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Mechanisms of hop inhibition, tolerance and adaptation in *Lactobacillus brevis*

Jürgen Behr

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Vorwort

Abbreviations

2D	two-dimensional
A	ampere
Å	Angström
A23187	calcimycin
ADI	arginine deiminase
AI	acid stress inducible protein
ATCC	American type culture collection, Manassas, Virginia, USA
ATP	adenosintriphosphate
BCECF	2('),7(')-bis(carboxyethyl)-5,6-carboxyfluorescein
BLAST	basic local alignment search tool
BLM	bilayer lipid membrane
Caps	N-cyclohexyl-3-aminopropanesulfonic acid
CCCP	carbonyl cyanidem-chlorophenylhydrazone
CDCF	5(6)-carboxy-2,'7'-dichlorofluorescein
cdLTA	chemically deacylated lipoteichoic acid
CDS	coding sequence
cFDASE	carboxyfluorescein succinimidyl ester
Da	Dalton
DAD	diode array detector
DCI	2,6-dichlorophenol-indophenol
DDBJ	DNA Data Bank of Japan

DDM	dodecyl maltoside
DNA	desoxyribonucleic acid
DTT	dithiothreitol
E.	*Escherichia*
EDTA	ethylenediaminetetraacetic acid
F	Faraday constant
F	farad
FA	fatty acid
Fig.	figure
G	conductance
g	gram, g-force
GP	general polarization, phosphatidyl glycerol
h	hour
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	hop stress inducible protein
HO	hop stress overexpressed protein
HPLC	high-performance liquid chromatography
HR	hop stress repressed protein
IEF	isoelectric focussing
IPG	immobilized pH gradient
ITT	indicator time test
k	kilo (10^3)

L	liter
L.	*Lactobacillus*
LC-ESI MS/MS	liquid chromatography coupled to tandem mass spectrometry with electrospray ionization
m	milli (10^{-3}), meter
M	mega (10^6), molar
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
MDR	multidrug resistance
Mes	2-[N-morpholino]ethansulfonic acid
MIC	minimal inhibitory concentration
min	minute
mRNA	messenger ribonucleic acid
Mw	molecular weight
MZP	mean zero current potential
n	nano (10^{-9})
NAD(P)	nicotinamide adenine dinucleotide (phosphate)
NCBI	National center for Biotechnology Information
NHE	normal hydrogen electrode
NRAMP	natural resistance-associated macrophage protein
OD	optical density
OX	oxidized species, electron acceptor
PAGE	polyacrylamide gel electrophoresis

Pa	Pascal
PC	phosphatidyl choline, personal computer
pI	isoelectric point
pmf	proton motive force
ppm	parts per million
R	gas constant
RAPD	random amplified polymorphic DNA analysis
RED	reduced species, electron donor
RNA	ribonucleic acid
s	second
SDS	sodium n-dodecylsulfate
SNAFL-calcein	seminaphthofluorescein-calcein
T	temperature, cycle time
Tab.	table
TCA	tricarboxylic acid
TE	Tris, EDTA
TMW	Technische Mikrobiologie Weihenstephan
Tris	tris (hydroxymethyl) aminomethan
TTFB	tetrachlorotrifluoromethylbenzimidazole
U	units
V	volt
voltammetry	volt-amperometry

v/v	volume / volume
w/v	mass / volume
w/w	mass / mass
ϕ	electrode potential
λ	wavelength
μ	Micro (10^{-6})

Contents

1 Introduction

1.1 Beer spoiling *Lactobacillus brevis*

Beer is an unfavorable medium for many microorganisms. It contains only low amounts of nutritive substances (sugars and amino acids) and exhibits hurdles for bacterial growth, because of its extremely low content of oxygen (<0.1 ppm), the low pH (3.8-4.7), and the presence of ethanol (0.5-10 % w/w), carbon dioxide (approx. 0.5 % w/v) and hop compounds (approx. 29-95 µM of iso-α-acids) (Sakamoto and Konings 2003). Despite these adverse growth conditions a few bacteria are capable to grow in and spoil beer. Among them, lactic acid bacteria most frequently cause beer spoilage. The most prominent beer spoiling species is *Lactobacillus* (*L.*) *brevis*, which causes more than 50 % of spoilage incidents in breweries (Back 2004). *L. brevis* is a Gram-positive, non-sporeforming, nonmotile, short, rod-shaped bacterium, which grows optimal at 30 °C in the pH range from 4 to 6. It is an obligately heterofermentative bacterium, which uses hexoses via the 6-phosphogluconate pathway, producing lactic acid, carbon dioxide and ethanol and/or acetic acid in equimolar amounts (Kandler 1983). Pentoses enter this pathway at the stage of xylulose-5-phosphate and are fermented without carbon dioxide production (Wood and Holzapfel 1995). *L. brevis* has been isolated from many environments, e.g. faeces (origin of the type strain), foods (sourdough, beer) and fermented herbs. The species *L. brevis* is considered as a rather heterogeneous group, which can be subdivided in several clusters according to e.g. DNA-RAPD patterns (Behr 2002) or their ability to degrade carbohydrates and amino acids (Back 1994). Despite this heterogeneity, generally all strains exhibit a tolerance to hops (Sakamoto and Konings 2003; Suzuki et al. 2004).

1.2 Definition of hop tolerance, hop resistance and adaptation

In general, tolerance in bacteria is characterized by a decreased mortality with regard to sensitive cells in the presence of an antibiotic agent. The total number of bacteria remains constant over time. After a prolonged incubation of the bacteria in the presence of the antibacterial agent the mortality can decrease and a slow growth could be recognized. These incubated bacteria exhibit a higher resistance to the antibacterial drug. Resistant bacteria are unaffected by the antimicrobial drug and thus exhibit an unimpaired growth (Wiuff et al. 2005). The step from tolerance to resistance can be contributed or achieved by adaptation. This adaptation is defined as the adjustment of the microorganism to its environment, with concomitant increase in robustness. In literature, which covers the part of beer spoilage the

term hop resistance is normally used, since the investigations are related to bacteria, which grow in the presence of hop compounds and thus are designated as hop resistant. However, it is know that beer spoiling *L. brevis* strains sometimes show no growth in beer over several months until a subsequent increase in cell count can be detected (Suzuki et al. 2002; Sakamoto and Konings 2003). Accordingly the first phase of beer spoilage can be assigned to hop tolerance, while a hop resistance characterizes the second phase. In order to precisely delineate involved mechanisms, definitions of both terms as they are used in thesis are given first. As non-growing bacteria are hard to investigate and adaptation and thus the transition from hop tolerance to resistance is a dynamic property, an alternative definition, which is analytically accessible, has to be used. Accordingly, in this thesis hop tolerance is defined as state, where the growth rate of the bacterium is dependent on hop concentration in the growth medium within a definite range. Consequently, the increase of hop concentration above a certain value within this range will render the culture bacteriostatic and indicate that the bacterium is hop tolerant, but not yet resistant. Hop resistance is therefore defined as the stage when the growth rate is virtually unaffected by the hop concentration in this definite range. The terms "hop tolerant" and "non-adapted" as well as "hop resistant" and "hop adapted" are used as synonyms.

1.3 Hop plant and hop compounds

The tradition to use the inflorescences of the hop plant *Humulus lupulus* for beer brewing was documented first in the sixth century B.C. Since 1516, as Wilhelm IV introduced the German purity law for beer, hop compounds were extensively used. The constituents of the resinous granules, derived from the female inflorescences, called hop cones, can be divided in hexane soluble soft resin (10-25 % of hop cones) and insoluble hard resin (3-5 %). The soft resin consists of α- (humulone) and β-fraction (lupulone), which account for the bitter taste of hops. As hop cones are added to wort, during boiling, the humulone fraction undergoes an isomerization, while the lupulone fraction does not. The isohumulones or iso-α-acids exhibit enhanced water solubility in comparison to the original compounds and an elevated bitter taste. The β-fraction shows a poor solubility in aqueous solution, which is further decreased at low pH, and thus it is not transferred to beer. The major constituents comprising the iso-α-acids are isohumulone (see figure 1), isocohumulone and isoadhumulone, occurring as stereoisomers in their cis- or trans-form (Belitz et al. 2001; Garcia-Villalba et al. 2006). These compounds as well as their precursors are known to be highly reactive. During drying,

processing and storage they can undergo isomerization-, oxidation- and polymerization
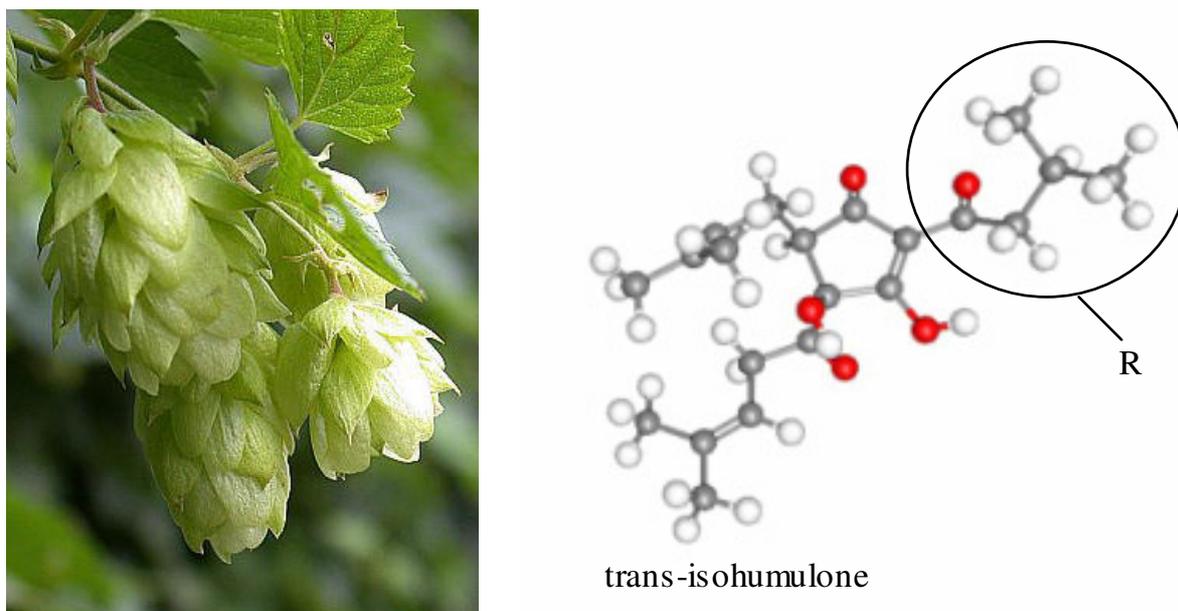
trans-isohumulone

Fig. 1 Hop cones and structure of isohumulone. Structure of isohumulone was generated with Ghemical (http://www.bioinformatics.org/ghemical) and optimized in a solvate box. Grey atoms = C; red atoms = O; white atoms = H; residue R: isohumulone, R = -OCH$_2$CH(CH$_3$)$_2$; isocohumulone, R= -OCH(CH$_3$)$_2$; isoadhumulone, R = -OCH(CH$_3$)CH$_2$CH$_3$.

reactions (personal communications Roland Schmitt, Nateco2 GmbH u. Co. KG, Mainburg, Hallertau, Germany), leading to various reaction products. This reactivity can be so pronounced, that hop stocks burn down by hops spontaneous combustion. Accordingly, hops are a multi-component mixture including hop-derived substances as well as their reaction products.

1.4 Hop inhibitory effect on bacteria

In addition to the comfortable bitterness of hop compounds in beer, they exhibit an inhibitory effect on bacteria. The antibiotic and bacteriostatic properties of hops were discovered about 70 years ago. From Shimwell in 1937 to Simpson in the 90's (Shimwell 1937; Simpson 1993) several factors of inhibitory mechanism of hop constituents were uncovered. Namely permeability changes of the bacterial cell wall (Shimwell 1937), leakage of the cytoplasmic membrane and a subsequent inhibition of respiration and protein, DNA and RNA synthesis (Teuber and Schmalreck 1973) as well as changes in leucine uptake and proton ionophore activity (Simpson 1993). This antibacterial activity was found to be restricted to Gram-positive bacteria, while Gram-negatives were nearly not inhibited (Shimwell 1937). A dependence of the inhibitory effect of hops on pH value and the decrease and increase of

antibacterial activity in the presence of divalent cations, such as Mn^{2+} and monovalent cations as K^+, respectively, was found by Shimwell and Simpson (Shimwell 1937; Simpson 1993). In summary of these literature data, hop inhibition has been described as the effect of hop compounds to act as ionophores, which dissipate the pH gradient across the cytoplasmic membrane and reduce the proton motive force (pmf). Consequently, the low intracellular pH interferes with essential enzyme reactions and the pmf-dependent nutrient uptake is hampered, resulting in cell death of hop sensitive strains (Simpson 1993; Sakamoto and Konings 2003; Yansanjav et al. 2004). The proton influx is thereby mediated by a proton/divalent cation exchange across the bacterial cytoplasmic membrane (Simpson 1993). However, the expected cross resistance to other proton ionophores could not be detected (Fernandez and Simpson 1993). A clear complication of hop inhibition research is the complexity of chemical composition of hops due to compound variation on account of consecutive chemical conversions as well as the variations of the natural product (Simpson and Smith 1992).

1.5 Principles of hop resistance described in literature

The tolerance to hops is a prerequisite for the capability of lactic acid bacteria to grow in beer and thus cause beer spoilage. Several mechanisms involved in hop resistance of lactobacilli have been described (Simpson and Fernandez 1994; Sami et al. 1998; Ulmer et al. 2000; Hayashi et al. 2001; Sakamoto et al. 2001; Ulmer et al. 2002; Sakamoto and Konings 2003; Suzuki et al. 2004; Suzuki et al. 2004; Suzuki et al. 2005). Proteins contributing to hop resistance include multidrug resistance (MDR) transporters that excrete the hop compounds into the outer medium (Sakamoto et al. 2001; Suzuki et al. 2002), and proton export systems that maintain the intracellular pH. HitA is a putative divalent cation transporter, whose gene is present in most beer spoiling lactobacilli (Hayashi et al. 2001). An alteration of teichoic acids in the cell wall (Yasui and Yoda 1997) and a changed lipid composition of the cytoplasmic membranes (Sakamoto and Konings 2003) might additionally contribute to the hop resistance (see figure 2). However, in contradiction to MDR transport mechanisms it was also proposed, that in hop resistant bacteria, the inability of hop compounds to reach their target is not responsible for hop resistance (Simpson 1993). Thus, the hop resistant cells apparently developed a strategy to survive and grow in the presence of hop compounds. The mechanism of this type of hop resistance in beer spoiling lactobacilli is not understood. Some potentially hop-resistant strains cannot grow in beer unless they have first been exposed to subinhibitory concentrations of hop compounds, but the mechanisms of adaptation are not understood

(Simpson and Fernandez 1992). The specific roles of single hop resistance mechanisms
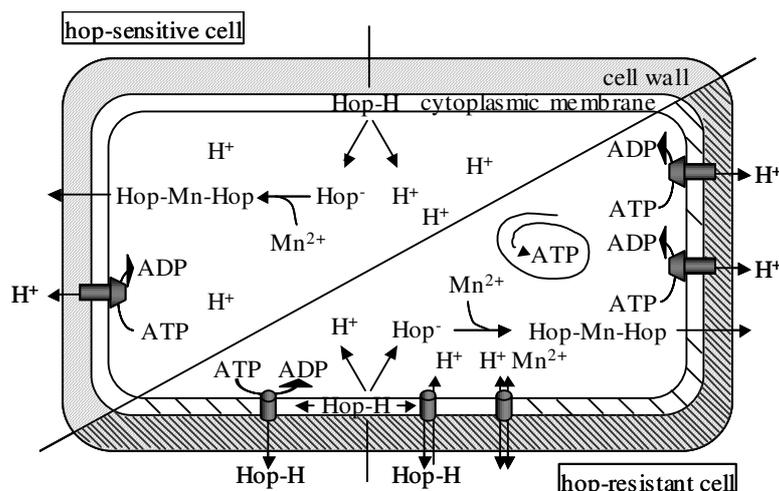
Fig. 2 Hop resistance mechanisms in *Lactobacillus brevis* as reported in literature. Hop compounds are described as ionophores, which dissipate the pH gradient across the cytoplasmic membrane and reduce the proton motive force (pmf). The low intracellular pH interferes with essential enzyme reactions and the pmf-dependent nutrient uptake is hampered, resulting in cell death of hop sensitive strains (Simpson 1993; Sakamoto and Konings 2003; Yansanjav, Siegumfeldt et al. 2004). The proton influx is mediated by a proton/divalent cation exchange upon the bacterial cytoplasmic membrane (Simpson 1993). Proteins contributing to hop resistance include pmf and ATP driven multi-drug-resistance (MDR) transporters that excrete the hop compounds into the outer medium (Sakamoto, Margolles et al. 2001; Suzuki, Sami et al. 2002), and proton export systems that maintain the intracellular pH. HitA is a putative divalent cation transporter present predominantly in beer spoiling lactobacilli (Hayashi, Ito et al. 2001). An alteration of teichoic acids in the cell wall (Yasui and Yoda 1997) and a changed lipid composition of the cytoplasmic membrane (Sakamoto and Konings 2003) might additionally contribute to the hop resistance. Adopted from Sakamoto et al. (Sakamoto and Konings 2003).

described in literature is not fully understood, especially because none of them confers hop resistance in the absence of other (unknown) mechanisms of hop resistance. Apparently, hop resistance of lactobacilli requires multiple interdependent resistance mechanisms. This fits with the stress conditions acting on bacteria in beer, which mainly consist of the antimicrobial effect of the hop compounds (Simpson and Smith 1992) and acid stress flanked by ethanol stress and starvation.

1.6 Tools to investigate hop inhibitory mechanisms

As it is obvious that hop inhibition, tolerance and adaptation involve complex events, it is evident that related investigations must cover all accessible aspects of bacterial response and hop inhibitory potential. For the latter an approach for better understanding the stress on bacteria committed by hop compounds is required. This is inspired by the fact, that any antibiotic tested until now could not simulate the stress induced by hop compounds, even though proton ionophores or divalent cation exchangers were used, according to the suggested mechanisms of hop inhibition (Simpson and Smith 1992; Simpson 1993). Consequently, a

phenomenological investigation of the mode of antibacterial action of hop compounds is
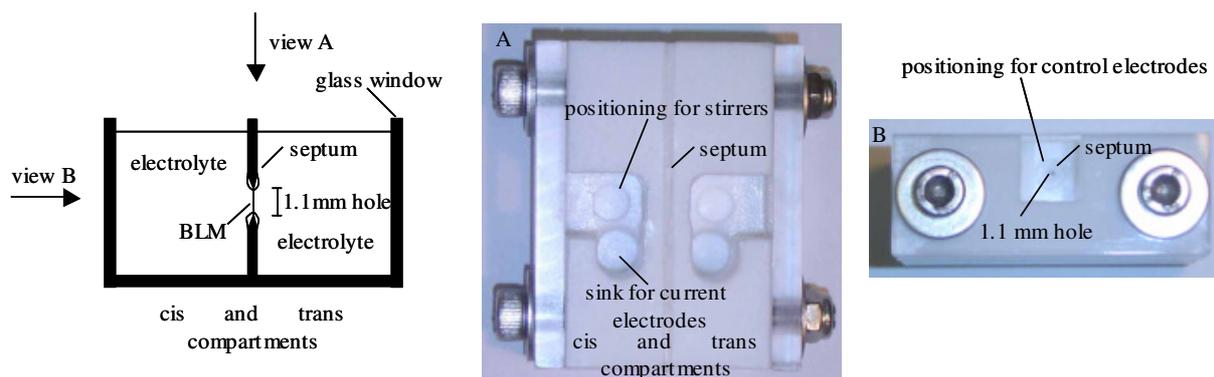
Fig. 3 Vessel for planar bilayer lipid membrane (BLM) formation (electrolytic cell). A schematic view is presented at the left side. The electrolytic cell was milled from a Teflon block. Sinks for stirrer and electrode positioning were shaped. The septum was milled off to 0.2 mm thickness and a hole of 1.1 mm in diameter was drilled. The parts were sealed with silicone and fixed by screws.

required. In this context, the term "phenomenological investigation" shall point to the fact that an empirical relationship of occurring phenomena (e.g. proton ionophore action and manganese binding of hop compounds) on account of definite experimental conditions is developed. These measurable phenomena may result from several parallel molecular events (cf. complexity of chemical composition of hops), which are basically unknown and for which no theory exists. This analytical approach, not commonly applied in microbial research is based on electrochemical investigations in reconstituted planar bilayer lipid membranes (BLMs), and is described below. As the inhibitory action of hop compounds on bacteria was found to be associated with the bacterial membrane (see above), the investigation of hop inhibition with membrane participation is required. Life processes taking place in cell membranes (e.g. membrane associated electron-, proton transfer in bioenergetics) comprise complex processes and thus, the employment of simpler membranes to evaluate function and membrane participation of the latter is necessary (Robinson and Cole-Hamilton 1991; Kalinowski 2005). BLMs are used as such "simpler" models for biological membranes (e.g. cytoplasmic membranes of bacteria) as they exhibit similar structures and physicochemical properties. They allow studies on parameters and functions of membrane components (e.g. phospholipids, incorporated membrane proteins), as well as on the impact of biological effective substances on membranes. In 1963 Mueller et al. invented a method for formation of planar BLMs (Mueller and Rudin 1963). These planar BLMs are formed in a vessel, which consists of two chambers, separated by a thin hydrophobic septum (0.2 mm thick) with a hole in the range of 0.1 to 3 mm in diameter (see figure 3). The chambers of this electrolytic cell are filled with a few milliliters of supporting electrolyte (e.g. 0.1 M NaCl or KCl in buffer).

The BLM formation is achieved by the so-called Mueller-Rudin method, which is based on a
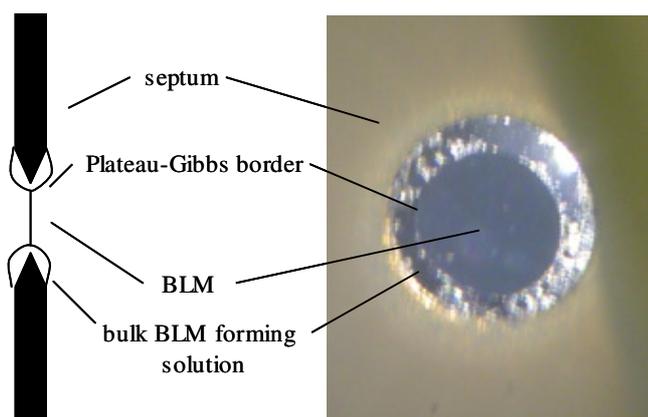
Fig. 4 Planar bilayer lipid membrane (BLM) and Plateau-Gibbs border. Membrane forming solution contained 20 mg phosphatidylcholine, 20 mg cholesterol in 1 ml decane. High amounts of membrane forming solution were infused in the hole in the septum, air bubbles were incorporated and the membrane formed. This way the normally very small Plateau-Gibbs border and the bulk membrane forming solution are visible and can be distinguished from the BLM.

solvent supported self-assembly process of amphipathic lipids, which results in a stable phospholipid bilayer in aqueous solutions. Therefore, the membrane forming solution (e.g. 20 mg phosphatidylcholine, 20 mg cholesterol in 1 ml decane) is brushed over the hole in the septum of the BLM chamber with a paintbrush or infused with a 10 µl tip. Subsequently the membrane self-assembly starts by a thinning process of the membrane and a visible Plateau-Gibbs border is formed, which represents the transition from the bulk membrane forming solution to the BLM (figure 4). The finished BLM is characterized by complete transparency, a thickness of about 60 – 90 Å, and a stability of several hours (Mueller and Rudin 1963). The major advantage of a planar BLM in comparison to other reconstituted membrane systems, like vesicle bilayers, which are commonly used in microbial research, resides in the accessibility of the membrane on both sides. Thus, changes in the solutions on opposite sides of the membrane can be precisely controlled, which is impossible for vesicle systems, where no measurements across the bilayer are possible (Robinson and Cole-Hamilton 1991). The opportunity of direct electrochemical measurements of transmembrane processes by immersing electrodes in both compartments of the electrolytic cell separated by the membrane implies some great advantages. No indicator substances, as fluorescent dyes, frequently used for vesicle bilayer systems (Robinson and Cole-Hamilton 1991), which could influence the measurements by their sole presence, are needed. Further, the same experimental setup allows to measure several parameters of the membrane, as e.g. their thickness and area (capacitance methods), membrane potentials (potentiometry), conductance (amperometry) and membrane associated redox properties (cyclic voltammetry) (Tien 1984; Kalinowski 2005). One clear disadvantage of planar BLMs with respect to vesicle bilayers is the small membrane area

investigated and the time consuming membrane forming process (range from seconds to nearly one hour). In addition to that, electrochemical investigations on BLMs do not allow using commercial equipment, as signals exiting the membrane during measurements must be as low as possible due to the fragile nature of the membrane (Kalinowski 2005). On account of this restriction, bioscientists do not frequently use the application of electrochemical BLM techniques (Tien 1984). However, on basis of innovations in electronics and electrochemical methods, the use of improvements in electronic analog and digital circuits simplifies the setup of BLM measurement instrumentations, which can undoubtedly contribute to the understanding of biological membrane processes (Kalinowski 2005).

phosphatidylcholine   fluid membrane   schematic membrane
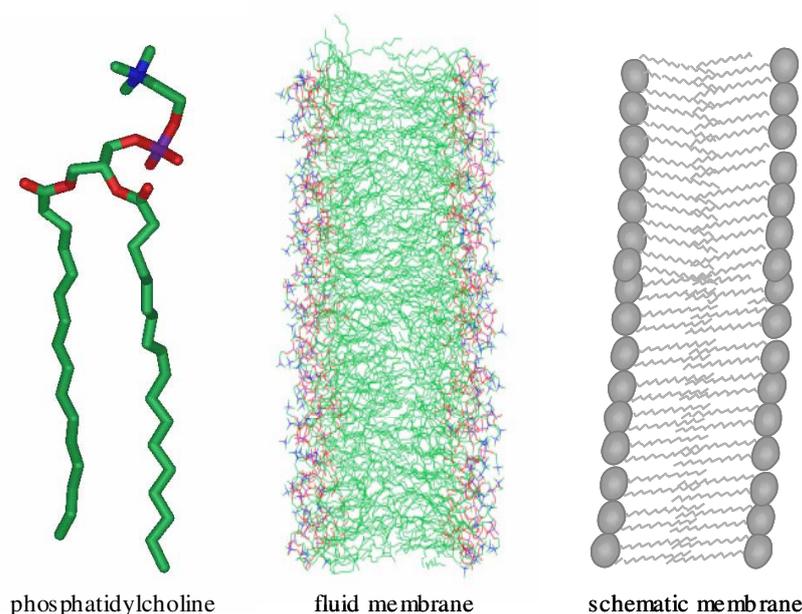
Fig. 5 Structure of a amphipathic lipid and a planar bilayer lipid membrane. Atoms in phosphatidylcholine: green atoms, C; red atoms, O; violet atom, P; blue atom, N. The fluid membrane model was downloaded from http://molvis.sdsc.edu/pe1.982/altas.htm#bilayers (original publication: Heller, Schaeffer et al. 1993). The schematic membrane displays the liquid hydrocarbon core (fatty acid residues, the tails, grey lines), sandwiched between two hydrophilic regions (the polar head groups, grey ovals).

