSMMLMIFGLLAYGIYLISTGVMSLGTLLGMMMYLMN 300
                :  **:**:***::*.: .*** : ** *:: :*.**   :  *.:.:***:.::***::

1.1282          MISPFAVLGQFMSDVAKASGSTTRIQALLQTHEEDRLTGTDLDIGDQTLQMNHVSFSYDQ 352
LmrA            LIGVVPTVATFFTELAKASGSTGRLTELLDEEQEVLHQGDSLDLEGKTLSAHHVDFAYDD 360
                :*. ...:. *::::******* *:  **: .:*    * .**: .:**. :**.*:**:

1.1282          HHPILSGVSFTAEPNSVIAFAGPSGGGKSTIFSLIERFYEPNEGSITIGNTNITDIQLAD 412
LmrA            SEQILHDISFEAQPNSIIAFAGPSGGGKSTIFSLLERFYQPTAGEITIGGQPIDSVSLEN 420
                 . ** .:** *:***:*****************:****:*. *.****.  * .:.* :

1.1282          WRQQIGLVGQDAAIMSGTIRYNLTYGLPGHFSDEQLWHVLEMAYATQFVQKMPRGLDTEV 472
LmrA            WRSQIGFVSQDSAIMAGTIRENLTYGLEGNFTDEDLWQVLDLAFARSFVENMPDQLNTEV 480
                **.***:*.**:***:**** ****** *:*:**:**:**::*:* .**::**  *:***

1.1282          GERGVKVSGGQRQRLAIARAFLRNPKILMLDEATASLDSESEMMVQKALDQLMANRTTLV 532
LmrA            GERGVKISGGQRQRLAIARAFLRNPKILMLDEATASLDSESESMVQRALDSLMKGRTTLV 540
                ******:*********************************** ***:***.** .*****