In order to delineate the basics of these electrochemical measurements the electrical properties of a BLM are described first. The BLM consists of a liquid hydrocarbon core (fatty acid residues, the tails) sandwiched between two hydrophilic regions (the polar head groups, see figure 5). The liquid-crystalline structure (Heller et al. 1993) of the BLM is an excellent insulator, which exhibits an electrical resistance up to several hundreds of mega ohms per cm^2 (Tien 1984). Another effect committed by the structure of solutions separated by a planar BLM, is their function as capacitor. Here the electrolyte solutions are the plates, whereas the membrane is the dielectric (see figure 6). Therefore, planar BLMs may be treated as a parallel

plate capacitor, whereas its capacitance increases with higher plate area (equivalent
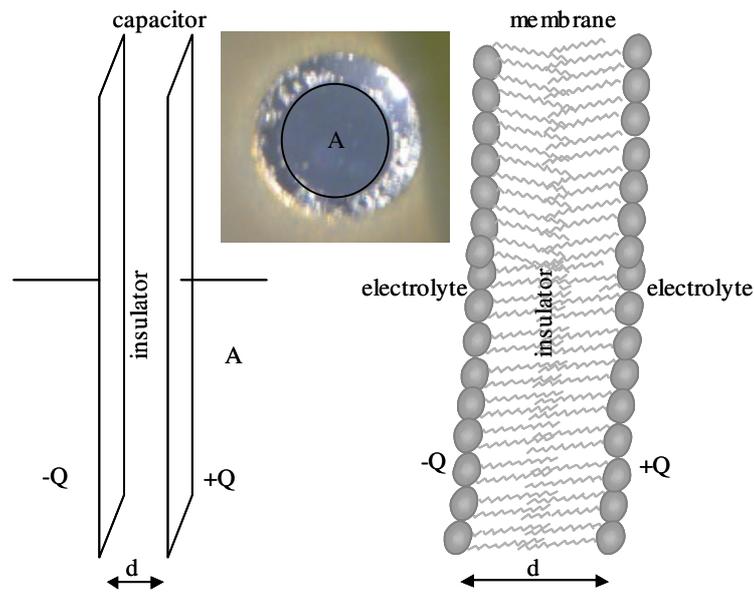
Fig. 6 A planar bilayer lipid membrane as parallel plate capacitor. The electrolyte represents the wires connecting the membrane as parallel plate capacitor to the current source, which charges the membrane. The higher the area and lower the thickness, the higher the capacitance. A= area; Q = charge; d = thickness of dielectric.

membrane area) and lower thickness of the dielectric (equivalent membrane thickness) (White and Thompson 1973). Typical specific capacitances measured at BLMs are in the range of 0.3 to 1 μFcm^{-2} (Kalinowski 2005). Due to membrane resistance and capacitance, the electrical equivalent of a planar BLM is a resistor connected in parallel with a capacitor. Subsequently, brief descriptions of general BLM measurement methods, their basics and their measurement setup are given.

One of the most commonly used techniques to investigate these BLMs is the measurement of the membrane potential. Cellular membrane potentials are essential for life processes. In normal cells this potential is negative inside the cell in comparison to the environment and reaches values up to 100 mV and above (Dolowy 1984; Simpson 1993). The origin of these potentials is the selective transport of ions across the membrane, which leads in result to a charge separation upon the membrane and durable potential differences. If only one type of ion or at least two types of ions are transferred across the membrane one refers to it as the Donnan potential and diffusion potential, respectively (Dolowy 1984). The Donnan potential is generated by a difference of concentration or more correct activity of one type of ion upon a membrane. The membrane exhibits semipermeable characteristics for this type of ion. The difference in activity of the ions on both sides of the membrane will drive an ion beam from the side of higher to the side of lower activity. At the same time a potential difference is set up

mediated by the charge separation upon the membrane. This potential slows down the latter ion beam and drives a second ion beam in opposite direction (see figure 7). If both beams are in equilibrium the Donnan potential can describe the situation by the following equation:

$$\Delta\phi = \frac{RT}{z_i F} \ln \frac{a_i^{cis}}{a_i^{trans}}$$

where $\Delta\phi$ is the potential difference, R is the gas constant, T is the temperature, F is the Faraday constant, z_i is the charge of the ion and a_i are the activities of the ions on the
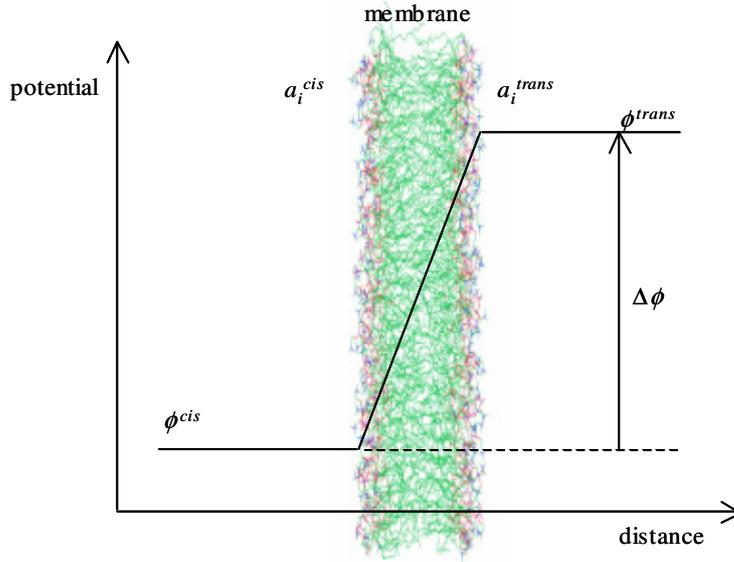
Fig. 7 Generation of potential across a bilayer lipid membrane. The activity of one type of ions is $a_i^{cis} > a_i^{trans}$ and results in the generation of a Donnan potential of $\Delta\phi$. Adopted from (Kalinowski 2005).

respective side of the membrane. In consequence, this means, that a 10-fold difference in concentration of one type of ion upon a semipermeable membrane results in a Donnan potential of 59 mV (at 25 °C, $z_i = 1$). The diffusion potential (upon a membrane) is generated by the transport of several types of ions across the membrane. In the simplest case it is mediated by a concentration difference of the same electrolyte in the aqueous solutions separated by the membrane. This will drive beams of cations and anions through the membrane, which is assumed to be permeable for both types of ions, resulting at equilibrium in the diffusion potential, described by the Henderson equation (Dolowy 1984):

$$\Delta\phi = -\frac{RT}{F} \frac{\sum_i z_i v_i (c_i^{cis} - c_i^{trans})}{\sum_i z_i^2 v_i (c_i^{cis} - c_i^{trans})} \ln \frac{\sum_i z_i^2 v_i c_i^{cis}}{\sum_i z_i^2 v_i c_i^{trans}}$$

where c_i is the concentration of one ion at the respective side of the membrane and v_i is the mobility of the ion (other symbols see above). As BLMs are excellent insulators, the permeability for ions can be mediated by incorporation of ionophores (cf. iso-α-acids) or channels (e.g. gramicidin). The ion selectivity of the latter determines whether the Donnan- or the diffusion potentials are the more appropriate model. The measurement of either potential in solutions separated by a BLM is basically simple. It can be achieved by immersing two e.g. silver/silverchloride electrodes in opposite compartments of the electrolytic cell connected to a potentiometer (see figure 8).
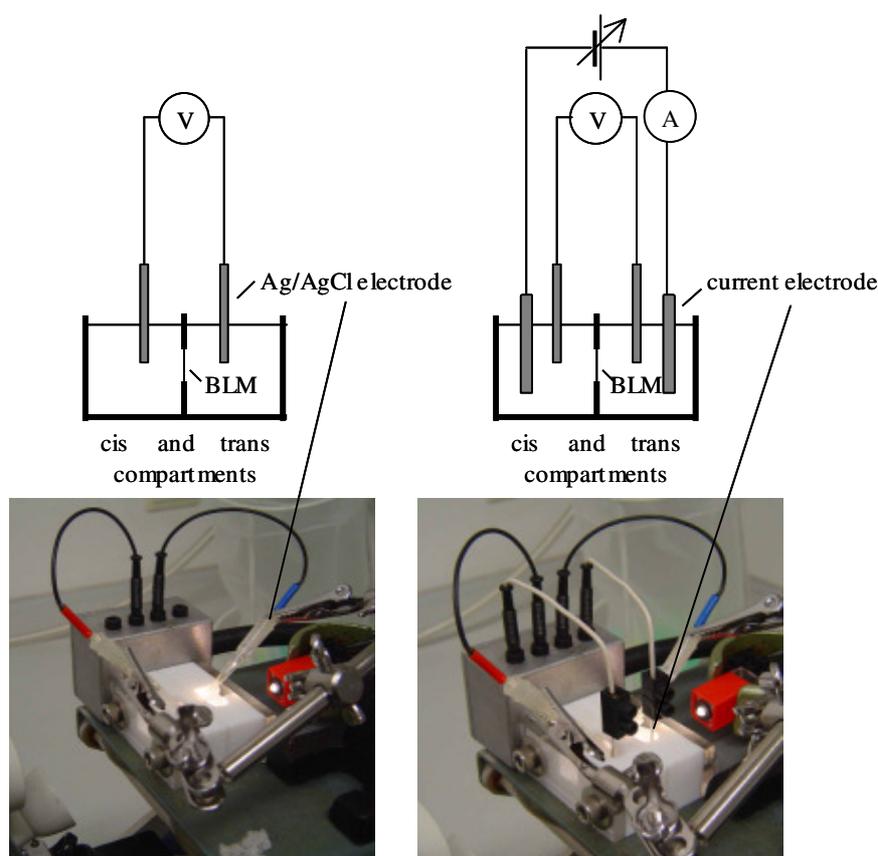
Fig. 8 Measurement setups for bilayer lipid membrane (BLM) experiments. Membrane potential measurements (left side) can be achieved by immersing two reference electrodes (e.g. Ag/AgCl electrodes) on opposite sides of the BLM, connected to a voltmeter. Potentiostatic measurements (right side) involve two additional current electrodes (made from e.g. platinum or stainless steel). The current forced through the current electrodes is controlled by the potential difference at the reference electrodes and simultaneously measured with an ammeter. For both measurement setups the reference electrodes are placed as close as possible to the membrane.

A second discipline of BLM investigations in addition to the before mentioned potentiometry is the amperometry. As membranes in living organisms exhibit potential differences across their membranes, which alter transmembrane processes, it is necessary to be able to apply specific potentials upon the BLM. The therefore developed potentiostats enable the determination of important membrane parameters as membrane resistance and conductance for e.g. investigations on ionophore committed transmembrane transport processes

(Kalinowski 2005). As the transport of ions across a membrane comprises a flowing current, the measurement of this current at a fixed membrane potential characterizes the charge translocating ionophore or channel. In general the equations describing this charge transport are highly complex (Schmickler 1996). This is due to the fact that a current through the membrane depends on membrane characteristics as e.g. the potential electrostatic energy barrier and the presence of unstirred layers at the membrane-solution interfaces, the membrane area and thickness, as well as on characteristics of the charge translocator species like charge, dissociation constants, bulk solution concentrations, membrane partition coefficient and intramembranous diffusion coefficient and so forth (Schoch et al. 1979). If assumptions concerning the relevance of parameters are made, it is often possible to simplify those expressions. As an example a simplified equation describing the conductance mediated by the proton ionophore CCCP (Foster and McLaughlin 1974) is displayed below:

$$G \propto \left[A^{TOT} \right]^2 \frac{\left[H^+ \right]}{\left(K_{HA} + \left[H^+ \right] \right)^2} \exp\left(\frac{F\psi}{RT} \right)$$

where G is the conductance, A^{TOT} is the total uncoupler concentration, K_{HA} is the dissociation constant of CCCP and ψ is the potential within the membrane with respect to the bulk aqueous solutions (other symbols see above). The pre-exponential factor stands for the concentration of charge transporting species at respective pH of the aqueous solutions, while the Boltzmann expression corrects this value to the concentration present immediately at the charged membrane-solution interface (for details consult Foster and McLaughlin (1974)). In consequence it can be understood, that even simple conductance models can get very complex if more than one uncoupler species is present at the same time (cf. complex chemical composition of iso-α-acids). However, basic information related to the general mechanism of charge transport as the class of uncoupler can be gained. Uncouplers of class I exhibit a linear dependence of membrane conductance on the ionophore concentration (LeBlanc 1971). For uncouplers of class II the dependence is quadratic, e.g. as predicted by the equation for CCCP conductance (see above). In this case the transfer of charge is attributed to a proton hopping or a bimolecular process, where a complex is formed between a neutral molecule and an uncoupler anion (Smejtek et al. 1976). Even though the complication of the interpretation of conductance data, basic phenomena of ionophore behavior can be studied. The measurement of such transmembrane charge transport processes at a fixed membrane potential is more demanding than the potentiometry described above. Usual two- or three-electrode potentiostats exhibit measurement errors (up to approximately 20 % of applied voltage), since

they do not compensate for undesired voltage drops at electrodes as well as at the supporting electrolytes. Thus, the use of a four-electrode system developed by (Kalinowski and Figaszewski 1995), which consists of two current electrodes and two control electrodes, is required. The current electrodes force the current through the membrane, while the control electrodes measure the potential close to the membrane. The latter regulate via feed back loop the current passing through the electrodes (see figure 8). Thus, a measurement of transmembrane charge transport at a fixed membrane potential can be achieved.
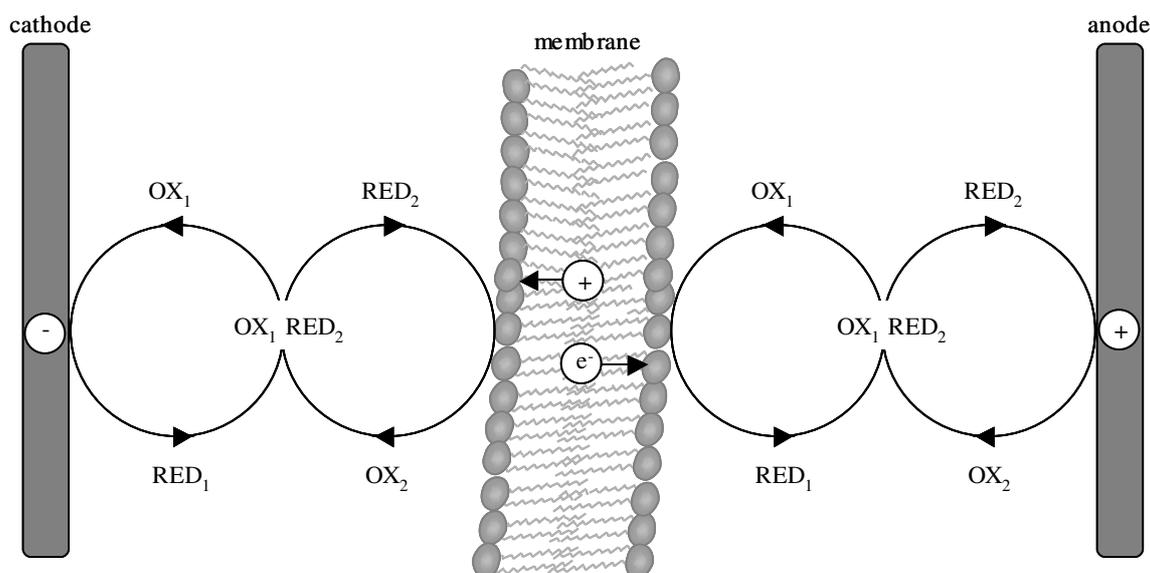
Fig. 9 Double-electrode behavior of a planar bilayer lipid membrane. Reactions in solutions: $OX_a + RED_b \leftrightarrows RED_a + OX_b$. Reactions at the phase transitions: $OX_i + ne^- \leftrightarrows RED_i$. OX, electron acceptor; RED, electron donor; +, positive hole, - or e-, electron. Adopted from (Bhowmik, Dutta et al. 1987).

Under the above-mentioned conditions, a potential scan between two potentials of interest applies cyclic voltammetry (<u>volt</u>-<u>ampero</u><u>metry</u>) as analytic tool to the BLM system. Cyclic voltammetry is an elegant tool for the study of transmembrane redox reactions in electron-conducting BLMs. These transmembrane redox processes play a central role in life's membrane bioenergetics (Tien 1984). The possibility of the application of cyclic voltammetry to a BLM resides in the double-electrode behavior (see figure 9) of the planar membrane (Bhowmik et al. 1987). The electron transport process through the BLM implies electron transfers at both solution-membrane interfaces, as well as an electron transfer through the BLM. Consequently, part taking redox couples must be located at the membrane-solution interfaces, as well as inside the membrane in their oxidized and reduced form. These redox couples in solutions and membrane are not necessarily the same. Nevertheless, for transmembrane electron transfer proximity between the redox potentials of both is required (Shiba et al. 2003). If the above-mentioned premises are fulfilled, cyclic voltammetry is

helpful to characterize occurring redox processes, which can be described by the following equation:

$$OX + ne^- \underset{k_b}{\overset{k_f}{\longleftrightarrow}} RED$$
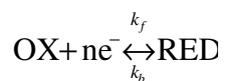
where OX and RED are the oxidized and reduced redox species and k_f and k_b denote the forward and backward rate constants. If the redox reaction is reversible, $k_f \approx k_b$. For cyclic voltammetry experiments the potential at the BLM is scanned between two potentials of interest, whereas scan rates in the range of 0.1 to 100 mV per second, depending on reaction kinetics, are used. During the scan the current passing through the working electrode (BLM) is measured and plotted against the applied voltage. The resulting graph is called voltammogram, which is characterized by several parameters (Tien 1984; Schmickler 1996). To delineate the processes occurring during the voltage sweep, a voltammogram (see figure 10) of a simple redox reaction (see equation above) is described subsequently. The scan is
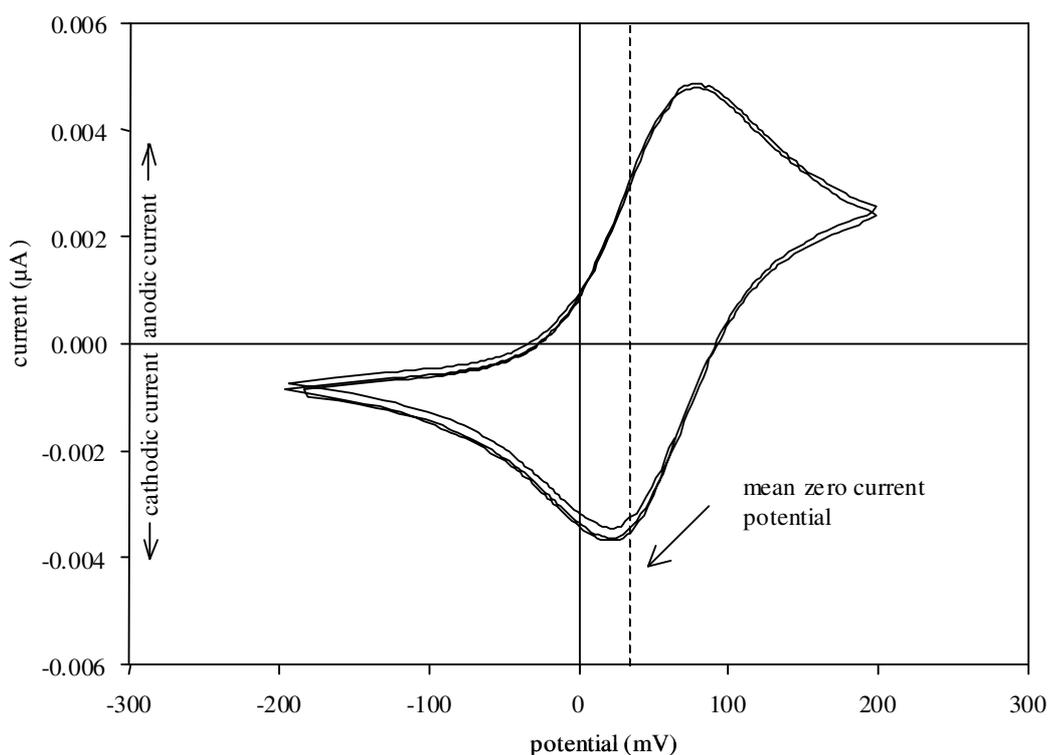
Fig. 10 Typical voltammogram of a simple redox reaction calculated with ModSim (http://echempp.sourceforge.net). Parameters: semi-infinite diffusion model (single electrode, boundary index: 0); triangular cyclic voltammetry excitation function (3 scans), range ± 200mV; reaction mechanism: A <=> B + e-; b<0>; model parameters: temperature = 293.15 K; electrode area = 6e-06 m2; reactant A: concentration 0.0001 M, diffusion coefficient = 1e-09 m2 s-1; reactant B: concentration 0 M, diffusion coefficient = 1e-09 m2 s-1; E0 =0.05 V, alpha = 0.5, kh =0.01m s-1.

explained from the negative to the positive switching potential. At about -200 mV the potential is below the mean zero current potential and a cathodic current is recognized. The concentration of the oxidized species at the working electrode is lower than in the bulk solution. With increasing potential, the total overvoltage is reduced with concomitant reduction of the cathodic current. The concentrations of oxidized and reduced species are further decreased and increased, respectively. At a potential below the mean zero current potential, the current readout crosses the x-axis and an anodic current arises. With higher potential the anodic reaction is enhanced and the current is strongly increased. At the same time the concentration of the reduced species at the working electrode is decreased and the anodic current reaches a maximum. Subsequently, the reduced species nearly vanishes at the electrode surface, which diminishes the anodic current. At this particular point, the scan is normally reversed. For reversible reactions analogous considerations are true for the backward scan. The characteristic shapes of such voltammograms are influenced by redox potentials and local concentration differences of the reactants and thus diffusion processes, as well as charge transfer reactions (Schmickler 1996). The measurement setup for cyclic voltammetry in electron-conducting BLMs is principally identical to that used for amperometry (see above). The only difference resides in the use of an additional programmable voltage generator connected to the potentiostat. With this any kind of voltage profiles can be applied to the BLM. Normally triangular signals are used.

Such triangular signals are also obtained in capacitance measurements of a BLM. As described above, planar BLMs may be treated as a parallel plate capacitor, whereas its capacitance depends on membrane area and membrane thickness. For that reason capacitance measurements allow to estimate the BLM's thickness and area and thus are an excellent tool for the analysis of the stability, quality and reproducibility of membrane formation (Kalinowski 2005). As the membrane area of a BLM is very small compared to those used in vesicle bilayer experiments, which can reach combined areas 10^{10} times greater than the largest BLM, it is evident, that the reproducibility and quality control of the BLM is of highest importance for significant measurement results (Robinson and Cole-Hamilton 1991). Several methods for membrane capacitance measurement, like capacitance current measurement, bridge- and pulse methods, as well as capacitance-to-frequency transduction exist (Kalinowski and Figaszewski 1995). The latter method implies some great advantages as it is possible to determine the capacitance in dependence on the polarization potential (electrostriction analysis (McLaughlin et al. 1971; Schoch et al. 1979)). The method consists of a cyclical charging and discharging of the BLM by a constant current intensity within a

fixed range of potential (amplitude approximately 10 mV). The cycle time is therefore proportional to the capacitance of the BLM and the potential amplitude as described by following equation:

$$C = T \frac{i}{2\Delta U}$$

where C is the membrane capacitance, T is the cycle time, i is the current and ΔU is the potential amplitude (Kalinowski and Figaszewski 1995). The measurement circuit is based on the TLC555 (Texas Instruments, Dallas, Texas, USA), which produces an alternating output signal (±5 V) of period proportional to the membrane capacitance (for details consult Kalinowski and Figaszewski (1995)). This signal can be recorded with commercial frequency measurement equipment. In order to achieve comparable BLMs for subsequent measurements, before each analysis the membrane forming process can be controlled by measurement of the membrane capacitance (cf. appendix). The measurement setup is comparable to that used for amperometry, where four electrodes are used. In this case two electrodes are the source of the constant current, while additionally two electrodes are used to measure the resulting membrane potential.
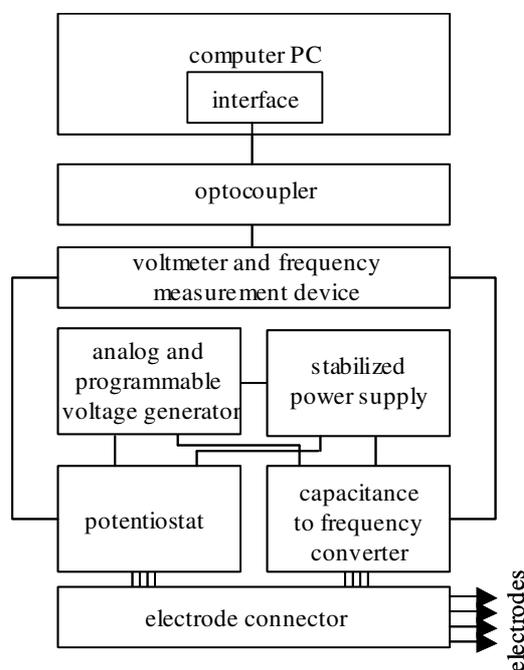
Fig. 11 General signal-processing scheme of bilayer lipid membrane measurement device. The apparatus consists of a stabilized power supply, analog and programmable voltage generators, a capacitance to frequency converter and a potentiostat. The potentiostat and capacitance to frequency converter outputs are connected to a high quality digital voltmeter and frequency measurement device coupled to a PC.

To get an overview, the whole modular BLM measurement system is displayed below. The general signal-processing scheme is depicted in figure 11. At the electrolytic cell the electrodes are connected to the measurement system via 4-electrode connector, with built-in voltage followers (headstage), used for signal stabilization. The stabilized signals are transferred via shielded multicore cable between headstage and BLM measurement device.
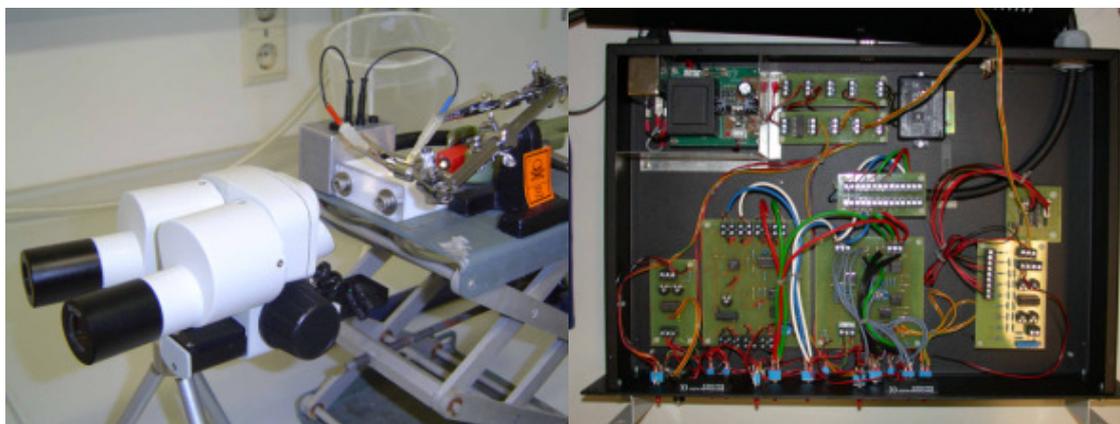
Fig. 12 Measurement equipment for bilayer lipid membrane experiments. The measurement cell with headstage containing amplifiers mounted as close to the electrodes as possible to stabilize the signal, electrodes and microscope for visual control (left side). The measurement apparatus: Circuit boards on top are the stabilized power supplies (± 5V, 9 V, 12V). In the middle are the electrode connector (center) and the controller (PIC 16F690, Microchip Technology Inc., Chandler, Arizona, USA; right). At the bottom (from left to right) are the analog voltage generator, the capacitance to frequency converter, the potentiostat and the 8-bit R-2R digital to analog converter.

The output data acquisition is realized by opto-electronic isolation between PC and measurement device and for convenience visualized by Excel macros. The BLM measurement equipment is displayed in figure 12. The measurement apparatus consists of a stabilized power supply, analog and programmable voltage generators, a capacitance to frequency converter, a potentiostat and a multicore cable connector with selectable inputs and outputs. The measurement device outputs are connected to a high quality digital voltmeter and frequency measurement device coupled to a PC. Signal noise protection is guaranteed by constructive precautions (Kalinowski 2005).

Taken together, BLM measurement systems can contribute to studies on the inhibitory effect of hop compounds in artificial bilayer lipid membranes by providing the possibility of acquisition of multiple membrane parameters.

1.7 Tools to investigate bacterial stress response

In addition to investigations on the inhibitory effect of hop compounds, analytical methods, which cover the part of hop stress response and adaptation of the bacterium, are used. The adaptation and stress response could imply e.g. alterations on the biochemical level, the

intracellular pH, the transmembrane transport and the transcriptome and the proteome of the bacterium. Methods for the determination of adaptation and stress response of bacteria are e.g. high performance liquid chromatography (e.g. metabolite analysis), gas chromatography (e.g. membrane fatty acid analysis), one dimensional gel electrophoresis (e.g. lipoteichoic acid analysis), fluorescence based methods (e.g. intracellular pH-, membrane fluidity-, transport analysis), microarrays (transcriptome analysis, not accessible without genome sequence) and two dimensional gel electrophoresis coupled to e.g. mass spectrometry (proteomics). In the subsequent section the fluorescence-based methods and the methods of proteomics are described exemplarily.

1.7.1 Fluorescence based methods

Fluorescence-based methods are of great value due to the fact that cellular parameters can be monitored at the time when the cell responds to environmental stresses. These non-invasive measurements are used to assess e.g. the bacterial transmembrane transport activity for bactericidal drugs, changes in cellular barriers as the cytoplasmic membrane and the effect of drugs, which alter the bacterial intracellular pH (pH_{in}). The general principle of these measurements resides in the positioning of the fluorescent dye in a specific site within the bacterial cell (e.g. intercalated in the DNA, intracellular or inside the cytoplasmic membrane) and the detection environmental changes in this location (e.g. dye concentration, dye complexation, pH value, polarity or fluidity of the environment). The fluorophore can be positioned by binding to a target molecule (e.g. DNA for ethidium bromide and Hoechst 33342), the use of amphiphilic fluorescent probes for membrane inclusion (e.g. Laurdan) or the use of nonfluorescent membrane-permeable molecules (e.g. acetoxymethyl esters), which are rendered membrane-impermeable and fluorescent by cleavage through intracellular enzymes. When the dye is loaded a beam of light of a specific wavelength (normally $\lambda > 250$ nm) is applied to the sample. The fluorescent dye absorbs the light (energy), which results in a transition from the molecule's ground electronic state to the exited state. The return to ground state is achieved be emitting energy in form of fluorescence. A simplified excitation/emission scheme is displayed below:

$$S_0 + photon \xrightarrow{10^{-15}\,s} S_1$$
$$S_1 \xrightarrow{10^{-9}-10^{-6}\,s} S_0 + photon$$
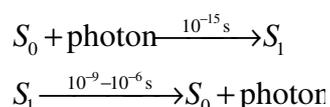
where S_0 and S_1 are the ground- and excited states, respectively (Fritz and Schenk 1989). The emitted light is of longer wavelength than the absorbed light (Stokes' shift), due to a small

loss of energy. The latter is attributed to relaxation-, solvent effects, excited state reactions, complex formation and energy transfer. At low fluorophore concentrations the fluorescence intensity is proportional to the dye concentrations. A nonlinear behavior of this relation is
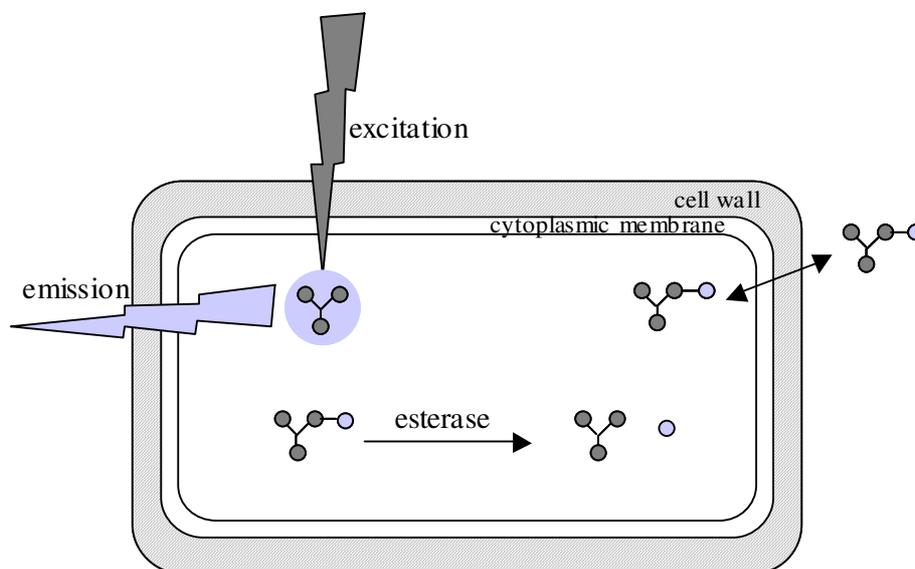
Fig. 13 Principle of calcein fluorescence measuerments. Calcein acetoxy methyl ester is membrane-permeable and non-fluorescent. A cleavage by intracellular esterases renders it fluorescent and membrane-impermeable.

monitored at higher fluorophore concentrations, due to quenching. This fluorescence quenching refers to any process, which decreases the fluorescence emission. The origin of the before mentioned quenching at higher dye concentration is a result of fluorophore deactivation by a collisional energy transfer between excited molecules (Fritz and Schenk 1989). Thus opposite molecular events as the decrease and increase in fluorescent dye concentrations can result in a similar fluorescence data readout. In this context a careful interpretation of fluorescence data combined with suited control experiments is required. The applications of fluorescence measurements comprise e.g. membrane protein transport studies (Ulmer et al. 2002), determination of the fluidity and polarity of the bacterial cytoplasmic membrane (Parasassi et al. 1990) and determination of the intracellular pH of bacteria (Yansanjav et al. 2004). The principle of membrane protein transport determination is the measurement of an energy dependent efflux of the fluorophore. This can be achieved by the setup of a fluorophore gradient across the cytoplasmic membrane in the presence and absence of a source of energy. The difference in the measured fluorescence intensities will determine the rate of energy dependent drug efflux. It can be assessed with fluorophores as e.g. ethidium bromide and Calcein AM as substrates (Lecureur et al. 2000; Ulmer et al. 2000). However, the choice of transport substrate has to be evaluated for each single bacterial strain. The principle of ethidium bromide and Calcein AM measurements is described briefly. Ethidium bromide as well as Calcein AM are membrane-permeable molecules. Calcein AM is a non-

fluorescent transport substrate for multidrug resistance transport proteins (Homolya et al. 1993), which are located in the cytoplasmic membrane of the bacterium. The detection reaction of Calcein AM implies a cleavage by intracellular esterases to calcein, which exhibits fluorescence (see figure 13). Thus a bacterial Calcein AM transport to the outer medium will decrease the intracellular dye concentration and accordingly decrease the measured fluorescence. Ethidium bromide is a fluorescent bacterial efflux pump substrate (Borges-Walmsley et al. 2003). The detection of intracellular accumulated ethidium bromide is due to a strong increase in fluorescence if ethidium bromide is intercalated in the bacterial DNA (Le Pecq and Paoletti 1967). In consequence a bacterial transport activity is indicated by a decreased fluorescence. However in both cases the dye concentrations have to be adjusted in the range, where no quenching, which would corrupt the measurement results, occurs.

The measurement of cytoplasmic membrane fluidity and polarity by Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) fluorescence is used to study phase coexistence and phase interconversions in bacterial cell membranes. A shift in emission spectra of Laurdan fluorescence is recognized on account of a transition of the dye surrounding membrane from liquid-crystalline to gel phase. The quantization of lipid phases is obtained using generalized polarization (GP), as described by the following equation:

$$GP = \frac{I_{440nm} - I_{490nm}}{I_{440nm} + I_{490nm}}$$

where I is the relative fluorescence (excitation: 360 nm) at the respective wavelength (Parasassi et al. 1990). High GP values are associated with low membrane fluidity and low polarity, while a low GP value is the opposite. A change in GP values can be related to e.g. changes in the cytoplasmic membrane fatty acids composition in the saturated/non-saturated fatty acids ratio (Guffanti et al. 1987; Krulwich et al. 1987) or changes in divalent cation concentrations near the membrane (Asai et al. 2000; Riske et al. 2002), which both alter the fluidity of the cytoplasmic membrane. Thus in combination with membrane composition data, Laurdan fluorescence is suggested to provide useful information on physical properties of membranes involved in regulating biological activities (Yu et al. 1996).

Another regulatory parameter of biological activity is the intracellular pH (Padan and Schuldiner 1987). The intracellular pH measurements include the loading of the bacterial cell with the fluorophore, a calibration of the fluorescence intensity to the pH value, which is achieved by equilibrating the intracellular and extracellular pH by proton ionophores (e.g.

CCCP or valinomycin/nigericin) and the actual experiment (Molina-Gutierrez et al. 2002). Several fluorescence-based methods exist for determination of the intracellular pH of bacteria (Zhou et al. 1995; Siegumfeldt et al. 2000; Molina-Gutierrez et al. 2002; Yansanjav et al. 2004; Nedergaard et al. 1990). In this context virtually all available fluorescence probes (e.g. BCECF, cFDASE, SNAFL-calcein) show a useful pH measurement range around pH 7.0. These dyes can be applied to many neutrophil bacteria, such as *E. coli*, which is unable to grow if the pH_{in} is below 6.6. In contrast, lactic acid bacteria can exhibit intracellular pH values from pH 7 to below 4.5 (Nannen and Hutkins 1991). As a result of available fluorescent pH indicators the pH_{in} measurements of beer spoiling strains in the presence of hop compounds were performed at a pH of 5.8 (Yansanjav et al. 2004). As beer commonly exhibits a pH (3.8-4.7) far below this pH value, the associated results have to be considered with caution. One indicator of intracellular pH, which covers this lower pH range of 4-5 is CDCF (Nedergaard et al. 1990). However fluorescent pH indicators, which cover the full range of lactic acid bacteria intracellular pH values are not yet available.

The instrumentation for fluorescence measurements implies a source of light, the excitation monochromator, the emission monochromator and the detector. The excitation and emission beam are arranged in an angle of 90°. The advantages of fluorescent measurements reside in the speed of the measurement and the application under difficult measurement conditions, such as high hydrostatic pressures (Molina-Gutierrez et al. 2002; Kilimann et al. 2005). The clear disadvantages are the above-mentioned possible influence of the fluorescent probe on the measurement target itself and effects as fluorescence quenching, which have to be taken into account. However, fluorescence-based analytical methods allow an insight view on stress-induced changes in cellular parameters immediately at the stage, when they occur and thus are indispensable in bacterial stress response research.

1.7.2 Proteomics

Another analytical tool, which allows a look at the bacterial enzymatic profile at a definite point of stress response, is proteomics. It is applied to monitor the bacterial stress response and adaptation on virtually all kinds of environmental stresses. Proteomic studies, which imply the used of high resolution two-dimensional gel electrophoreses cover the topics of acid stress response, oxidative stress resistance, cold and heat stress response, osmotic stress, bile tolerance, starvation stress response and so forth (van de Guchte et al. 2002). Accordingly the two-dimensional gel electrophoresis is the working horse in stress response and adaptation research. This is due to the universality of the method, which allows discovering stress

responses on almost all cellular levels and thus detecting even unexpected bacterial resistance mechanisms. Additionally, two-dimensional gel electrophoresis is the only routinely applied technique for parallel quantitative expression profiling of complex protein mixtures, resolving about 2000 proteins simultaneously on a single gel (Görg et al. 2004). The resolution power of the method resides in a separation of the proteins according to their isoelectric point in the first dimension, while the second dimension separates according to the molecular sizes of the proteins. The proteomics workflow comprises protein isolation and optional pre-fractioning of the sample, rehydration of immobilized pH gradient (IPG) strips, isoelectric focusing on IPGs, equilibration of IPGs, SDS-polyacrylamide gel casting and electrophoresis, protein staining, detection and quantification and a subsequent protein identification by Edman-sequencing or mass spectrometry. This complex workflow clearly implies the problem of reproducibility of the method, since comprehensive handling is required. However the introduction of immobilized pH gradients in two-dimensional electrophoresis (Görg et al. 1988), which resolved the problem of carrier ampholytes-based pH gradient (O'Farrell 1975) drift, contributed to reproducibility, handling and resolution. In the subsequent section, the basic steps of two-dimensional electrophoresis (see figure 14) with regard to this work are described briefly.

IPG- strip (Immobilized pH-gradient)

Isoelectric focusing (Harder 2001)

Protein loading with SDS (Harder 2001)

IPG – strip transfer to the second dimension (Harder 2001)

Run of the second Dimension

Colloidal-Coomassie stained gel
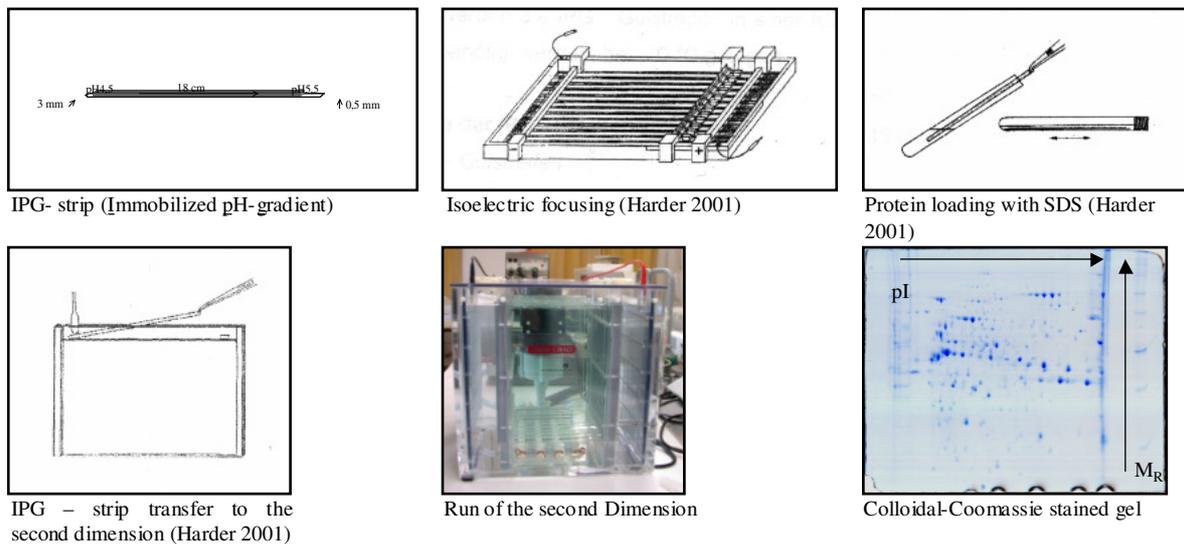
Fig. 14 Workflow of two-dimensional gelelectrophoresis.

Prior protein separation an adequate protein isolation protocol has to be established. This can be considered as the critical step of the procedure, as the separation result can only be as good as the starting material. Hence a simple, robust and reproducible sample preparation protocol is required, which can only be developed empirically. Before the protein extraction it is sometimes required to determine the optimum of growth state of the bacterium for protein

isolation, since the protein expression is altered during growth cycle. Normally the bacterial proteins are extracted during the exponential growth phase. After a washing step to remove possible growth medium derived contaminations, the cells are disrupted. For slow growing lactic acid bacteria as *L. brevis*, which exhibit tough cell walls, a combination of e.g. enzymatic lysis (e.g. lysozyme) and mechanical disruption (e.g. ultrasonic treatment) is required. Protein solubilization is achieved by the use of chaotropic reagents (e.g. urea, thiourea), which disrupt hydrogen bonds, detergents (e.g. SDS, CHAPS, DDM), which disrupt hydrophobic interactions and reducing agents (e.g. DTT), which disrupt disulfide bonds. Sometimes carrier ampholytes are included in the solubilization/denaturation buffer in order to prevent protein precipitation during isoelectric focusing. To avoid protein decay or modification, proteinase inhibitors are included and the sample temperature is kept below 37 °C to avoid protein carbamylation (McCarthy et al. 2003; Görg et al. 2004). High salt concentrations, as they are present in lactic acid bacteria (Archibald and Duong 1984), can be removed by dialysis or directly in gel during isoelectric focusing.

The principle of isoelectric focusing resides in the charged groups of the proteins, which take up or loose protons as result of the surrounding pH value. The pH value at the point of zero net charge is termed isoelectric point of the protein (pI). If an electric field is applied on a protein within a pH gradient (e.g. IPG strip) the protein will migrate to the electrode of opposite charge. If the pH of the pI of this protein is located on the way to the electrode, the protein is discharged as it approaches and the speed of migration is slowed down until it stops at the pH value of its pI. A diffusional transport away from this point will result in the uptake of charge and thus the protein is back-drawn to place of its pI again and therefore focused in this position. The focusing requires high voltages (e.g. 3500 – 10000 V), which cannot be achieved in highly conducting samples with high salt contents. Thus the applied voltage is ramped from low (e.g. 50 V) to high voltage (e.g. 3500 V) and the salt is removed by replacement of the electrode filter paper strips (Görg et al. 2004). Under these conditions sample cup-loading is preferred over in-gel rehydration. Commonly used IPG's exhibit pH ranges from e.g. 3-10, 4-7, 6-11 and are used for an overview of expressed proteins in complex samples and ranges from e.g. 4.5-5.5, 5.5-6.7 (zoom-in gels) are used for separation of unresolved protein spot clusters. Normally separation gel dimensions of 18 or 24 cm are used.

After isoelectric focusing, the proteins exhibit no net charge and thus have to be charged with SDS for the second dimension by equilibration of the IPG strips. In addition, a reduction and

alkylation of the proteins is achieved during equilibration. The equilibration buffer (50 mM Tris-HCl, pH 8.8) contains urea, SDS, glycerol and DTT in the first or iodoacetamide in the second step. Each equilibrations step is carried out for 15 min (Görg et al. 1988).

Subsequently the IPG strips are placed of top of a SDS polyacrylamide gel (PAG) for the separation of the now negative charged proteins according to their molecular sizes. The IPG gel functions as a stacking gel. Commonly, the SDS-PAG exhibits total acrylamid concentrations (T) from T10 to T15 % and Tris-glycine electrode buffers are used (Laemmli 1970). This allows a separation of proteins in the range from about 10–250 kDa. In vertical SDS-PAGE systems 10 gels can be run in parallel. This is advantageous for comparison of proteins expressed under multiple stress conditions, as differences in the location of the spots on the gels on account of individual gel runs, are avoided. Running conditions for 10 gels are e.g. 200 V, 150 mA at 20°C (Görg et al. 2000).

The visualization of the separation result can be achieved by several methods, including e.g. negative staining (e.g. zinc or copper staining), positive staining (e.g. silver or coomassie blue staining) or fluorescence staining (e.g. SYPRO ruby). Commonly coomassie blue staining is used, as it is cheap, offers a high dynamic range and is compatible to a subsequent mass spectrometric analysis. However the detection limit is high in comparison to silver or fluorescent staining (Görg et al. 2004). Thus a combination of coomassie blue staining and sensitive silver staining can resolve this problem. Therefore high abundant proteins can be quantified by coomassie blue staining. Subsequent the gel is destained (e.g. with fixing solution of the silver staining protocol) and the low abundant proteins (if not masked by high abundant proteins) can be detected by silver staining (Blum et al. 1987). After scanning the stained gel, with e.g. a calibrated document scanner, the image analysis can be performed with computational aid (Mahon and Dupree 2001). However, the software is expensive and manual spot editing is usually necessary. A simple method for fast detection of inhibited proteins, which disappeared from the gel or induced protein, which appear on the gel, is a fast switching between two gels of interest (e.g. compressed images are placed on two subsequent slides in a PowerPoint presentation, the curser keys are used to switch between the slides). The two images are fused to one gel, due to the inertness of the human eyes. "Flashing" protein spots can be assigned to one of the both afore-mentioned protein categories.