1.1282          IAHRLSTITNADEIYFIENGRVTGQGTHQQLVKTTPLYREYVKNQSATSNG 583
LmrA            IAHRLSTIVDADKIYFIEKGEITGSGKHNELVATHPLYAKYVSEQLTVGQ- 590
                ********.:**:*****:*.:**.*.*::** * *** :**.:* :..:
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Alignment of the translated functional *horA* genes found in beer spoiling *Lactobacillus brevis* strains. Given is the TMW number. TMS (green letters) and ABS (blue letters) were determined with a comparison with the sequence from LmrA.

```
1.317                MLHHHHHHQAQSKNNTKFNFKTFMGLINRIHPRYWQLLFGFFLGVVATAMQLMVPGIAKG 60
1.483                MLHHHHHHQAQSKNNTKFNFKTFMGLINRIHPRYWQLLFGFFLGVVATAMQLMVPGIAKG 60
1.313                MLHHHHHHQAQSKNNTKFNFKTFMGLINRIHPRYWQLLFGFFLGVVATAMQLMVPGIAKG 60
1.1282               MLHHHHHHQAQSKNNTKFNFKTFMGLINRIHPRYWQLLLGFFLGVVATAMQLMVPGIAKG 60
                     **************************************:*********************

1.317                IINSIGHSMDVGLIVAVILLFVFSTIIGASSGSILGFFGEDVVYKLRTTLWDKILTLPVG 120
1.483                IINSIGHSMDVGLIVAVILLFVFSTIIGASSGSILGFFGEDVVYKLRTTLWDKILTLPVG 120
1.313                IINSIGHSMDVGLIVAVILLFVFSTIIGASSGSILGFFGEDVVYKLRTTLWDKILTLPVG 120
1.1282               IINSIGHSMDVGLIVAVILLFVFSTIIGAFSGSILGFFGEDVVYKLRTTLWDKILTLPVG 120
                     ***************************** ******************************

1.317                YFDQTKSGEITSRLVNDSTQVKELLANSVPKTATSILQLVGALVLMLIMDWRMTIIMFIA 180
1.483                YFDQTKSGEITSRLVNDSTQVKELLANSVPKTATSILQLVGALVLMLIMDWRMTIIMFIA 180
1.313                YFDQTKSGEITSRLVNDSTQVKELLANSVPKTATSILQLVGALVLMLIMDWRMTIIMFIA 180
1.1282               YFDQTKSGEITSRLVNDSTQVKELLANSVPKTATSILQLVGALVLMLIMDWRMTIIMFIA 180
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1.317                VPLVLICLLPIVRQSHKVARARQDALADLNGKAGEMLGEVRLVKSSTAENLERTAGDKRM 240
1.483                VPLVLICLLPIVRQSHKVARARQDALADLNGKAGEMLGEVRLVKSSTAENLERTAGDKRM 240
1.313                VPLVLICLLPIVRQSHKVARARQDALADLNGKAGEMLGEVRLVKSSTAENLERTAGDKRM 240
1.1282               VPLVLICLLPIVRQSHKVARARQDALADLNGKAGEMLGEVRLVKSSTAENLERTAGDKRM 240
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1.317                YRLYRIGLKEAIYDSIAGPVMGMVMMAMVLGILGYGAIRVREGAIDIGTLFSFLMYLVQM 300
1.483                YRLYRIGLKEAIYDSIAGPVMGMVMMAMVLGILGYGAIRVREGAIDIGTLFSFLMYLVQM 300
1.313                YRLYRIGLKEAIYDSIAGPVMGMVMMAMVLGILGYGAIRVREGAIDIGTLFSFLMYLVQM 300
1.1282               YRLYRIGLKEAIYDSIAGPVMGMVMMAMVLEILGYGAIRVREGAIDIGTLFSFLMYLVQM 300
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1.317                ISPFAVLGQFMSDVAKASGSTTRIQALLQTHEEDRLTGTDLDIGDQTLQMNHVSFSYDQH 360
1.483                ISPFAVLGQFMSDVAKASGSTTRIQALLQTHEEDRLTGTDLDIGDQTLQMNHVSFSYDQH 360
1.313                ISPFAVLGQFMSDVAKASGSTTRIQALLQTHEEDRLTGTDLDIGDQTLQMNHVSFSYDQH 360
1.1282               ISPFAVLGQFMSDVAKASGSTTRIQALLQTHEEDRLTGTDLDIGDQTLQMNHVSFSYDQH 360
                     ************************************************************
                                  ______Walker A______
1.317                HPILSDVSFTAEPNSVIAFAGPSGGGKSTIFSLIERFYEPNEGSITIGNTNITDIQLADW 420
1.483                HPILSDVSFTAEPNSVIAFAGPSGGGKSTIFSLIERFYEPNEGSITIGNTNITDIQLADW 420
1.313                HPILSDVSFTAEPNSVIAFAGPSGGGKSTIFSLIERFYEPNEGSITIGNTNITDIQLADW 420
1.1282               HPILSGVSFTAEPNSVIAFAGPSGGGKSTIFSLIERFYEPNEGSITIGNTNITDIQLADW 420
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1.317                RQQIGLVGQDAAIMSGTIRYNLTYGLPGHFSDEQLWHVLEMAYATQFVQKMPRGLDTEVG 480
1.483                RQQIGLVGQDAAIMSGTIRYNLTYGLPGHFSDEQLWHVLEMAYATQFVQKMPRGLDTEVG 480
1.313                RQQIGLVGQDAAIMSGTIRYNLTYGLPGHFSDEQLWHVLEMAYATQFVQKMPRGLDTEVG 480
1.1282               RQQIGLVGQDAAIMSGTIRYNLTYGLPGHFSDEQLWHVLEMAYATQFVQKMPRGLDTEVG 480
                     ************************************************************
                                      ___Walker B___
1.317                ERGVKVSGGQRQRLAIARAFLRNPKILMLDEATASLDSESEMMVQKALDQLMANRTTLVI 540
1.483                ERGVKVSGGQRQRLAIARAFLRNPKILMLDEATASLDSESEMMVQKALDQLMANRTTLVI 540
1.313                ERGVKVSGGQRQRLAIARAFLRNPKILMLDEATASLDSESEMMVQKALDQLMANRTTLVI 540
1.1282               ERGVKVSGGQRQRLAIARAFLRNPKILMLDEATASLDSESEMMVQKALDQLMANRTTLVI 540
                     ************************************************************

1.317                AHRLSTITNADEIYFIENGRVTGQGTHQQLVKTTPLYREYVKNQSATSNW 590
1.483                AHRLSTITNADEIYFIENGRVTGQGTHQQLVKTTPLYREYVKNQSATSNG 590
1.313                AHRLSTITNADEIYFIENGRVTGQGTHQQLVKTTPLYREYVKNQSATSNG 590
1.1282               AHRLSTITNADEIYFIENGRVTGQGTHQQLVKTTPLYREYVKNQSATSNG 590
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Lebenslauf

Holger Teichert

Geburtsdatum:	10.02.1977
Geburtsort:	Bayreuth
Nationalität:	deutsch
1984-1988	Grundschule Hollfeld
1988-1993	Staatliche Gesamtschule Hollfeld, gymnasialer Zweig
1993-1997	Wirschaftswissenschaftliches und mathematisch naturwissenschaftliches Gymnasium Bayreuth, naturwissenschaftlicher Zweig
1997	Allgemeine Hochschulreife
07/1997-02/1999	Wehrdienst
03/1999-08/1999	Industriepraktikum
09/1999-02/2005	Studium Technologie und Biotechnologie der Lebensmittel, Technische Universität München; Diplomarbeit „Identifizierung und Charakterisierung Trocknungsstress induzierter Stressproteine von *Shigella sonneii* und *Salmonella enterica* ssp. Enterititis“
02/2005	Studienabschluss als Diplom Ingenieur (Univ.)
02/2005-06/2008	Wissenschaftlicher Mitarbeiter am Lehrstuhl für Technische Mikrobiologie Weihenstephan, Technische Universität München Beginn der Dissertation
Seit 07/2008	Angestellter bei Campbell's Germany GmbH, Lübeck