After identification of protein spots of interest, the proteins can be blotted for subsequent Edman-sequencing or cut out of the gel for mass spectrometric (MS) analyses. For the latter a e.g. tryptic digest can be performed in-gel and analysis of the derived peptides by MALDI-

TOF MS (peptide mass finger printing), LC-ESI MS/MS or ESI MS/MS (*de novo* sequencing) can be performed. In general MALDI-TOF MS and LC-ESI MS/MS protein identification is based on a comparison of measured peptide masses with database stored theoretic digestion masses of the peptides. In MS/MS experiments the peptides (parent ions) are fragmented by collision-induced dissociation (CID) and additional information in form of a short sequence tag is generated. As several amino acid sequences can constitute the same peptide mass, this additional information can be used to refine the protein identification. However, MS/MS based protein identification, still works best for peptides that exactly match the database-stored masses, which is normally the case if the complete genome sequence of the target organism is available (Johnson et al. 2005). If no exact matches are present in the database, the programs using peptide mass matching (or filtering) will discard a considerable part of gained information and thus are not able to significantly identify this protein. In this context it has to be mentioned that none of the beer spoiling bacteria genomes is sequenced until now. Thus routine identification of beer spoiling bacteria (homologous) proteins is difficult using MS/MS (Johnson et al. 2005). The alternative is Edman- or *de novo* sequencing coupled to subsequent reverse genetics, which is not practical for large protein numbers. Another approach is the use of *de novo* sequencing programs (e.g. Lutefisk) and a consecutive sequence homology-based search algorithm as e.g. FASTA or BLAST (Pearson and Lipman 1988; Gish 1996; Johnson and Taylor 2002). However, the de novo sequencing programs require huge computational resources and are accordingly not implemented in routine mass spectrometry's dataflow. But for special applications as the identification of proteins from organisms whose genomes are not sequenced, "automated" *de novo* sequencing, combined with the other above-mentioned techniques is the state of the art (Shevchenko et al. 2001).

Two dimensional gel electrophoresis, previously described by Görg et al. (1988; 2000; 2004; additionally, a manual by Görg et al. is available at http://www.wzw.tum.de/proteomic), coupled to Edman-sequencing, mass spectrometric analysis and newly developed computational possibilities of protein identification (Shevchenko et al. 2001) provide powerful tools to investigate hop resistance in beer spoilage lactobacilli for which no genome sequences are available. The publication of the recently finished genome of *Lactobacillus brevis* ATCC 367 is helpful for this proteomic research, using current innovations in available databases and analytic instrumentation (see appendix).

The combination of the afore-mentioned analytical techniques, as e.g. biochemical and metabolic characterizations and proteomics, applies a powerful set of tools covering several essentials of bacterial life to investigations on hop resistance in beer spoiling lactobacilli.

1.8 General stress response in lactic acid bacteria

Bacterial stress response is based on the coordinated expression of genes, which are involved in several cellular processes (e.g. cell division, energy metabolism, amino acid metabolism, DNA metabolism, membrane composition, transport, etc.) in order to improve the bacterial stress tolerance (Stortz and Hengge-Aronis 2000). Best-studied stress responses of lactic acid bacteria are acid, heat and cold stress responses. However, no general stress response seems to exist and the stress-specific response varies among the species or even strains (van de Guchte et al. 2002). Accordingly, possible stress responses of lactic acid bacteria to acid stress and starvation stress, which can be directly linked to the conditions present in beer, are delineated subsequently.

The growth of lactic acid bacteria is associated with the production of organic acids as lactate and acetate (Kandler 1983), which results in a considerable acidification of their environment (final pH about 4.0-3.0). In lactic acid bacteria at least two states of acid tolerance increase are recognized: the logarithmic growth adaptive response, which is induced by sub-lethal acid concentrations and the stationary phase response, which is part of a general stress protein expression (Hartke et al. 1996). A third strategy contributing to acid tolerance is growth in biofilms (Li et al. 2001). In general, biochemical, proteomic and genetic analysis point to the involvement of the synthesis of a variety of proteins in acid tolerance resulting in several acid tolerance mechanisms. These mechanisms imply energy dependent proton expulsion, cation transport for pmf generation, the production of basic compounds, the electrogenic transport of end products, an alteration of the cell envelope, as well as the repair mechanisms for damaged DNA or proteins (van de Guchte et al. 2002). The contribution of the arginine deiminase pathway (ADI), as source of basic compounds, which elevate the environmental pH, in acid tolerance is described exemplarily. The ADI pathway consists of three enzymes, the arginine deiminase, the ornithine carbamoyl-transpherase and the carbamate kinase and catalyzes the conversion of arginine to ornithine, with concomitant formation of two moles NH_3, one mole CO_2 and one mole ATP. An arginine/ornithine antiporter allows the import of arginine at no energy cost. The produced NH_3 exhibits and pKa value of 9.37 and thus is easily protonated in the pH range of the lactic acid bacterial growth. This proton consumption elevates the environmental pH value and thus prolongs the time for the bacterium to react on acid stress

with additional cellular improvements (see above). The ADI pathway is known to be present in several lactic acid bacteria strains, however its association with acid tolerance has not always been demonstrated. Furthermore, it is suggested, that several factors are involved in ADI pathway regulation, as arginine availability, energy depletion, catabolite repression and oxygenation, while its importance in acidurance may vary among the lactic acid bacteria species (van de Guchte et al. 2002).

Another stress acting on bacteria in beer is starvation. Bacterial growth is normally associated with substrate depletion and starvation. Nutrient starvation can be regarded as the most common stress acting on bacteria. In result growth is limited and shifted into stationary growth phase. However, habitats exist, which are generally low in nutritious substances or due to an acidified environment, nutrient uptake is hampered by a decreased activity of transport proteins. The acidified environment as well as the low amount of nutritive substances is both found in beer. To overcome starvation, many bacteria developed strategies as spore-forming to ensure long-term survival (Schlegel and Zaborosch 1992). However lactic acid bacteria developed other strategies. As lactic acid bacteria comprise a heterogeneous group of bacteria, with regard to growth conditions and nutrient requirements, it is obvious, that no general starvation response seems to exist (van de Guchte et al. 2002). However an important common aspect of lactic acid bacterial survival during starvation seems to be the maintenance of a metabolic active state (Poolman et al. 1987). Under carbohydrate starvation, the importance of amino acid catabolism for survival was described in several studies (van de Guchte et al. 2002). In this context, again the arginine metabolism was always found to provide a source of energy. In *E. faecalis* starvation was associated with the expression of proteins involved in the modulation of the metabolism of the bacteria, as e.g. pyruvate metabolism (Giard et al. 2001). These investigations demonstrated, that only a small overlap between stress-specific and starvation regulons exist and general stress proteins are rare.

In conclusion, these observations suggest, that the molecular basis of stress response and adaptation in lactic acid bacteria is at least species or even strain specific. Thus more information is required on the diversity of lactic acid bacterial stress response. With the increase of genomic information future transcriptome or current proteome studies on several species of lactic acid bacteria will contribute to the understanding lactic acid bacterial stress response. E.g. *L. brevis* for which no comprehensive stress studies exist until now (cf. chapter 1.5). In this context the identification of crucial stress related genes might be helpful in

modulating bacterial growth (promote or limit), the development of screening tools and the evaluation of fitness and level of adaptation of a culture (van de Guchte et al. 2002).

1.9 Objectives of the work

It was the aim of this thesis to investigate the mechanisms of hop inhibition, tolerance and adaptation in *L. brevis*. The understanding of these mechanisms can contribute to the development and improvement of new and current detection methods, respectively, for beer spoiling lactic acid bacteria during beer production, as well as offer targets for their elimination by mild preservation techniques. In this work should be investigated the diversity in metabolism, transmembrane transport, membrane composition and fluidity, cell wall associated lipoteichoic acids, gene expression, proteome, cross resistance, cellular manganese levels and intracellular pH of *L. brevis* and its adapted hop resistant variant in order to establish a model on the interaction of the multiple resistance mechanisms and their respective role in hop adaptation. Furthermore, the mode of hop inhibition should be characterized. Thus, several parameters of bacterial hop inhibition, adaptation and tolerance could be individually evaluated, as for analytical convenience, the whole cell is usually separated into cytoplasm, membranes and cell wall and their dedicated functions are explored. However, in the living cell one shades into another and they are all interdependent in function and formation (adapted from Rogers (1988)). Consequently the challenge in hop resistance research is to reassemble the various defense mechanisms on all cellular levels to see the overall functions. Beyond the specific model of hop tolerant bacteria, these investigations should provide general insight on the role of electrophysiology and ion homeostasis in bacterial stress responses and tolerance development to membrane active drugs.

2   Material and Methods

2.1   Characterization of isomerized hop extract

The chemical composition of the isomerized hop extract was determined by Roland Schmidt via HPLC-DAD (Nateco2 GmbH u. Co. KG, Mainburg, Hallertau, Germany). A titration curve of the isomerized hop extract (Isohop, Nateco2 GmbH u. Co. KG, Mainburg, Hallertau, Germany) at a concentration of 500 µM iso-α-acids in 0.2 M KCl in bidestilled water was generated by a conventional method (Wannowius and Plenio 2005). The changes of pH were measured with a pH meter (761 calimatic, Knick GmbH, Berlin, Germany) under stirring. As titrants 0.1 M HCl or 0.1 M NaOH were used. The degree of protonation was calculated as previously described (Wannowius and Plenio 2005). Cyclic voltammetry and electrochemical ITT (indicator time test) (Sobiech et al. 1998) was used to assess the redox properties of hop compounds at various pH values with and without the addition of $MnCl_2$. Iso-α-acids were dissolved at concentrations of 100 µM in buffer (50 mM acetic acid, potassiumphosphate and 5 mM Caps, 0.5 gL^{-1} KCl). The pH values covered the range from 4 to 11 in steps of 1 pH unit. If indicated, $MnCl_2$ was added at concentrations of 200 µM. All buffers and samples were degassed and stored under nitrogen. The redox analysis was performed at the chair for Technologie der Brauerei II (Weihenstephan, Germany) with a modified analytic automaton APP 5003 (ME Instrument, Trappenkamp, Germany), as previously described (Sobiech et al. 1998). In short, cyclic voltammetry was performed with a starting potential of 0.0 V and a switching potential of 1.1 V versus NHE (scan rate 0.4 Vs^{-1}). The zero current potentials for forward and reverse scans were determined and the mean zero current potentials were calculated. The ITT was measured with a working potential of 0.625 V versus NHE and the DCI (2,6-dichlorophenol-indophenol) reaction time was set to 5 min. Reagent solutions of 0.005 M DCI and 0.001 M ascorbic acid and an equimolar amount of oxalic acid were used (dosage 0.55 ml per 8.80 ml of sample). The reductone-ascorbic acid ratio was calculated as previously described (Sobiech et al. 1998).

2.2   Microorganism, media and culture conditions

L. brevis TMW 1.465 was selected as the most potent beer-spoiling organism among 31 strains of lactobacilli. The variant of this strain adapted to hop stress (see below) was designated *L. brevis* TMW 1.465A. The properties of *L. brevis* TMW 1.465A reflect those of *L. brevis* TMW 1.465 (cf. RAPD patterns in the appendix) except that the MIC of iso-α-acids was elevated to > 100 µM (highest tested 103.2 µM) at pH 4.0 after 48 h. *L. brevis* TMW

1.465 was grown in mMRS4 (A growth medium containing fructose was chosen, because mannitol was detected by HPLC in spoiled beers. Additionally, this mMRS4 medium was optimal for growth of *L. brevis* TMW 1.465.) (Stolz et al. 1993) at pH 6.0 (reference conditions) or at pH 4.0 (acid stress conditions). *L. brevis* TMW 1.465A was grown at pH 4.0 in the presence of isomerized hop extract (Isohop, Nateco2 GmbH u. Co. KG, Mainburg, Hallertau, Germany) added to a concentration of 86 µM iso-α-acids. Cultures were grown at 30°C. Unless otherwise stated, cells were harvested by centrifugation (2500 x g for 15 min) for each condition at the optical density (OD) $OD_{590nm}= 0.4$ in an early exponential growth phase.

2.3   Determination of MDR-transport activity.

MDR transport activity of *L. brevis* TMW1.465 and TMW1.465A was assessed with ethidium bromide, Hoechst 33342 and Calcein AM as substrates according to previously established protocols (Lecureur et al. 2000; Ulmer et al. 2000).

2.4   Adaptation conditions and measurement of hop resistance

Cultures of *L. brevis* TMW 1.465 were subcultured in media with increasing concentrations of iso-α-acids. The inoculation density in each case was $OD_{590nm} = 0.4$. The concentrations of iso-α-acids were increased from 17.2 µM to a final concentration of 86 µM within a period of 60 days. Growth curves were measured at days 15, 30 and 60 of the adaptation period. Growth challenges were carried out in microtiter plates with hop extract dilution series from 17.2 µM to 103.2 µM in steps of 8.6 µM iso-α-acids. Media were inoculated to an OD_{590nm} of 0.15. A layer of sterile paraffin was applied to ensure anaerobic conditions and the OD_{590nm} was measured over a period of 200 h. 50 % of the cultures did not survive the adaptation procedure. The hop resistant variant of *L. brevis* TMW 1.465 adapted to 86 µM iso-α-acids was named *L. brevis* TMW 1.465A.

2.5   Acquisition of metabolic data

Cultures were grown to stationary phase at reference-, acid stress- and hop stress conditions and the culture supernatant was obtained by centrifugation (5000 x g, 10 min). Metabolites were determined by high-performance liquid chromatography (HPLC). Maltose, glucose, fructose, mannitol, lactic acid, acetic acid, and ethanol were separated on a polyspher OA KC column (Merck, Darmstadt, Germany), amino acids in the supernatants were determined using an AminoPac PA10 column (Dionex, Idstein, Germany) as previously described (Thiele

et al. 2002; Korakli et al. 2003). Two external amino acid standards containing arginine, ornithine, lysine, citrulline, glutamine, asparagine, alanine, threonine, glycine, valine, serine, proline, isoleucine, leucine, methionine, histidine, phenylalanine, glutamate, aspartate, cystine, cysteine, tyrosine and norleucine as internal standard were used. Samples from independent cultures were prepared in duplicate and the means were calculated.

2.6 Growth challenges in the presence of amino acids

L. brevis TMW 1.465 and TMW 1.465A were grown in mMRS4 medium (pH 4.0 and 86 µM iso-α-acids) supplemented with different concentrations of arginine, ornithine, alanine, asparagine, leucine, glutamate, methionine and phenylalanine. The amino acids were added in concentrations from 20 mM to 35 mM in steps of 5 mM and pH was adjusted to 4.0 again. Growth challenges were carried out in microtiter plates. The inoculation density was set to OD_{590nm} 0.15. A layer of sterile paraffin was applied. The growth was measured photometrically (590nm).

2.7 Contribution of membrane associated transport protein to hop resistance

The contribution of membrane associated transport proteins to hop resistance was determined upon selective inactivation of membrane transport systems by high pressure treatment (Ulmer et al. 2000; Ulmer et al. 2002). Cells from *L. brevis* TMW 1.465 and *L. brevis* TMW 1.465A were subjected to a pressure treatment at 300 MPa and 20°C for 0 to 30 min as previously described (Ulmer et al. 2000). The treated cells were subsequently incubated at 30°C in mMRS4 media (pH 4.0) containing 0 to 68.8 µM iso-α-acids and the OD_{590nm} of the cultures was determined in 1 h intervals over 28 days. The detection times were determined as the incubation times required to increase the OD_{590nm} by 0.2.

2.8 Determination of membrane composition

Cells were grown under reference-, acid stress- and hop stress conditions as described above in 50 ml of mMRS4 to stationary growth phase. Cells from each condition were lyophilised, packed under N_2 atmosphere and sent to Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) for analysis. Membrane fatty acids were extracted, transesterified, and analyzed by gas chromatography.

2.9 Measurement of the membrane fluidity

The membrane fluidity of *L. brevis* TMW 1.465 grown under reference-, acid stress- and *L. brevis* TMW 1.465A grown under hop stress conditions was determined by Laurdan

fluorescence (Parasassi et al. 1990). The cells were washed twice with phosphate buffer (50 mM, pH 6.5, 10 mM glucose) and resuspended to an OD_{590nm} 1.0 in the same buffer. A stock solution of Laurdan (2 mM in ethanol) was added to a concentration of 40 µM. Cells were stained at 30 °C for 30 min in the dark. Afterwards the cells were washed twice with phosphate buffer (50 mM, pH 6.5, 10 mM glucose) and resuspended again in phosphate buffer (50 mM, pH 6.5, 10 mM glucose). The fluorescence spectra (λ_{Ex}: 360nm, λ_{Em}: 380-550nm) were measured in a Luminescence Spectrometer LS 50B (Perkin Elmer, Rodgau - Jügesheim, Germany) in steps of 1 nm at a temperature of 5, 10, 15, 20, 25, and 30°C. The Laurdan general polarization (*GP*) was calculated as follows: *GP* = [I(440 nm) – I(490 nm)] / [I(440 nm) + I(490 nm)], I= relative fluorescence at the respective wavelength (Parasassi et al. 1990). To measure the effect of the hop compounds on the membrane fluidity, the phosphate buffer was replaced with sodium acetate buffer (50 mM, pH 4.0) containing 86 µM iso-α-acids. The measurement was performed at 30 °C.

2.10 Preparation and analysis of lipoteichoic acids

Cells were grown under reference-, acid stress- and hop stress conditions as described above to stationary growth phase. Cells were harvested by centrifugation (5000 x g, 20 min, 4°C) and washed once with cold 0.01 M sodium-acetate (pH 4.7) containing 0.9 % NaCl and resuspended at a concentration of 0.4 g cells/ml (Jenni and Berger-Bächi 1998). Cells were broken with an ultrasonic treatment (HD-70/Bandelin, 5 cycles of 30 s; power 90 %, cycle 30 % and intermediate cooling). The dry weight of the broken cell suspension from each condition was determined and used to normalize analytical data. The lipoteichoic acid (LTA) was extracted and purified on an octylsepharose column essentially according to Fischer et al. and Jenni et al. (Fischer et al. 1983; Jenni and Berger-Bächi 1998). The purification of lipoteichoic acids was controlled online (BioLogic Optics Module II OM-11, Bio-Rad, USA) for protein and DNA contaminations photometrically. Lipoteichoic acids were concentrated by lyophilisation. For characterization, the LTA was chemically deacylated (cdLTA) by mild alkali treatment with 0.1 M NaOH for 1 h at 60°C (Pollack et al. 1992) and the cdLTA was analyzed by polyacrylamide gel electrophoresis (PAGE) as described (Neuhaus and Baddiley 2003). The cdLTA was visualized by a combined alcian blue and silver stain (Moller et al. 1993). For determination of the glycerolphosphate content, the cdLTA was hydrolysed with 2 M HCl for 2 h at 100°C, the buffer was neutralized with NaOH and the glycerolphosphate concentration was measured enzymatically (Nishihara and Koga 2000).

2.11 Expression analysis of hop resistance genes on the mRNA level

For the expression analysis of hop resistance genes a total RNA extraction from cells in all three stress conditions (see above) was performed according to Aiba et al. (Aiba et al. 1981). The RNA was purified and reverse transcription was performed with random primers and MLV-reverse transcriptase according to the instructions of the supplier of the reagents (Promega, Mannheim, Germany). The nucleotide sequences for *arcA*, *arcB*, *arcC* and phosphoketolase were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/). The primers for the hop resistance genes were constructed with the Dnasis software (Hitachi Software Engenieering Co, Yokohama, Japan) according to the instructions of the manufacturer of the LightCycler (Roche Diagnostics GmbH, Mannheim, Deutschland). The cDNA was quantified using the QuantiTect SYBR Green Mastermix (Quiagen, Hilden, Germany). The efficiency of each primer pair was determined with a dilution series of chromosomal DNA. The expression analysis (Pfaffl 2001) was normalized for each experiment using phosphoketolase as a housekeeping gene. Template RNA was included in the LightCycler runs to exclude contamination of the RNA preparation with DNA. The primers and annealing temperatures for the amplification reactions are shown in table 1.

Tab. 1 Primers used for expression analysis of hop resistance genes via LightCycler PCR

Primer	Sequence (5´ to 3´)	Annealing temperature (°C)
hitA_V	TAGCACGGTCGGGCGATTCGTTG	59
hitA_R	CCTGACAGCGTCCCGGTAATCGTG	59
arcA_V	CAAGAGTCATTTTGACAAGGTTATTG	52
arcA_R	GAATCAAATCTAAATCGTCAAGGTTC	52
arcB_V	AAATTACTTGTTACCGACGACTTAGC	52
arcB_R	CCATTTCAGTAATACCGTACTTTCC	52
arcC_V	GACCACAAGTTGGTAATTTACTGTTG	52
arcC_R	CTTAATCTCAGCTTCTGTGTAGAACG	52
keto_V	TTCTACATTGGTGGTCCTGGTC	52
keto_R	GTTGCGTGGGAAAGTGAGTAAC	52

2.12 Evaluation of resistance based on MDR transport systems, barriers or metabolism

The respective role of the hop resistance mechanisms in the phenotype of *L. brevis* TMW 1.465A were evaluated by measurements of changes in the intracellular pH of cells that were exposed to different concentrations of hop compounds to study passive hop barriers as well as HorA independent transport processes (Suzuki et al. 2002). *L. brevis* TMW 1.465A was grown under hop stress conditions as described above. The effect of hop compounds on the intracellular pH can only be measured in cells with "normal" starting pH_{in}. Accordingly, the cells were subcultured once in the same medium without hop compounds to restore their intracellular pH for the measurement. Cells were harvested by centrifugation and washed

twice with phosphate buffer (50 mM, pH 6.5) and resuspended in the same buffer at OD_{590nm} =1.0. The intracellular pH was measured with the fluorescent dyes cFDASE (pKa 6.5) according to a previously described method (Molina-Gutierrez et al. 2002) and with Calcein AM (Invitrogen GmbH, Karlsruhe, Germany). Calcein AM exhibits pKa values (pKa_1=2.1, pKa_2=2.9, pKa_3=4.2, pKa_4=5.5, pKa_5=10.8 and pKa_6=11.7), which makes it suitable for measuring in the pH range from pH 3.0 to 6.5. 4 µM Calcein AM were added to the samples. After 3 h of incubation at 30°C to allow for dye internalization and hydrolysis by cellular esterases the cells were washed twice with sodium acetate buffer (50 mM, pH 4.0), resuspended at OD_{590nm} =1.0 and cooled down to 4°C to achieve a low permeability of the bacterial membrane for hop compounds until the measurement starts (Simpson 1993). Hop compounds were added to the samples to achieve a concentration of 0, 2, 20 and 80 µM iso-α-acids. For energized samples 10 mM arginine or glucose were added. The ionophores valinomycin (1 µM) and nigericin (1 µM) were used as controls. The fluorescence (λ_{Ex}: 485 nm, λ_{Em}: 520 nm) was measured in black microtiter plates in a spectrafluor microtiter plate reader (Tecan, Grödig, Austria) at 30°C for 120 min with shaking before and between measurements. For pH_{in} calibration the pH_{in} and pH_{ex} were equilibrated by addition of valinomycin (1 µM) and nigericin (1 µM) (Molina-Gutierrez et al. 2002) (data not shown). It was verified that calcein is not a substrate for MDR transport in *L. brevis* TMW 1.465A (data not shown). Furthermore, it was excluded that the divalent cations Ca^{2+}, Mg^{2+} and Mn^{2+}, from which the latter is known to be present in high concentrations in lactic acid bacteria (Archibald and Duong 1984), affect calcein fluorescence when added to the buffer to concentrations ranging from 16 µM to 10 mM (data not shown). To take into account that no pH independent fluorescence could be measured for calibration, it was ascertained that a loss of dye was not responsible for the decrease of fluorescence. The recovery rate of fluorescence for pH_{in} = 6.5 equilibrated cells quantified before and after each measurement (as described above) was 97 % ± 3 %.

2.13 Extraction of whole-cell protein

Cells were washed twice with phosphate buffer (50 mM, pH 7). For standardization a cell mass corresponding to 7 mg cell dry weight was used for all conditions for subsequent protein extraction. The cell walls were digested with 318 kU lysozyme in TE-DTT buffer (10 mM Tris-HCl, 0.01 M EDTA, 6 mM DTT, pH 8.0) for 45 min at 37 °C. Digested cells were centrifuged (5000 x g for 5 min) and supernatant was discarded. The pellet was resuspended in 200 µl SDS buffer (0.9 % SDS, 0.1 % Pefabloc, 100 mM Trisbase, pH 8.6) and disrupted

by sonication (HD-70/Bandelin, 3 cycles of 30 s; power 90 %, cycle 30 % and intermediate cooling). The suspension was diluted 3.5 fold with thiourea lysis buffer (6.10 M urea, 1.79 M thiourea, 65.06 mM DDM, 1 % w/v DTT, 0.5 % v/v Pharmalyte 3-10) and sonicated again. The proteins were solubilized by shaking for 20 min. The remaining cell wall fragments were removed by centrifugation at 17500 x g and 4 °C for 30 min. The clear supernatants were stored at –80 °C (Harder 2001).

2.14 Two dimensional (2D) electrophoresis

Two dimensional electrophoresis was performed with samples from reference-, acid stress- and hop stress conditions according to Görg et al. (Görg et al. 2000). IEF was carried out using a Multiphor II DryStrip kit system (Amersham Biosciences Europe GmbH, Freiburg, Germany) with 24 cm IPG 4.5-5.5 strips (Amersham Biosciences) at 20 °C. The IPG strips were rehydrated with an excess of rehydration solution (6.10 M urea, 1.79 M thiourea, 8.13 mM DDM, 0.2 % w/v DTT, 0.2 % v/v Pharmalyte 3-10). In total, 300 µl protein extract were applied by sequential anodic cup loading. Initial IEF was run for 24 h at 50 V and subsequently 12 h at 150 V. IEF to steady state at 3500 V was carried out according to the micropreparative IEF protocol (Görg et al. 2000). SDS-PAGE was performed on a vertical system with gels of total acrylamide concentration of T11 % at 15 °C. The proteins were visualized by colloidal coomassie staining (Roti-Blue, Carl Roth GMBH & Co, Karlsruhe, Germany) and quantified as previously described (Mahon and Dupree 2001). For analysis of reproducibility of protein expression, it was monitored on 18 cm IPG strips 3-10, 4-7 and 4.5-5.5 by at least two independent experiments. Protein quantification on high resolution 24 cm IPG 4.5-5.5 strips is based on electrophoretic analysis of two independent experiments.

2.15 Semidry-blotting and N-terminal sequence analysis

The transfer of proteins from the 2D gel to a PVDF-membrane (Immobillon^{TM}-PSQ, Millipore) and their visualisation was performed as previously described (Baker and Dunn 1994). For N-terminal sequencing of 3 chosen proteins, the blot was sent to Prosequenz Bioanalytik (Ludwigsburg, Germany) and analyzed with the Proteinsequinator Modell 476 (Applied Biosystems, ABI) according to the instructions of the supplier.

2.16 Mass spectrometric protein characterization

The colloidal coomassie (Roti-Blue, Roth) stained proteins were excised from the gel and sent to the Zentrallabor für Proteinanalytik (Ludwig-Maximilians-Universität München, Germany) for MALDI TOF-MS, LC-ESI MS/MS and ESI MS/MS analysis (cf. appendix).

2.17 Protein identification

For identification of proteins from non-genome sequenced *L. brevis* TMW 1.465A, a protein database was created according to Johnson et al. (Johnson et al. 2005). All available protein sequences from *Lactobacillus* and *Lactococcus* species, especially from *L. brevis* ATCC 367, were obtained from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). The CDS translations of the genome draft sequences from *L. casei* ATCC 334, *L. delbrueckii* subsp. *bulgaricus* ATCC BAA-365, *L. gasseri* ATCC 33323, *L. reuteri* JCM 1112, *L. reuteri* 100-23 and *Lactococcus lactis* subsp. *cremoris* SK11, which were produced by the U.S Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/draft_microbes), were downloaded. Three hundred contaminant proteins including keratins and proteases were added into the database (Zhu et al. 2004). For protein identification, the NCBI database and the created database, which contained a total 34401 sequences, were searched by the Matrix Science Mascot software (Perkins et al. 1999) and the MS-BLAST version (Shevchenko et al. 2001) of the WU-BLAST2 program (Gish 1996). For the latter, "Sequest dta formatted" (Yates et al. 1995) files derived from LC-ESI MS/MS analysis were processed to peptide sequences with LutefiskXP (Taylor and Johnson 1997). A program written in C generated and submitted multiple permutations and catenations of peptide sequences to MS-BLAST (cf. appendix).

2.18 Growth challenges in the presence of divalent cations

L. brevis TMW 1.465A was grown in mMRS4 medium prepared without $MgSO_4$ and $MnSO_4$ (pH 4.0 and 86 µM iso-α-acids) and supplemented with different concentrations of $MnSO_4$, $MgSO_4$ or $CaSO_4$. The divalent cations were added in concentrations from 0 mM to 70 mM in steps of 10 mM and pH was adjusted to 4.0 again. Growth challenges were carried out in microtiter plates. The inoculation density was set to OD_{590nm} 0.10. A layer of sterile paraffin was applied. The growth was measured photometrically (590nm) after 48 h.

2.19 Growth challenges with calcimycin and valinomycin and nigericin

Growth challenges with *L. brevis* TMW 1.465 and TMW 1.465A were done in mMRS4 medium (without supplementation of $MgSO_4$ and $MnSO_4$) with different pH values and additions of the antibiotics calcimycin (Abbott et al. 1979) or valinomycin and nigericin. The pH values were set from 4.0 to 5.0 in steps of 0.2. The antibiotics were each added in concentrations of 5 µM. Controls were performed without addition of antibiotics. In controls without antibiotics 1% (v/v) ethanol was added, which was the amount that was brought into

the growth medium by the addition of the antibiotic stock solutions. Growth challenges were carried out in microtiter plates. The inoculation density was set to $OD_{590nm} = 0.10$. A layer of sterile paraffin was applied. The growth was measured photometrically (590nm).

2.20 Measurement of cellular manganese

Cells were grown to late stationary phase, washed and divided in two aliquots. One aliquot was resuspended in mMRS4 (without supplementation of $MgSO_4$ and $MnSO_4$ and pH 4.0) (control sample) and the other in the same medium with 86 µM iso-α-acids and incubated at 30 °C for three days. During this period the growth medium was changed every 12 h. Cells were harvested by centrifugation, washed, controlled for cell lysis and lyophilised. 50 mg of each sample were reduced to ashes at 800 °C for 2 h. The cellular minerals were dissolved in 600 µl nitric acid (15 %) and 10 µl phosphoric acid. 50 mg periodic acid were dissolved and the mixture was incubated for 10 min at 100 °C. A subsequent addition of 50 mg periodic acid and incubation at 100 °C (10 min) ensured a complete oxidation of manganese to permanganate. The solution was made up to 1 ml and the permanganate was measured photometrically at 525 nm.

2.21 Measurement inhibition of enzyme reactions by hop compounds

The enzyme activity of glucose-hexokinase and glucose-6-phosphate dehydrogenase was measured photometrically principally according to the instructions of the supplier of the enzymes (Roche, Mannheim, Germany) in the presence of hop compounds. Glucose-6-phosphate dehydrogenase activity was determined with excess of glucose-6-phosphate (0.4 mM) and $NADP^+$ (0.1 mM) as limiting factor. Glucose–hexokinase activity was determined with access of glucose (1 mM) and Mg-ATP (0.1 mM) as limiting factor. For the latter reaction a subsequent conversion of glucose-6-phosphate by glucose-6-phosphate dehydrogenase (1 mM $NADP^+$ present) as detection reaction was used to assess the glucose-hexokinase activity. Hop compounds were added to the enzyme assays in concentrations ranging from 0 to 100 µM of iso-α-acids. As divalent cation Mg^{2+} was added at a concentration of 100 µM.

2.22 Phenomenological investigation of mode of action of hop compounds via bilayer lipid membrane techniques

L. brevis TMW 1.465 was grown in 10 L mMRS4 at 25°C to a transient to stationary growth phase. Daily the pH of the growth medium was adjusted to 6.0. The cells were harvested by

centrifugation at 25°C and 2500 x g for 90 min and washed twice with phosphate buffer (50 mM, pH 7.0). The natural membrane lipids from *L. brevis* TMW 1.465 cells were extracted and purified with modifications (personal communications Teichert, TMW) as previously described (Ames 1968; Margolles et al. 1999). In detail, the washed cells were frozen (-20 °C) and thawed, resuspended in phosphate buffer (20 mM, 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^{-1} lysozyme and 0.01 mg ml^{-1} DNase. Following steps were carried out under nitrogen atmosphere to avoid lipid oxidation. One volume of chloroform and 2 volumes of methanol were added to the suspension and stirred overnight at 4 °C. Cell debris was removed by centrifugation at 2000 x 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 room temperature. The phase separation was permitted overnight at 4 °C. The chloroform phase was evaporated in a rotary evaporator at 40 °C and redissolved in chloroform at a concentration of 0.1 g ml^{-1}. Aliquots containing 1 mg of *L. brevis* membrane lipids were vacuum dried and stored under nitrogen at -80 °C. Further egg phosphatidyl choline (E PC S, Lipoid GmbH, Ludwigshafen, Germany) and synthetic phosphatidyl glycerol 16:0/16:0 (DPPG, Lipoid GmbH, Ludwigshafen, Germany) were used. Membrane forming mixture contained 20 mg lipids and 20 mg cholesterol (Mueller et al. 1963) per 1 ml decane. The bilayer lipid membrane (BLM) was formed in a 1.1 mm hole in a 0.2 mm thick Teflon partition by a conventional method (Mueller et al. 1963) using a symmetrical 2 ml Teflon BLM chamber with glass windows on both sides. Sintered Ag/AgCl electrodes (In Vivo Metric, Healdsburg, CA, USA; cf. appendix) were connected to the electrolyte solution through 10 µl pipette tips filled with 2 % agarose in 0.2 M KCl. Capacitance-, open circuit-, chronoamperometric- and cyclic voltammerty measurements were performed with a custom-made microchip-controlled two or four electrode capacitance measurement and potentiostat/galvanostat system (cf. circuit boards in the appendix) built on the basis of previous descriptions of Kalinowski et al. (Kalinowski and Figaszewski 1995; Kalinowski and Figaszewski 1995). The formation of the membrane was controlled visually by a microscope and by the measurement of its electric capacitance. The measurements were performed thermostatic (Multitemp III, Amersham Biosciences Europe GmbH, Freiburg, Germany) at 23 °C. Isomerized hop extract (Joh. Barth & Sohn GmbH & Co., Nuernberg, Germany), ionophores calcimycin and CCCP and salts of divalent cations $CaCl_2$, $MgCl_2$ and $MnCl_2$ were used. Unless otherwise stated, KCl (0.2 M) was used as supporting electrolyte. Electrolyte solutions were buffered with Tris, Mes, citric acid (5 mM) or potassium phosphate

(20 mM). pH gradients were set by addition of a 1 M HCL. All solutions were stored under nitrogen.

3   Results

3.1   Characterization of isomerized hop extract

The chemical composition of the isomerized hop extract is shown in table 2. To understand the role of solution pH and acid/base properties of hop compounds from an isomerized hop

Tab. 2 Determination of iso-α-acids and α-acids in isomerized hop extract via HPLC-DAD (diode array detector) analysis.

% (w/v)	iso-humulone	humulone	lupulone
co-compound	6.4	0.1	0.1
n-compound	14.3	0.2	0.0
ad-compound	3.6	0,0	0.0
sum	24.3	0.3	0.1

extract a titration curve was established. Figure 15 depicts the degree of protonation P = [HA]/([A⁻]+[HA]) of isomerized hop compounds as a function of the pH value. The isomerized hop extract showed buffer capacities between pH 2.2 and pH 6.0 in accordance with the published pKa values for e.g. trans-iso-humulone (3.1), humulone (5.0) and
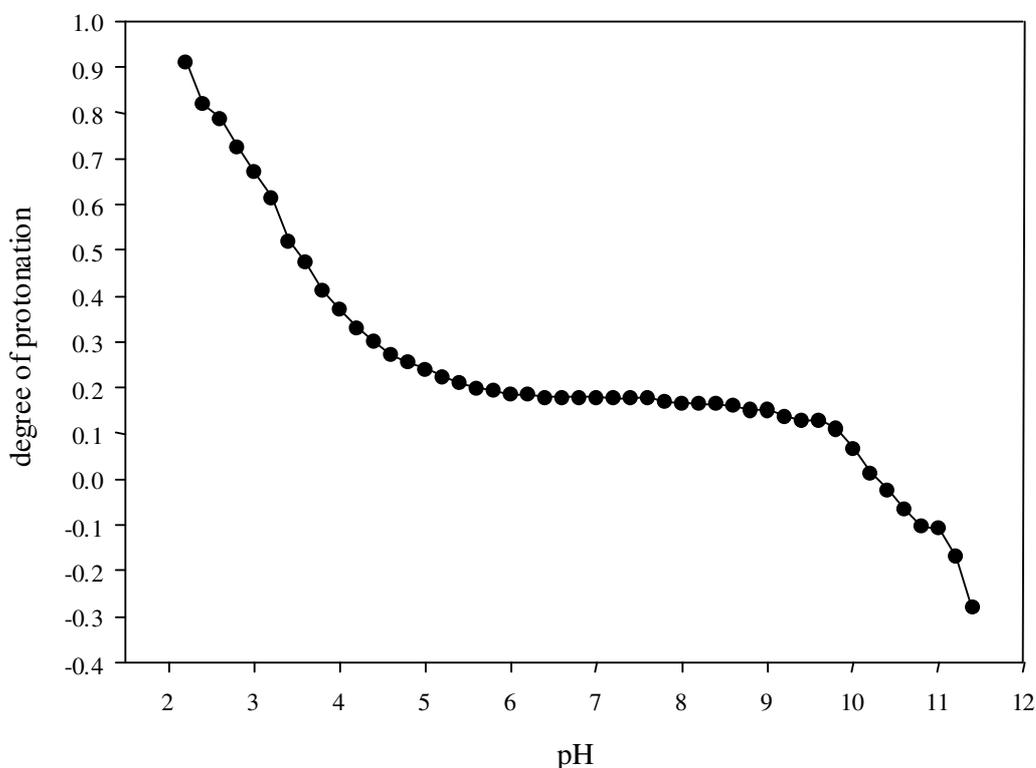
Fig. 15 The degree of protonation P = [HA]/([A⁻]+[HA]) of isomerized hop compounds (500 µM) related to the pH value, determined by titration according to Wannowius et al. (Wannowius and Plenio 2005).

colupulone (6.1) (Simpson and Smith 1992). A second buffer range was determined between pH 9.7 and 11.4. The negative values obtained beyond pH 10.2 can be attributed to hop

compounds with buffer capacity (in addition to that displayed in table 2), which were not included in the 500 µM iso-α-acids (value used for calculation) of titrated hop compounds. As a drift of measured pH was observed (Simpson and Smith 1992) and hop compounds are known to be highly reactive substances (Belitz et al. 2001) an influence of chemical conversions of hop compounds on the titration result cannot be excluded. For dosage of hop compounds the molarity of the iso-extract was calculated on the basis of the molecular weight of trans-iso-humulone. As the iso-extract contained 98.4 % (w/v) iso-α-acids, the terms hop compounds and iso-α-acids are subsequently used as synonyms.
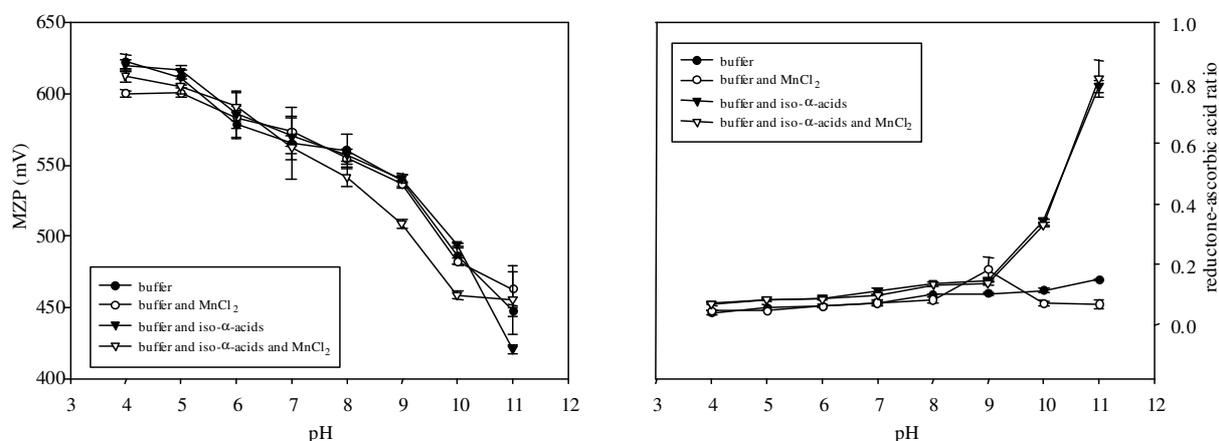
Fig. 16 Cyclic voltammetry determination of mean zero current potentials (MZP) at pH indicated (left). Reductone-ascorbic acid ratios determined by electrochemical ITT (indicator time test) at pH indicated (right). As buffers 50 mM acetic acid/potassiumphoshate and 5 mM Caps, 0.5 g/L KCl at pH indicated were used. Concentrations of iso-α-acids and $MnCl_2$ were 100 µM and 200 µM, respectively (Sobiech, Neumann et al. 1998).

To determine the redox properties of hop compounds in dependence of pH value and the presence of $MnCl_2$, automated cyclic voltammetry and electrochemical ITT analysis (equipment made available by the chair for Technologie der Brauerei II, Weihenstephan, Germany) were performed. The mean zero current potentials (MZP; cyclic voltammetry) and the reductone-ascorbic acid ratios (electrochemical ITT) of hop compounds at the respective pH values were determined. The cyclic voltammograms from hop samples (data not shown) showed the typical shape of beer cyclic voltammograms. The calculated MZP is assumed to deliver some information about the redox condition of the sample, where high MZP is associated with a more oxidizing condition and low MZP is the opposite (Sobiech et al. 1998). Figure 16 (left) depicts the MZP values of 100 µM iso-α-acids in buffer and 100 µM iso-α-acids and 200 µM $MnCl_2$ in buffer. As controls the sole buffer and buffer with 200 µM $MnCl_2$ were used. In general a decrease of MZP with raising pH value is obtained for all samples. A comparison of iso-α-acids in the presence of $MnCl_2$ and pure iso-α-acids shows, that the

MZPs in the presence of $MnCl_2$ are mainly below that found for pure iso-α-acids, indicating the iso-α-acids in the presence of $MnCl_2$ as the more reducing condition. Figure 16 (right) shows the electrochemical ITT analysis of the same samples used for cyclic voltammetry. In this measurement the reductone level of the sample is determined and standardized with a known amount of ascorbic acid as reducing agent. Thus, a high reductone-ascorbic acid ratio is correlated with a more reducing condition of the sample. Figure 16 (right) obviously shows no significant differences in the reductone level of iso-α-acids in the presence or absence of $MnCl_2$ in the pH range from 4 to 9. Beyond pH 9 an elevated reducing power for iso-α-acids as well as iso-α-acids in the presence of $MnCl_2$ is monitored.

3.2 Adaptation of *L. brevis* TMW 1.465 to hop compounds

The minimal inhibitory concentration (MIC) of iso-α-acids towards the unadapted *L. brevis* TMW 1.465 was 17.2 µM at pH 4.0 after 48 h of incubation. To document the progress of adaptation of *L. brevis* TMW 1.465 to high concentrations of hop compounds, the hop resistance was measured in three stages of adaptation. The starting iso-α-acids concentration of 17.2 µM reflected the hop tolerance of the unadapted *L. brevis* TMW 1.465. After 15 days the hop tolerance was increased to 51.6 µM iso-α-acids and after 45 days to the maximum test concentration of 103.2 µM (figure 17). Upon adaptation, the lag-phase and the growth rate of
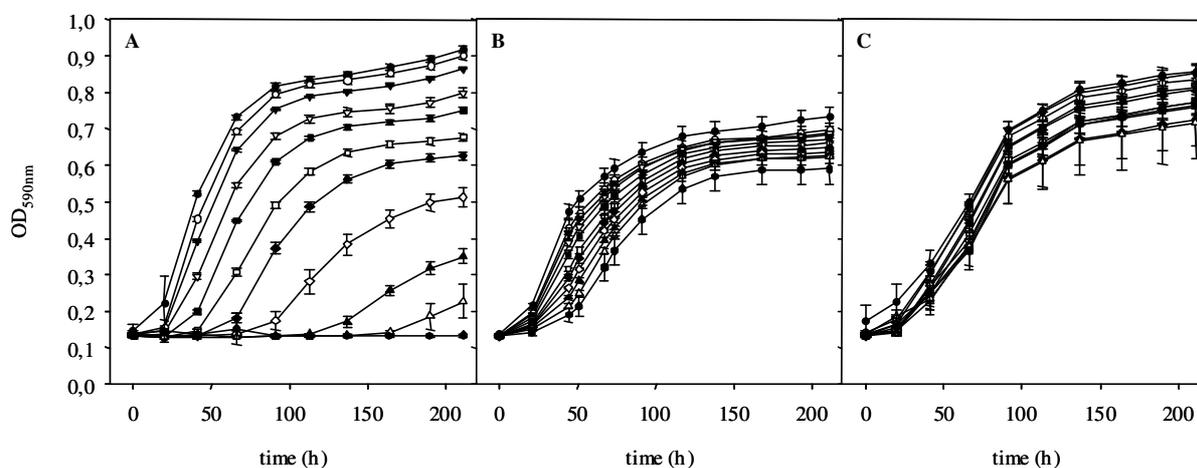
Fig. 17 Adaptation of *L. brevis* TMW 1.465 to increasing concentrations of iso-α-acids. The growth curves were measured after 15 d (A), 45 d (B) and 60 d (C) of adaptation. The cultures were inoculated on mMRS4 (pH 4.0) containing ●, 17.2; ○, 28.8; ▼, 34.4; ▽ 43.0; ■ 51.6; □ 60.2; ◆ 68.8; ◇ 77.4; ▲ 86.0; △ 94.6; ● 103.2 µM iso-α-acids. Shown are the means and standard deviations of four independent experiments.

the *L. brevis* TMW 1.465A were nearly independent of the iso-α-acids concentration in the growth medium. The adaptation to even higher hop stress levels was investigated, and *L. brevis* TMW1.465A grew at up to 172 µM iso-α-acids. These cultures showed an extended

lag phase and poor growth in comparison to the cultures grown under acid stress and optimal conditions.

3.3 Metabolism of *L. brevis* TMW 1.465 and *L. brevis* TMW 1.465A under reference- and stress conditions

The metabolites from maltose, glucose and fructose of *L. brevis* TMW 1.465 grown at reference-, and acid stress conditions, and of *L. brevis* TMW 1.465A grown under hop stress conditions are shown in table 3. A major change in the metabolism was observed when reference conditions (pH 6.0) were compared to the acid stress conditions (pH 4.0). This trend

Tab. 3 The sugar metabolism of *L. brevis* TMW 1.465 under reference-, acid stress- and *L. brevis* TMW 1.465A under hop stress conditions as measured via HPLC analysis.

mM	mMRS4	Reference (TMW 1.465)	Acid stress (TMW 1.465)	Hop stress (TMW 1.465A)
Maltose	31,5	1,1	21,4	22,1
Glucose	28,0	0,0	1,8	7,3
Fructose	28,4	0,0	1,8	1,2
Mannitol	0,0	8,6	19,6	24,3
Lactat	0,0	74,8	42,1	24,2
Acetat	0,0	11,1	13,2	18,8
Ethanol	0,0	80,2	31,6	10,8

continued with the higher stress level caused by additional hop, although to a lesser extent. Fructose was predominantly used as carbon source at reference conditions, but was used predominantly as electron acceptor at stress conditions with a concomitant increase of mannitol and acetate production. Lactate and ethanol production were reduced at stress conditions. One third of the maltose was not fermented under acid stress and hop stress conditions.

The amino acid metabolism under reference-, acid stress- and hop stress conditions was also examined. No detectable differences in amino acid composition of fermented supernatants and fresh growth medium were observed except for ornithine, which was formed from arginine. Ornithine was not detectable in fresh mMRS4. *L. brevis* 1.465A accumulated ornithine to 3.1 mM under hop stress condition, after incubation at reference and acid stress conditions, 1.4 and 1.7 mM ornithine were produced.

To evaluate a possible role of arginine metabolism to hop resistance, hop tolerant *L. brevis* TMW 1.465 and hop resistant *L. brevis* TMW 1.465A were grown in mMRS4 (pH 4.0) with 86 µM iso-α-acids that was additionally substituted with various amino acids. *L. brevis* TMW

1.465 grew only in medium substituted with arginine, and increased arginine concentrations resulted in a higher growth rate. Hop-containing medium that was substituted with any of the other amino acids remained lethal for *L. brevis* TMW 1.465. The hop resistant strain *L. brevis* TMW 1.465A showed a slightly faster growth with increasing concentrations of any of the amino acids.

3.4 Inactivation of membrane associated transport proteins via high pressure and determination of resulting decrease of hop resistance

The hop resistant as well as the hop tolerant strain harbor a functional *hitA* gene (Hayashi et al. 2001) and exhibit MDR transport activity when Calcein AM was used as substrate but not when ethidium bromide or Hoechst 33342 were used, which are accepted as substrates by HorA. To check HorA functionality, the *horA* gene (cf. DDBJ Accession AB167897) was cloned and sequenced to reveal a deletion in base 99, which leads to a stop codon after amino acid 36 and renders it non-functional. To determine the relevance of membrane associated
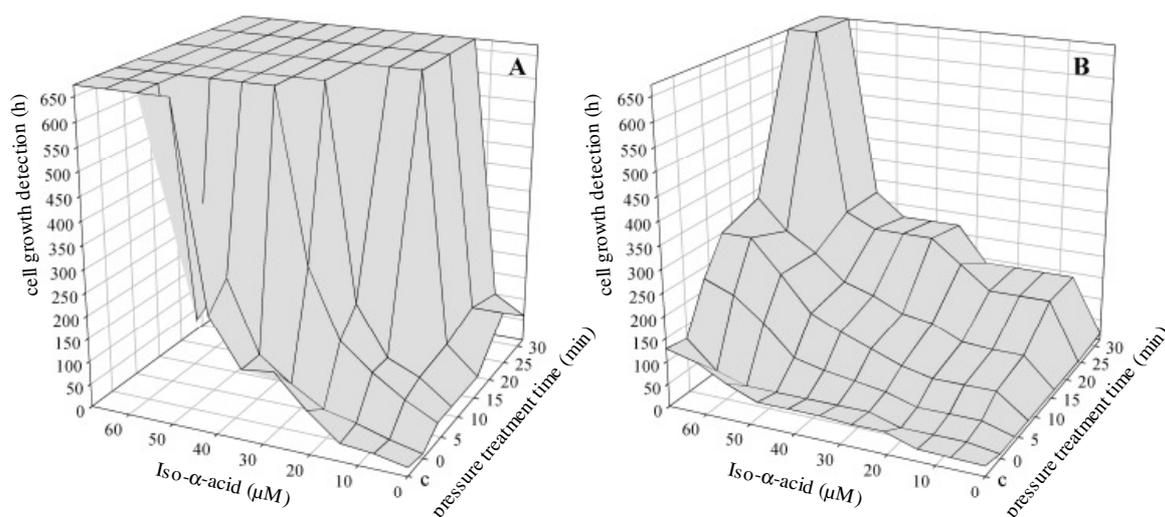
Fig. 18 Effect of sublethal high pressure treatment on the growth during subsequent storage in hopped media. *L. brevis* TMW 1.465 (A) and *L. brevis* TMW 1.465A (B) were treated at 300 MPa and 20°C for 0 to 30 min and subsequently incubated in mMRS4 media (pH 4.0) containing different concentrations of iso-α-acids. No pressure controls were designated "c". Cell growth detection times were calculated as the time required for an OD$_{590nm}$ increment of 0.2.

transport proteins on hop resistance, the transport proteins were inactivated by sublethal pressure treatment of *L. brevis* as previously described (Ulmer et al. 2002) and the hop resistance of pressure-treated *L. brevis* was assessed through the determination of detection times in media containing various levels of iso-α-acids. Pressure treated samples were diluted to obtain the same level of viable cell counts independent of pressure holding time (data not shown). This ensured a constant inoculation density for all preparations in the growth

experiment. Figure 18 shows that the addition of hop iso-α-acids to the growth medium delayed growth of pressure treated cells. However, for *L. brevis* TMW 1.465 and TMW 1.465A, 15 min and 24 min (lethal level), respectively, at 300 MPa were needed to inhibit the growth. For inactivation of MDR transport activity 30 s pressure holding time were sufficient (data not shown).

3.5 Determination of membrane composition

The fatty acid composition of the membrane of *L. brevis* TMW 1.465 under reference- and acid stress conditions and of *L. brevis* TMW 1.465A under hop stress conditions was determined (figure 19). Acid- or hop stress strongly affected the membrane composition. The most prominent shift upon acid stress was an increased level of C19 cyclopropane fatty acids
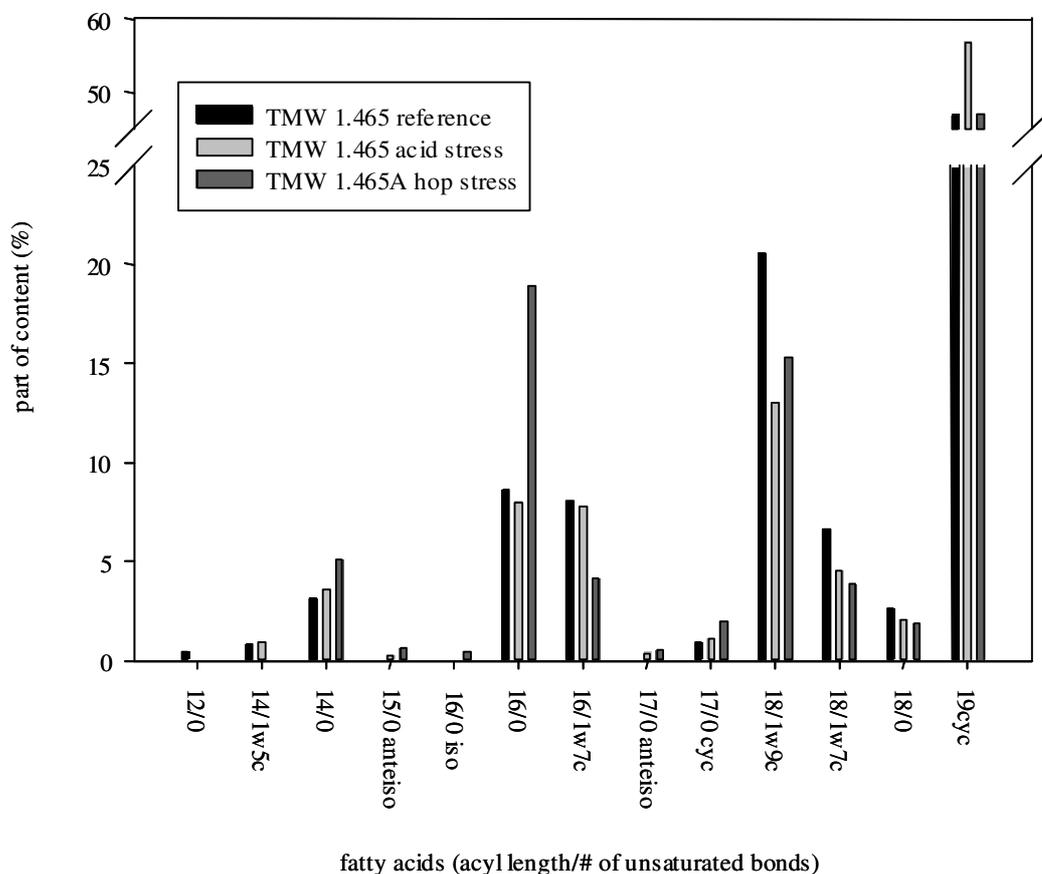
Fig. 19 Fatty acid composition of *L. brevis* TMW 1.465 grown at reference- and acid stress conditions, and *L. brevis* TMW 1.465A cytoplasmic membranes.

at the expense of 18/1 unsaturated fatty acids. Upon adaptation to hop stress, the content of palmitic acid (19 % of total FA) was strongly increased relative to reference- and acid stress conditions (8 %). Simultaneously, the 16/1 and 18/1 fatty acid contents were decreased. The ratio of the 16/0 to 16/1 fatty acid content, which is considered a "magic number" in proton

ionophore resistance (Krulwich et al. 1987) was elevated from 1.0 to 4.75 upon hop adaptation.

3.6 Membrane fluidity

To evaluate the change of fatty acid membrane composition, the fluidity and polarity of the cytoplasmic membrane of *L. brevis* TMW 1.465 grown under reference-, acid stress- and *L. brevis* TMW 1.465A under hop stress conditions was determined by using the Laurdan fluorescence. An increase of the *GP* value correlates to a reduced fluidity of the membrane (Yu et al. 1996). The influence of the hop compounds itself on the membrane fluidity was determined at 30°C. Hop addition induced an upshift of the *GP* value of approximately 0.06 +/-0.01 units, indicating a decrease in fluidity of the membrane (data not shown). The effect
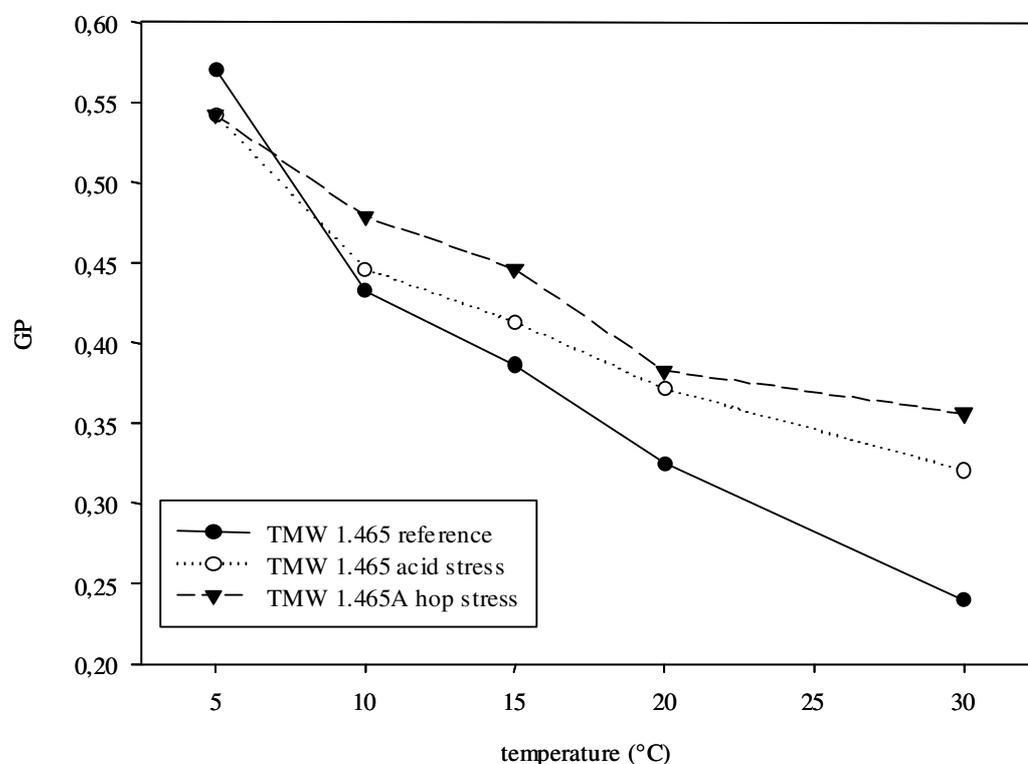
Fig. 20 *GP* value of Laurdan incorporated in the cytoplasmic membrane of *L. brevis* TMW 1.465 and *L. brevis* TMW 1.465A. Cells were grown to stationary growth phase under reference and acid stress conditions (TMW 1.465) or in the presence of hops (TMW1.465A). The data is representative for two independent experiments.

of the adaptation to acid- and hop stress on membrane fluidity is depicted in Figure 20. The cells grown under acid stress conditions showed a marked decrease of the membrane fluidity in the temperature range of 10 - 30°C when compared to cells grown at reference conditions. Upon hop adaptation, the membrane fluidity was further reduced. At 5 °C, a temperature at which the membrane is in the gel phase, the highest *GP* was observed in reference cells.

3.7 Lipoteichoic acids

The changes provoked by the presence of hop compounds in the growth media in the lipoteichoic acid content of the cell walls was measured under reference-, acid stress- and hop stress conditions. For a qualitative analysis, the lipoteichoic acid was isolated, chemically deacylated (cd) and separated by native PAGE (figure 21). As expected from its heterogeneity, the cdLTA migrated as a smear. Under reference conditions nearly no cdLTA was detected on the gel. The cdLTAs from acid adapted cells were visible as a smear in the

Fig. 21 Analysis of LTA isolated from *L. brevis* TMW1.465 and *L. brevis* TMW 1.465A. Electrophoretic separation of cdLTA purified from *L. brevis* TMW 1.465 grown under reference (lane 1), or acid stress conditions (lane 2), and of hop-adapted *L. brevis* TMW 1.465A (lane 3). Samples were loaded to represent equivalent cell dry masses for the various conditions and the analysis is representative for two independent LTA isolations.

middle of the gel. The cdLTA from hop-adapted cells formed a thick band in the upper third of the gel and a slight smear comparable to the acid stress conditions. To obtain quantitative information on the cdLTA levels, the cdLTA was hydrolyzed and the amount of glycerolphosphate, which forms the backbone of the LTA, was measured. In agreement with the PAGE analysis, the glycerolphosphate content of the cdLTA increased from 0.6 ± 0.0 mM g^{-1} to 2.9 ± 0.2 mM g^{-1} and finally to 6.4 ± 0.1 mM g^{-1}(cell dry weight) with each increasing stress level.

3.8 Expression analysis of hop resistance genes on the mRNA level

To determine the regulation of the hop resistance contributing mechanisms, the expression of the previously characterized hop resistance gene *hitA* was measured on the mRNA level. As the metabolite analysis of *L. brevis* TMW1.465A indicated a role of arginine metabolism in hop resistance, the genes of the arginine deiminase (ADI) pathway (*arcA*, arcB and *arcC*) were additionally quantified. Gene expression was normalized to phosphoketolase (*pta*) as a

housekeeping gene. The expression profile of the target genes is given in figure 22. The gene
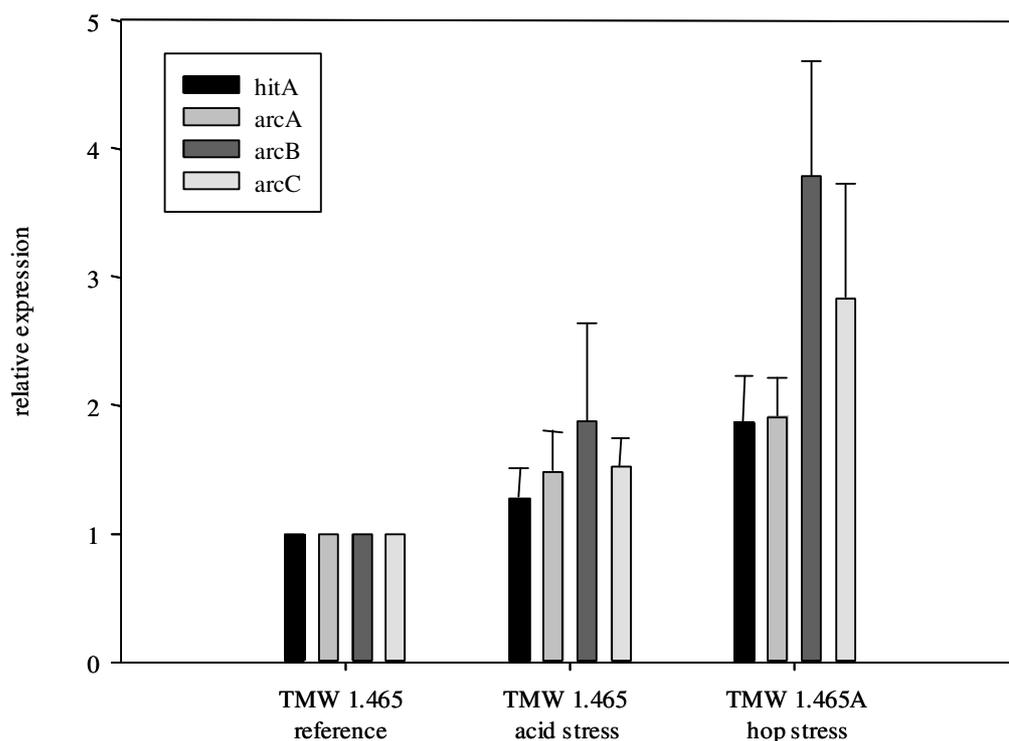
Fig. 22 Expression analysis of hop resistance genes *hitA*, *arcA*, *arcB* and *arcC* of *L. brevis* TMW 1.465 (reference and acid stress conditions) and *L. brevis* TMW 1.465A (hop adapted cells). mRNA levels were quantified by LightCycler PCR using cDNA as template and the *pta* as reference. Shown are the means and standard deviations of four independent experiments.

expression at the reference conditions was set to 1.0. Acid stress resulted in a 1.2 - 1.8 fold overexpression of all four genes whereas *arcB* and *arcC* were overexpressed 2.5 - 4 fold upon hop adaptation.

3.9 Evaluation of resistance based on MDR transport systems, barriers or metabolism

The intracellular pH was measured in *L. brevis* TMW 1.465A with the cFDASE method and with Calcein AM in order to determine whether barrier functions, transport or metabolism play the major role in the hop resistance of *L. brevis* TMW 1.465A. Measurements with the cFDASE and Calcein AM generally provided consistent results, however, based on the pKa values of the two dyes, calcein was the more appropriate choice. Therefore, only the values obtained from Calcein AM measurements are shown in figure 23. As reference for the experiments, cells from *L. brevis* TMW 1.465A without energy source and hop compounds were used. The decrease of fluorescence in these controls is mainly caused by a decrease of the pH_{in} upon the shift from the pH 6.5 phosphate buffer to the pH 4.0 acetate buffer. After 40 minutes, equilibrium was reached. Figure 23 A shows the rapid drop of intracellular pH in deenergized cells mediated by hop compounds. Even in hop-adapted cells, addition of 80 µM

iso-α-acids strongly decreased the intracellular pH, indicating that the alterations of the cell envelope failed to protect against hop compounds in the absence of a source of metabolic
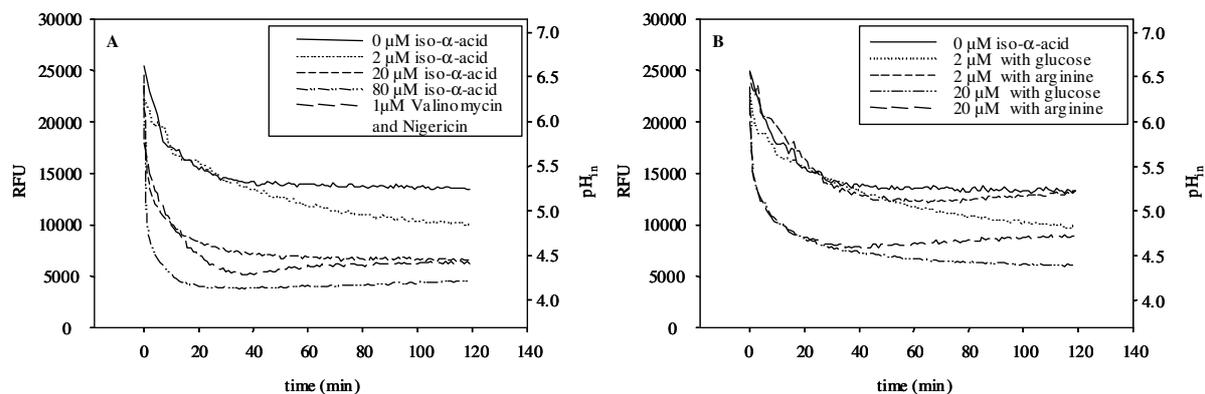
Fig. 23 Measurements of intracellular pH changes in cells of *L. brevis* TMW 1.465A after addition of different concentrations of hop compounds. Panel A. Exposure of de-energized cells to 0 - 80 µM of iso-α-acids or 1µM valinomycin and nigericin. Panel B. Exposure of cells to 2 and 20 µM of iso-α-acids with concomitant addition of glucose or arginine as energy source. The data are representative for two independent experiments.

energy. In figure 23 B, the intracellular pH of cells is depicted that were energized with glucose or arginine concomitant with their exposure to iso-α-acids. No differences in the curves of deenergized and energized cells were determined in the first 40 minutes of measurement (see figure 23 A and 23 B). In cells energized with arginine, but not in cells energized with glucose, a slow increase of the intracellular pH could be detected after 40 min.

3.10 Optimization and control experiments for reproducible 2D analysis of hop regulated proteins

A comparison of sequential protein extraction, as described by Molley et al. (Molloy et al. 1998), to detergent-based whole cell protein extraction, as specified above by 2D-PAGE (IPG 3-10), determined the latter as more robust and reproducible. The main reason for this observation resides in the tough cell walls of *L. brevis* TMW 1.465, which are variable in composition under different growth and stress conditions and very resistant to lysis. Accordingly, an extraction protocol was chosen that ensures high cell lysis efficiencies under all conditions investigated. Consecutively, the experimental error of protein spot quantification was determined principally according to Mahon et al. (Mahon and Dupree 2001). Total cell protein extracts were diluted in steps of 10 % with lysis buffer in the range from 10 % to 90 % and separated by 2D-PAGE (IPG 4-7). A linear behavior of individual protein volumes was observed with a maximum relative standard deviation of 16 %. To determine the pI range of interest, within which most of the stress regulated proteins are located, the protein extracts from all three growth conditions were focused on IPG strips 3-10,

4-7, 4.5-5.5 and 6-11. Changes in the protein patterns were noticed manly in the range from pI 4.8-5.4 and to a lesser extent from pI 9.8 to 11. However, the patterns in the range from 9.8 to 11 were difficult to reproduce and showed very small protein quantities on the gel. Higher protein loads did not separate properly. Thus, the IPG 4.5-5.5 which covered 86 % of all stress regulated proteins was chosen for incipient analysis. The dependence of growth phase on protein yield and expression level of stress inducible proteins was determined from exponential growth phases at OD_{590nm} = 0.3, 0.4, 0.5 and transient growth phase at OD_{590nm} = 0.9. Hop stress regulated proteins were detected with similar expression levels in all growth phases, and the extractable protein yield was maximal at an OD_{590nm} of 0.4. As a control for possible carryover of hop proteins, isomerized hop extract (860 µM iso-α-acid in lysis buffer) was separated by 2D-PAGE. The isomerized hop extract showed two rows of 4 proteins with pI 4.76 to 4.94 and 69 kDa and pI 4.99 to 5.10 and 57 kDa in each case, which were detectable only by sensitive silver staining. Hence, there was no detectable contamination in the protein samples derived from the addition of isomerized hop extract to the growth medium. Examples for IPGs used and the reproducibility of the 2D PAGE are displayed in the appendix.

3.11 Differential proteome analysis

Whole-cell protein was isolated from hop tolerant *L. brevis* TMW 1.465 under reference and acid stress conditions, and under hop stress conditions from *L. brevis* TMW 1.465A, its hop resistant variant (see appendix). High resolution IPGs with 24 cm separation distances in the range from pH 4.5-5.5 were used in the first dimension. The second dimension exhibited a separation range from 10-250 kDa. This combination resulted in the separation of high protein loads in the range of 4.74-5.46. The anodic edge was not exploitable for protein pattern comparison because it showed vertical streaks at the cup loading position. In total, 12 hop stress inducible proteins (HI), two acid stress inducible proteins (AI), 17 hop stress overexpressed proteins (HO) and one hop stress repressed protein (HR) were identified (figure 24). Acid stress inducible proteins were expressed under acid stress, but not overexpressed under the hop stress condition. For expression analysis, a reference map with proteins of *L. brevis* expressed at pH 6 (reference condition) was established and compared to those proteins expressed by the same strain under acid stress (pH 4.0) and the hop adapted variant under hop stress (pH 4.0 and 86 µM iso-α-acids). The expression values of hop-overexpressed proteins are depicted in figure 25.
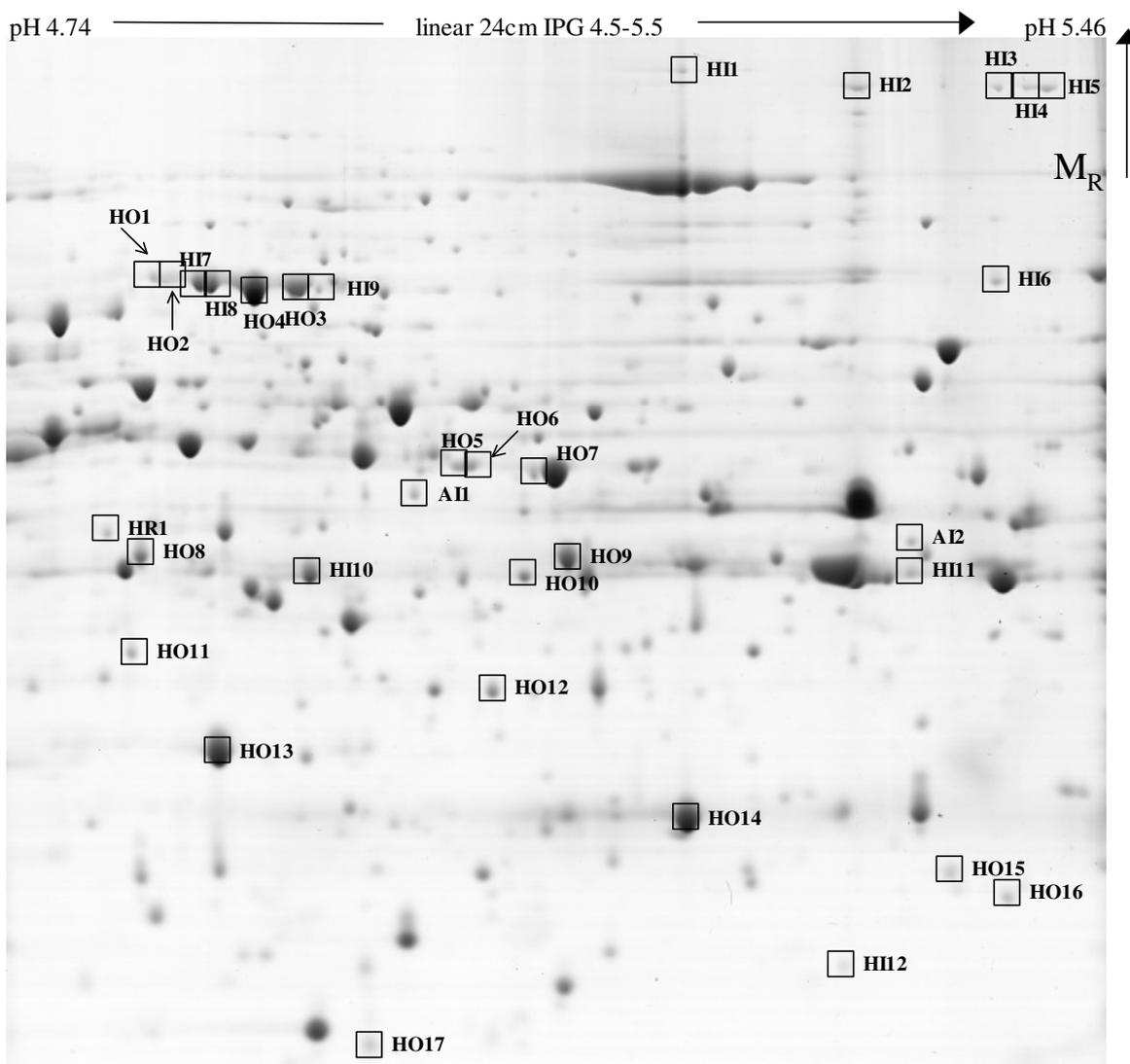
Fig. 24 Two-dimensional electrophoretic analysis of coomassie blue stained total protein from cells of *L. brevis* TMW 1.465A grown under hop stress. HI: proteins detected under hop stress conditions only. AI: proteins detected in acid stressed cells. HO: proteins overexpressed in hop stressed cells. HR: proteins repressed in hop stress cells.

3.12 Protein identification

The N-terminal sequence of HO13 was determined by Edman degradation as A(G/T)SNGKVAMVTGGXQ, which identified HO13 as member of the oxidoreductase group. Subsequent protein analysis was performed by mass spectrometry. All differentially expressed proteins were subjected to a MALDI-TOF MS and LC-ESI MS/MS analysis. Mass spectrometric data were interpreted by the Matrix Science Mascot software and searched against the NCBI and the custom database (see above). In parallel, LC-ESI MS/MS data were exported to Sequest dta files, which were processed by the *de novo* sequencing program LutefiskXP and MS-BLAST as described above (cf. appendix). The two methods provided

consistent results if good quality MS/MS spectra were obtained. However, the latter method
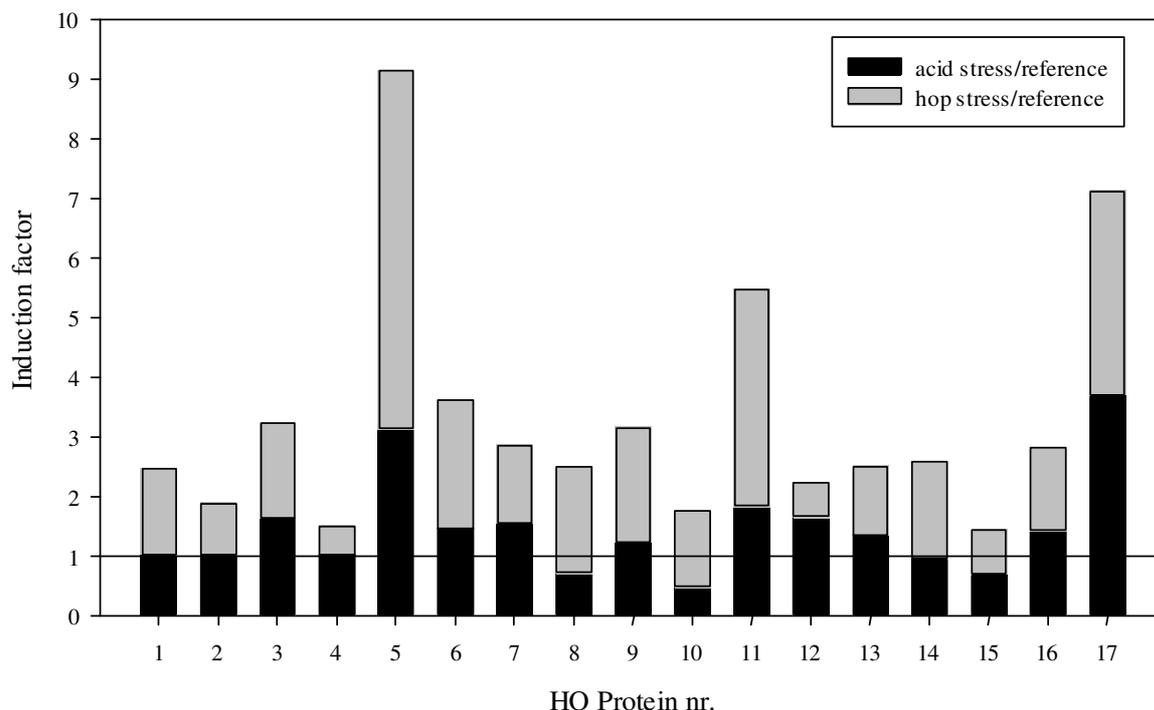
Fig. 25 Induction factors of proteins overexpressed under hop- and acid stress conditions. The normalized volumes of each protein spot were calculated using the Image Master2D Elite software. The induction factors were calculated by dividing each stress regulated protein spot volume by the corresponding spot volume of the reference condition. Shown are the means of two independent experiments.

was very sensitive (Huang et al. 2001) and enabled a higher degree of reliability by a higher sequence coverage in comparison to the Mascot analysis. For identification of HI10 (cf. appendix), which was strongly induced by hop stress (figure 26), nanospray sequencing ESI MS/MS was used. The spectra were manually interpreted and led to the identification of HI10
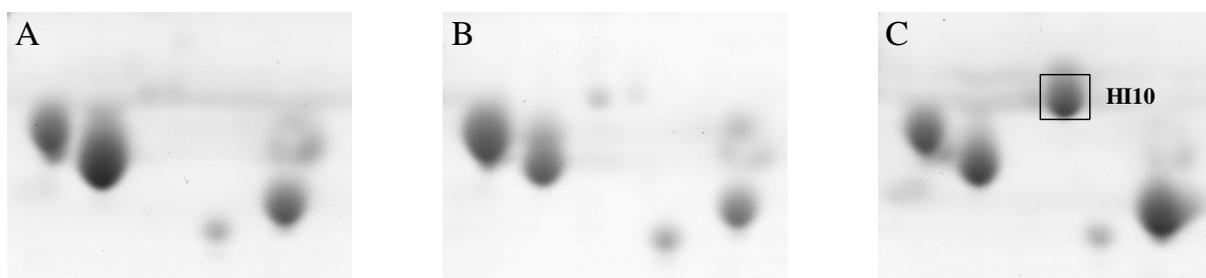
Fig. 26 Protein HI10 expression of *L. brevis* TMW 1.465 under reference- (A), acid stress (B) and of *L. brevis* TMW 1.465A under hop stress conditions (C).

as pyruvate carboxylase. Five proteins (HO2, HI1, HI5, HI6 and HI10) exhibited a different molecular weight in comparison to the corresponding database stored sequence. 27 out of 32 proteins investigated from non-genome sequenced *L. brevis* TMW 1.465A were identified by these techniques and are displayed in table 4.

The acid shift induced a cysteine sulfurase related enzyme and the cyclopropane-fatty-acyl-phospholipid synthase. The proteins upregulated under the hop stress condition were enzymes from glycolysis, reversed TCA cycle pathway, glycerol metabolism, amino acid metabolism and nucleotide metabolism. One transcriptional regulatory protein was found among them. Five forms of pyruvate kinase with pI´s ranging from 4.83 to 4.92 and molecular weight from 65 to 66 kDa, were identified. Two proteins differing in their molecular weight and pI were identified as phosphoglycerate kinases according to their LC-ESI MS/MS spectra.

Tab. 4 Molecular weights and isoelectric points of the proteins identified from 2D-electrophoresis. The proteins designated negative could not be identified by a peptide mass fingerprint and a LC-ESI MS/MS analysis. Accession numbers designated gi refer to the NCBI database entries, jgi to the U.S Department of Energy Joint Genome Institute database, which is followed by the designation of the strain. HI: proteins detected under hop stress conditions only. AI: proteins detected in acid stressed cells. HO: proteins overexpressed in hop stressed cells. HR: proteins repressed in hop stress cells.

protein	Mw (kDa)	PI	Accession nr.	function
HO1	66	4.83	gi|116333412|	pyruvate kinase
HO2	66	4.84	gi|116333309|	3-phosphoglycerate kinase
HO3	65	4.92	gi|116333412|	pyruvate kinase
HO4	65	4.89	gi|116333412|	pyruvate kinase
HO5	45	5.04	gi|116333309|	3-phosphoglycerate kinase
HO6	45	5.05	gi|116334175|	phosphopentomutase
HO7	44	5.08	gi|116334720|	glycerol dehydrogenase
HO8	38	4.82	-	negative
HO9	38	5.10	gi|116332816|	2-hydroxyacid dehydrogenase
HO10	37	5.08	jgi1730_lcasATCC334	transcriptional regulatory protein
HO11	33	4.81	gi|62463721|	malate dehydrogenase, NAD-dependent
HO12	31	5.05	gi|116333251|	RecR protein
HO13	29	4.87	gi|116332857|	acetoin dehydrogenase
HO14	29	5.18	gi|28379560|	phosphoglyceromutase
HO15	23	5.36	gi|116334174|	purine-nucleoside phosphorylase DeoD
HO16	21	5.40	-	negative
HO17	15	4.97	gi|116333447|	ATP-dependent protease HslVU (ClpYQ)
HI1	159	5.18	jgi3841_lcreSK11	formamidopyrimidine-DNA glycolase
HI2	140	5.30	-	negative
HI3	140	5.39	-	negative
HI4	140	5.41	-	negative
HI5	140	5.42	gi|116334767|	pyridoxal-dependent decarboxylase
HI6	65	5.39	gi|116333104|	3-hexulose-6-phosphate synthase related protein
HI7	65	4.86	gi|116333412|	pyruvate kinase
HI8	65	4.87	gi|116333412|	pyruvate kinase
HI9	65	4.94	gi|116333334|	glucosamine 6-phosphate synthetase
HI10	37	4.93	gi|42519197|	phosphoenolpyruvate carboxylase
HI11	37	5.33	gi|116334587|	ornithine carbamoyltransferase
HI12	19	5.29	gi|28378831|	peptidylprolyl isomerase
HR1	40	4.80	gi|116333957|	D-lactate dehydrogenase
AI1	43	5.00	gi|116334038|	cysteine desulfurase related enzyme
AI2	39	5.33	gi|116333366|	cyclopropane-fatty-acyl-phospholipid synthase

3.13 Growth challenges in hopped media supplemented with divalent cations

The impact of the divalent cation composition of the growth medium on hop resistance was tested by growth challenges of *L. brevis* TMW 1.465A in the presence of 86 µM iso-α-acids and the divalent cations Ca^{2+}, Mg^{2+} and Mn^{2+} (Simpson and Smith 1992). Growth challenges were performed in mMRS4 medium without the addition of hop compounds (which were the controls) and with hop compounds. The percentage growth with regard to control cells is depicted in figure 27. The supplementation with calcium did not accelerate the growth.
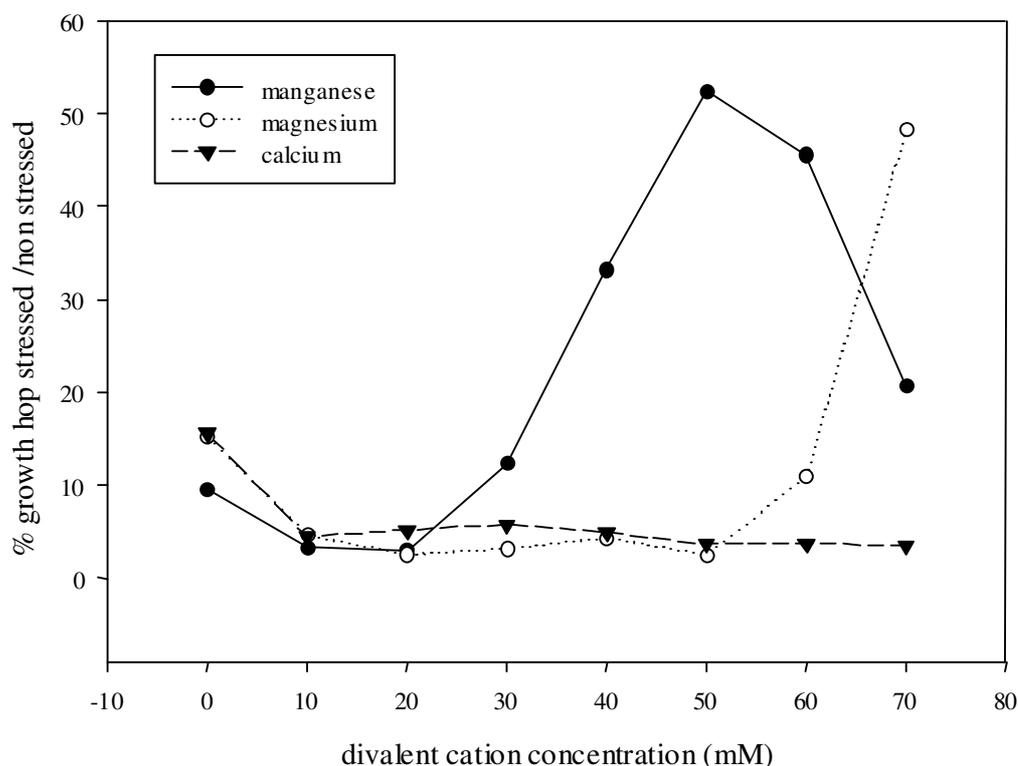
Fig. 27 Growth challenge of *L. brevis* TMW 1.465A in the presence of 86 µM iso-α-acids in mMRS4 (pH 4.0) supplemented with divalent cations Mn^{2+}, Mg^{2+} or Ca^{2+}. The growth was measured after 2 d. % growth was calculated with regard to control cells, which were grown without addition of iso-α-acids. Shown are the means of two independent experiments.

Magnesium only worked at high concentrations. On the other hand, manganese accelerates growth at a concentration above 30 mM (optimal 50 mM). The calculated enhanced percentage growth without supplementation can be attributed to the bad growth of the control cells.

3.14 Cross resistance to ionophores

To determine whether the origin of hop resistance of *L. brevis* TMW 1.465A compared to hop tolerance of *L. brevis* TMW 1.465 is more associated with the tolerance to a proton influx or a

manganese efflux, a growth challenge of both strains in the presence of a proton ionophore or
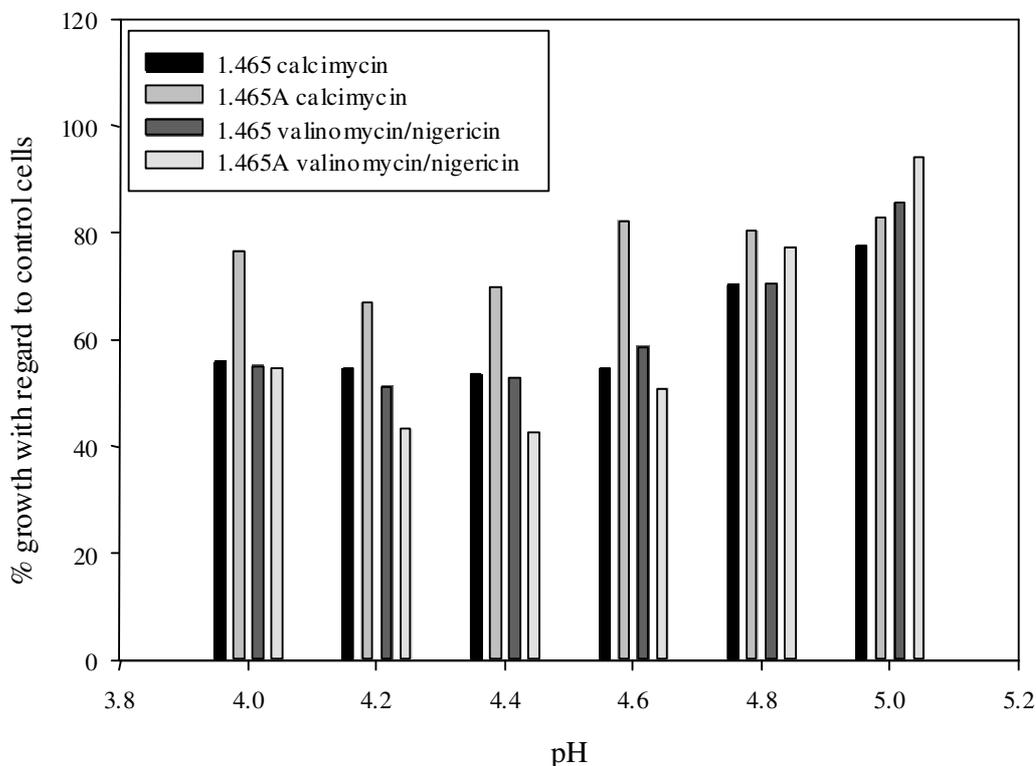
Fig. 28 Growth challenges of *L. brevis* TMW 1.465 and *L. brevis* TMW 1.465A in the presence of antibiotics (calcimycin or valinomycin/nigericin; 5 µM) at the pH indicated. The growth was measured after 8 d. % growth was calculated with regard to control cells, which were grown without addition of antibiotics. Shown are the means of two independent experiments.

a manganese/proton exchanger was carried out. Upon adaptation to hops the tolerance to proton ionophores (valinomycin/nigericin) was decreased, while the tolerance to the divalent cation exchanger calcimycin was increased at low pH values (figure 28).

3.15 Cellular manganese content under hop stress

Percentage cellular concentrations of Mn^{2+} with regard to control cells (incubation without hop compounds) after a hop incubation (86 µM iso-α-acids) were measured for *L. brevis* TMW 1.465 (5.9 %) and for *L. brevis* TMW 1.465A (8.0 %). The manganese content of the cells was reduced to a tenth part of the initial value and was the same in cells with high and low hop resistance. Within one subculture *L. brevis* TMW 1.465A restored the intracellular manganese content to 86 % of the value of the control cells.

3.16 Inhibition of enzyme reactions by hop compounds

The glucose-hexokinase, as well as the glucose-6-phosphate dehydrogenase require divalent cations for activity (Schomburg et al. 2002). The first enzyme stands for a group of ATP dependent enzymes, which can only bind to ATP if it is in complex with a divalent cation. The second enzyme directly binds to divalent cations, which trigger its activity. The percentage substrate conversion with addition of different amounts of iso-α-acids was calculated. The addition of 20 to 100 µM of iso-α-acids reduced the substrate conversion of the glucose-hexokinase to 47 ± 8 % and that of the glucose-6-phosphate dehydrogenase to 47 ± 11 % with respect to the substrate conversions measured without the addition of hop compounds. An addition of divalent cations restored the enzyme activity of the glucose-hexokinase to 88 ± 5 %, as well as that of the glucose-6-phosphate dehydrogenase to 90 ± 2 %. To check, that the reduced activity calculated for glucose-hexokinase was not due to the sole reduction of the detection reaction, Mg-ATP was added to the latter to identify, that iso-α-acids were capable to bind to Mg-ATP as well as to Mg^{2+}. The addition of Mg-ATP (which is not involved in the enzymatic reaction) restored the enzyme activity of glucose-6-phosphate dehydrogenase to 90 ± 14 %.

3.17 Phenomenological investigations of the mode of action of hop compounds in bilayer lipid membranes

3.17.1 Optimization and control experiments for reproducible bilayer lipid membrane measurements

To obtain basic information on the mode of action of hop compounds in biological membranes, a bilayer lipid membrane measurement system was set up. Correct function and reliability of the custom measurement system was tested with multiple resistor-capacitor combinations according to Kalinowski et al. (Kalinowski and Figaszewski 1995; Kalinowski and Figaszewski 1995). BLM measurements on mode of action of the ionophores calcimycin, nigericin, CCCP and ion channel gramicidin (Markin et al. 1975; Kasianowicz et al. 1984; Pohl et al. 1990; Kalinowski and Figaszewski 1995) were reproduced. Capacitance measurements during the membrane formation ensured similar initial conditions for all experiments. The influence of variation of the supporting electrolyte on the measurement result was negligible for NaCl, KCl and cholinechloride. As the solubility of high concentrations of hop compounds was best in KCl solution it was chosen as electrolyte at a concentration of 0.2 M. The influence of buffer composition was assessed for the buffer

compounds Hepes, Tris, Mes, histidine, phosphate and citric acid, which are generally used for BLM experiments. The choice of buffer did not significantly influence the measurement result (e.g. conductance data at pH 7.0 for 60 µM iso-α-acids in Hepes buffer: $1.4e^{-10}$ Ohm^{-1}, in histidine buffer: $1.7e^{-10}$ Ohm^{-1}). For large range pH variation experiments the composition Tris, Mes and citric acid (5 mM each) was chosen (Kasianowicz et al. 1984). For cyclic voltammetry Tris, Mes and citric acid (5 mM each) or phosphate buffer (20 mM) was used. Divalent cations were added as chlorides and sulfates resulting in no measurable influence of the counter anion at given concentrations. As lipids natural lipids isolated from *L. brevis* TMW 1.465, egg phosphatidyl choline (PC) and synthetic phosphatidyl glycerol 16:0/16:0 (GP; Lipoid GmbH, Ludwigshafen, Germany) were used. A comparison of membrane conductance in the presence of hop compounds showed that it increased in following order: *L. brevis* lipid, PC/PG (1/1) and PC. Potential measurements showed the same trend. The mode of action of hop compounds in all lipids was consistent, but with higher markedness for PC/GP and PC. PC was favored for experiments, when a change in membrane surface potential mediated by a screening effect of divalent cations on charged membranes (GP and possibly *L. brevis* lipid) should be avoided (McLaughlin et al. 1971). To assess the role of mono- and divalent cations on membrane potential in the presence of hop compounds, NaCl, KCl, $MnCl_2$, $MgCl_2$ and $CaCl_2$ were used. The experiments were recorded at pH 7.0 (Tris, Mes, cholinechloride) and a salt gradient of 1/100 in the presence of 86 µM iso-α-acids. Monovalent cations Na^+ and K^+ caused a membrane potential of 0.3 ± 1.6 mV and 1.0 ± 0.3 mV, respectively. Divalent cations Mn^{2+}, Mg^{2+} and Ca^{2+} resulted in potentials of 65.1 ± 5.5 mV, 28.2 ± 3.8 mV and 10.8 ± 2.2 mV, correspondingly. As Mn^{2+} showed the greatest effect, only $MnCl_2$ was used for further investigations.

3.17.2 Comparison of isomerized hop extract and A23187

As initial measurement a comparison of the mode of action of hop ionophores, which are described as proton/manganese exchangers (Simpson 1993), with the well characterized proton/divalent cation exchanger calcimycin was recorded (Antonenko and Yaguzhinsky 1983; Pohl et al. 1990; Jyothi et al. 1994). Calcimycin exhibits a high affinity for the divalent cations $Mn^{2+}>Ca^{2+}=Mg^{2+}$ (Abbott et al. 1979). The nonelectrogenic divalent cation/proton exchange can be monitored by equalizing the proton transport of the exchange by the addition of a proton ionophore as TTFB or CCCP (Antonenko Yu and Yaguzhinsky 1982; Antonenko and Yaguzhinsky 1983). Thus the divalent cation transport results in a potential that is

positive on the side of lower divalent cation salt concentration. The change in membrane
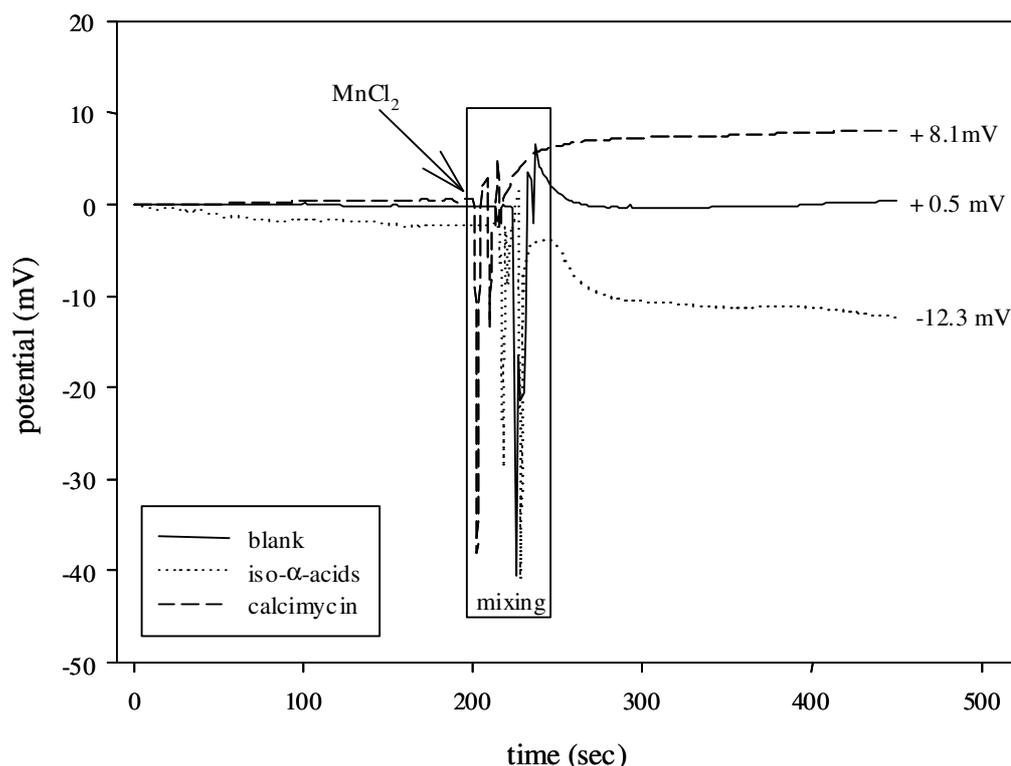
Fig. 29 Potential generation on BLM (*L. brevis* lipid) upon addition of $MnCl_2$ (5 mM) in one compartment of the electrolytic cell in the presence of 10 µM CCCP and 86 µM iso-α-acids or 5 µM calcimycin or no addition of an additional ionophore (blank). Composition of aqueous solutions: Tris, Mes, KCl (pH 7.0).

potential after addition of $MnCl_2$ is shown in figure 29. Under the same experimental conditions hop compounds cause a potential difference opposite in sign in comparison to calcimycin.

3.17.3 Conductivity data from BLMs in the presence of hop compounds

Conductance data of BLMs was recorded upon variation of the hop compound concentration, pH values and $MnCl_2$ concentration. Figure 30 shows the dependence of membrane conductivity upon the variation of the aqueous concentration of hop compounds with and without $MnCl_2$ addition at a pH of 7.0. A linear dependence of conductance versus log(hop compound concentration) is observed, which changes slope at a given hop compound concentration of 60 µM iso-α-acids for both conditions. The slope from 20 to 60 µM iso-α-acids indicates a monomeric charge transport process across the BLM. Above 60 µM iso-α-acids a dimer or oligomer in the presence of Mn^{2+} is the translocator species. The increase of conductance in the presence of Mn^{2+} is depicted in figure 31. A linear relationship between conductance and log (Mn^{2+} concentration) with a slope of about 0.5 was found, indicating that

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only one Mn^{2+} per two charge transporting species is involved (Toro et al. 1987). The
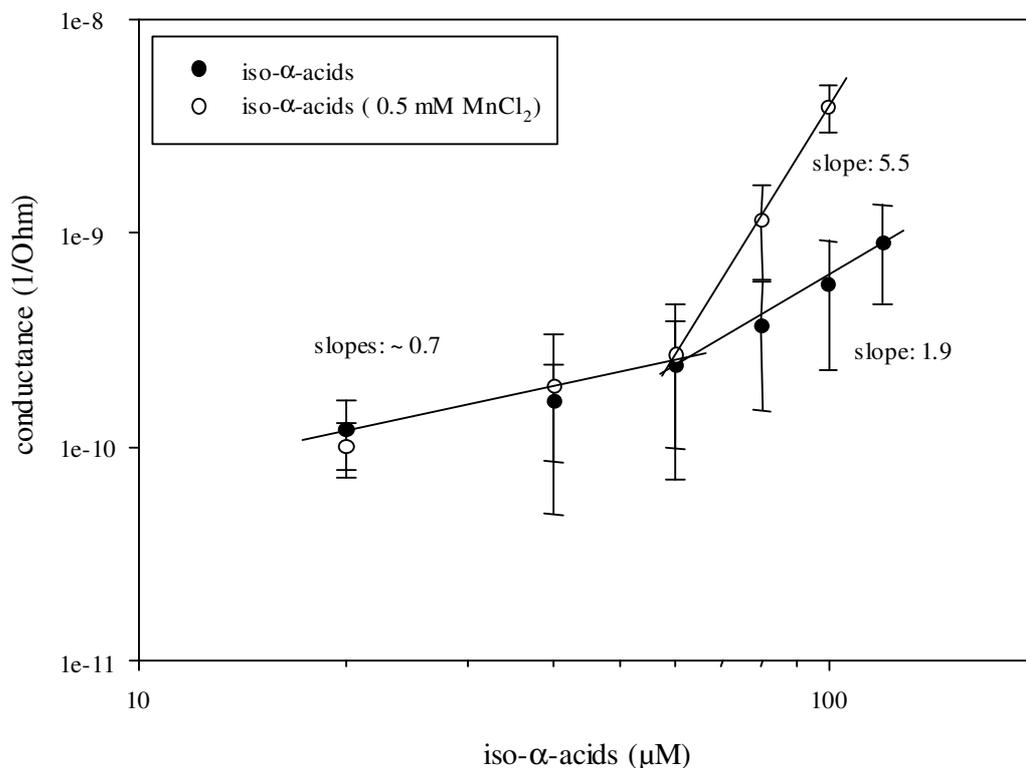
Fig. 30 Dependence of membrane (*L. brevis* lipid) conductivity at 50 mV upon the variation of the aqueous concentration of hop compounds with and without $MnCl_2$ (0.5 mM) addition to both compartments of the electrolytic cell. The slopes (change in log(conductance) per log(iso-α-acids concentration)) are indicated. Composition of aqueous solutions: Tris, Mes, KCl (pH 7.0).

dependence of conductivity on pH value of aqueous solution is shown in figure 32. The hop compounds concentration, which is needed to achieve a 10 fold increase in BLM conductance with respect to zero hop compound addition at respective pH value was calculated. A linear increase of log(hop compound concentration) over pH was found. The slope of change in BLM conductance on account of the hop compounds concentration is given in dependence of solution pH, indicating a combination of monomeric and dimeric charge transport process between pH 4.0 and 5.0 and between pH 8.0 and 11.0 and a mainly dimeric translocator species acting between pH 6.0 and 7.0. The influence of the lipid composition on BLM conductance was recorded. In order to obtain BLMs with different surface charge *L. brevis* lipid was mixed with the zwitterionic, but net neutral PC in ratio of 2/3 (PC/*L. brevis* lipid (w/w)) and PC was mixed with negative charged GP (1/1(w/w)). The respective BLM conductance was determined in the presence of hop compounds. The conductance decreased

with higher content of GP or *L. brevis* lipid with regard to the PC content of the membrane.
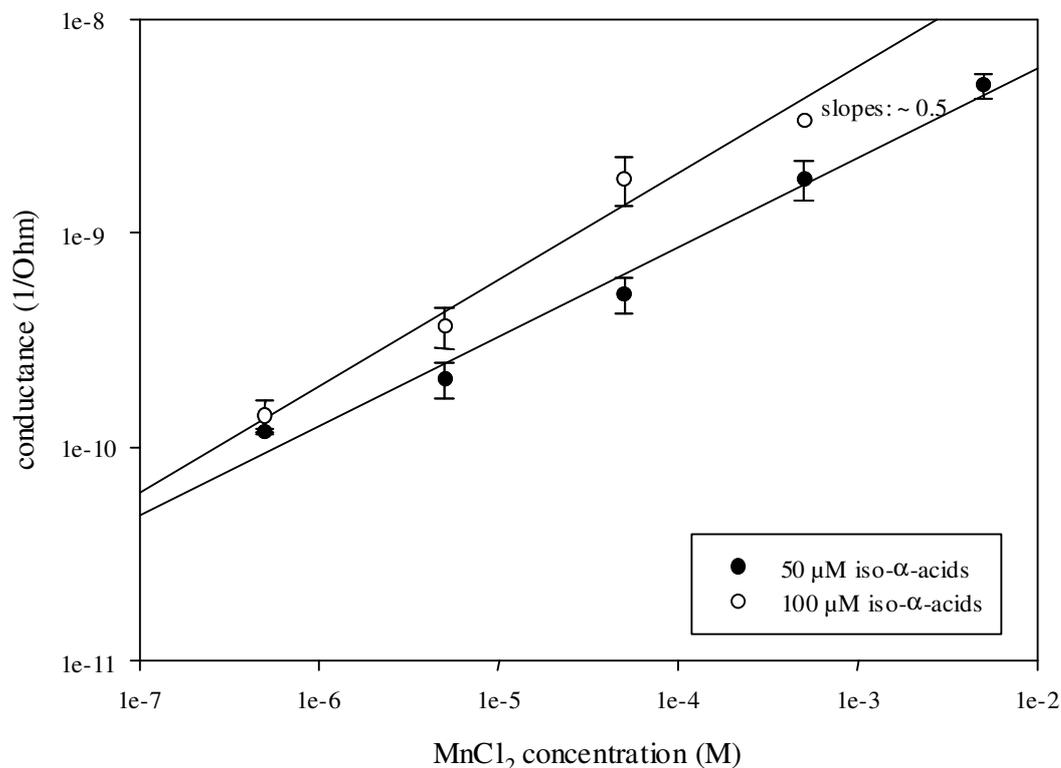
Fig. 31 Dependence of the membrane (*L. brevis* lipid) conductance at 50 mV mediated by iso-α-acids and $MnCl_2$ added to both compartments of the electrolytic cell. The slope (change in log(conductance) per log($MnCl_2$ concentration) is indicated. Composition of aqueous solutions: Tris, Mes, KCl (pH 7.0).

The conductance of PC doped *L. brevis* lipid membranes increased by 49 %. The conductance of GP/PC (1/1(w/w)) compared to PC membranes decreased by 33 % ± 4 %. This indicates that both, the *L. brevis* lipid as well as the hop charge translocator species exhibit negative charges.

3.17.4 Membrane potential data from BLMs in the presence of hop compounds

Membrane potential data were recorded upon variation of the hop compound concentration, pH values and $MnCl_2$ concentration. The membrane potentials were generated as follows: the solutions separated by the membrane exhibited a) the same pH and the hop compound concentration gradient was varied b) the same pH and hop compound concentration and the $MnCl_2$ concentration gradient was varied; c) the same hop compound concentration, but differing in a fixed ΔpH; d) the same pH and hop compound concentration, but differing in a

fixed $MnCl_2$ concentration gradient; e) the same hop compound and $MnCl_2$ concentration, but
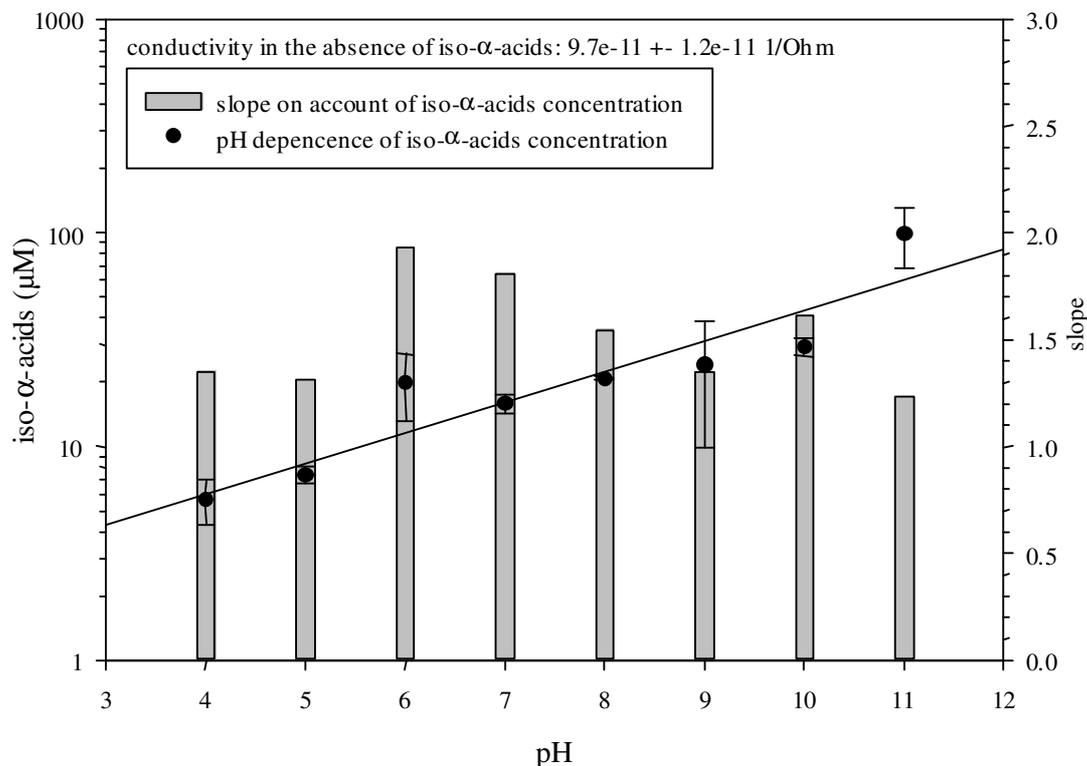
Fig. 32 pH dependence of iso-α-acids concentration, which is needed to achieve a 10 time increase in BLM (PC, 25 mV) conductance with regard to zero hop compound addition (full circles, left axis). The slope (log(conductance) per log(iso-α-acids concentration) of change in BLM conductance on account of the hop compound concentration is given in dependence of solution pH (gray bars, right axis). Composition of aqueous solutions: Tris, Mes, citrate, KCl at pH indicated.

differing in a fixed ΔpH. For case a) the potential generated is depicted in figure 33 and shows a linear dependence of potential versus log(hop compounds concentration). The potential increases for each order of magnitude of hop compound concentration by 3.5 ± 0.4 mV at pH 4.0, 5.2 ± 2.2 mV at pH 7.0 and 5.1 ± 1.4 mV at pH 10.0. Figure 34 shows the data for case b). For pH 4.0 and 7.0 the potential was linear dependent on log($MnCl_2$ concentration) with a increase of 37.5 ± 6.1 mV for pH 4.0 and 43.8 ± 3.9 mV for pH 7.0 per power of ten. At pH 10.0 a nonlinear behavior of $MnCl_2$ dependent membrane potential was monitored. An elevated hop compound concentration shifted the pH 10.0 potential curve towards the results derived from the other pH values. The data for case c) are depicted in figure 35. A pH gradient of −1 pH unit was set up for each pH and the iso-α-acids concentration was kept constant at 10 μM. From pH 4.0 to 9.0 the hop doped membrane worked nearly as perfect pH electrode. An average signal of 53.3 ± 9.3 mV (theoretic value 59 mV) was generated by a Δ

pH of 1.0. Above pH 9.0 the potential decreases to the final value of 8.4 ± 2.0 mV at a pH of
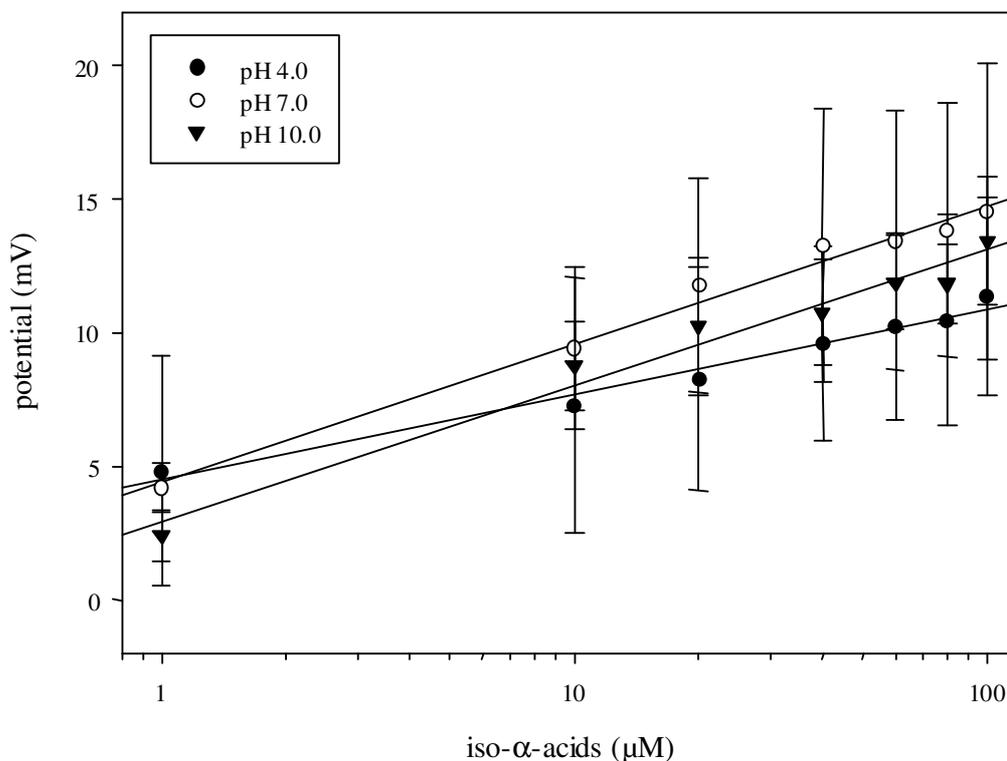
Fig. 33 Potential generation on BLM (PC) upon addition of iso-α-acids in one compartment of the electrolytic cell at pH 4.0, 7.0 and 10.0. The potential is positive on the side of iso-α-acids addition. Composition of aqueous solutions: Tris, Mes, citrate, KCl at pH indicated.

11.0. Case d) is shown in figure 35. At pH 4.0 a high membrane potential of 40.0 ± 1.0 mV was monitored. From pH 5.0 to 8.0 the potential was constant at 23.5 ± 1.3. Above pH 8.0 it decreased to 2.0 ± 2.9 mV. The highest membrane potentials were measured for case e) and are shown in figure 35. It has to be stressed that case e) is not an addition of the potentials measured for conditions c) and d). The aqueous solutions separated by the membrane contained each 10 µM of iso-α-acids and 250 µM of $MnCl_2$, which will not cause any potential difference across the membrane. The measured high potentials are due to the same ΔpH as in condition c) and the presence of low amounts of $MnCl_2$ on both sides of the membrane. The ΔpH = 1 caused potential across the membrane was 121.7 ± 46.4 mV at pH 4.0 and decreased nearly linear over pH up to the pH 8.0 condition. From pH 8.0 to 10.0 the measured potential coincides with the data gained for case c), indicating that the $MnCl_2$ addition does not influence the charge transport process in this range. Only for case e) high standard deviations were obtained in the acidic measurement range. The ΔpH as sole source of potential generation cannot explain the high measurement values observed at low pH.
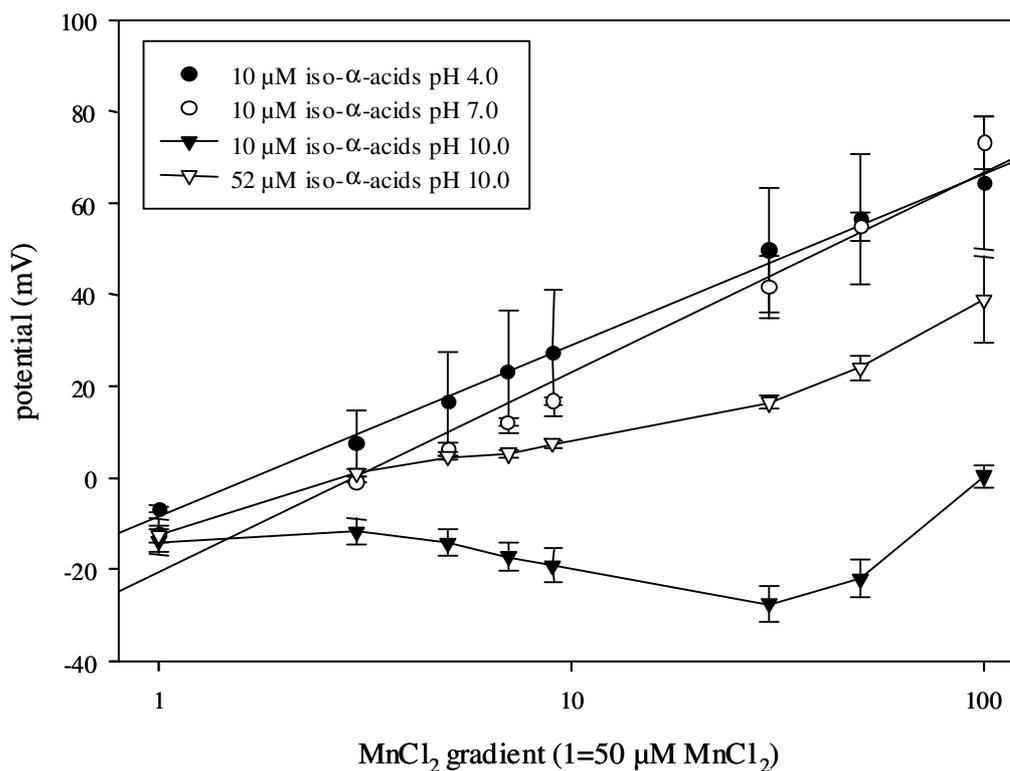
Fig. 34 Potential generation on BLM (PC) upon addition of $MnCl_2$ in one compartment of the electrolytic cell in the presence of iso-α-acids in both compartments of the cell at pH 4.0, 7.0 and 10.0. The potential is positive on the side of $MnCl_2$ addition. Composition of aqueous solutions: Tris, Mes, citrate, KCl at the pH indicated.
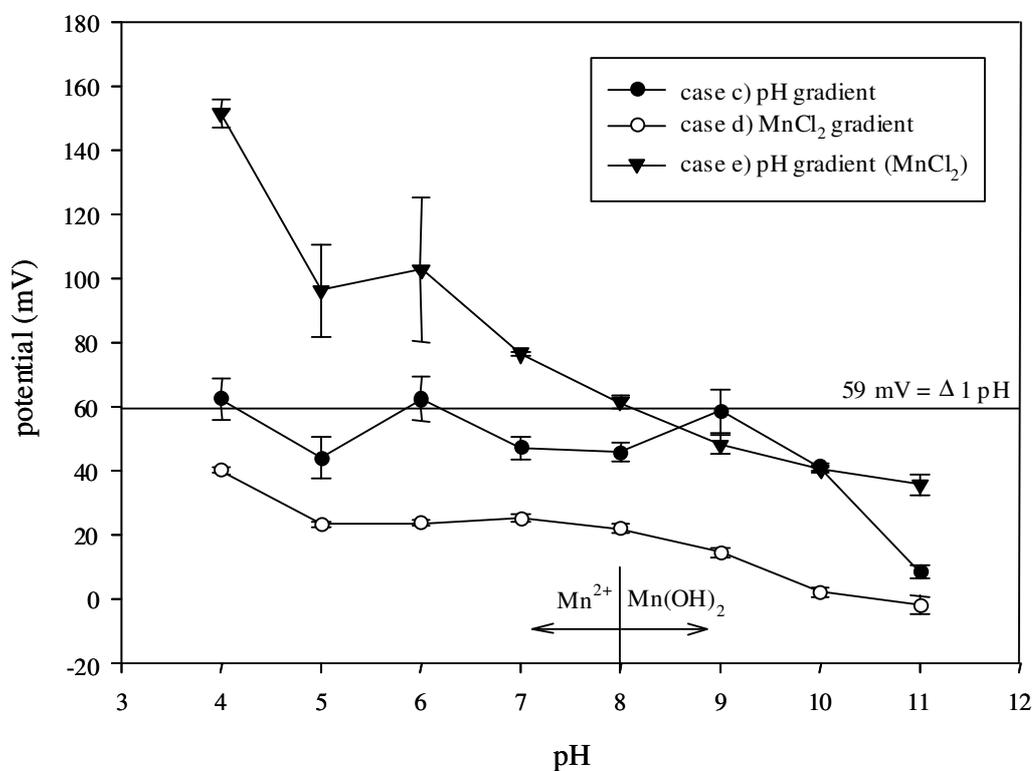
Fig. 35 Potential generation on BLM (PC) upon the setup of a ΔpH (1 pH unit; case c)), a $MnCl2$ gradient (case d) and a ΔpH (1 pH unit) in the presence of $MnCl2$ (250 µM in both compartments; case e)) between the compartments of the electrolytic cell in the presence of 10 µM iso-α-acids in both compartments of the cell at the pH indicated. The potential is positive on the side of higher pH (case c) and e)). The potential is positive on the side of $MnCl2$ addition (case d)). Composition of aqueous solutions: Tris, Mes, citrate, KCl at the pH indicated.

3.17.5 Cyclic voltammetry data from BLMs in the presence of hop compounds

Cyclic voltammetry data of BLMs were recorded in the presence of the hop compounds upon variation of pH values, $MnCl_2$ concentration and in the presence of membrane impermeable electron donors and acceptors in aqueous solutions. To prove the agreement of the model system (hop compounds in Tris, Mes, citrate or phosphate buffer, 0.2 M KCl) with beer a cyclic voltammogram of beer separated by the membrane was performed. According to Sobiech et al. and Tien (Tien 1984; Sobiech et al. 1998) voltammograms were recorded at a slow scan rate of 0.3 mVs^{-1} in the range of –100 mV to 100 mV, since the reaction time of beer associated redox couples (e.g. hop polyphenols and hop bitter acids) covers the range from 15 s to 150 min. The breakdown voltage of the BLMs limits the switching potential. For all conditions three subsequent scans were run. No separate peaks could be identified under all conditions tested (Sobiech et al. 1998). Cyclic voltammograms recorded at various pH values, with or without addition of $MnCl_2$ or membrane impermeable electron donors and acceptors, but without hop compounds in the aqueous solution, coincided with the x-axis on the scale used. Cyclic voltammograms of BLMs at pH 4.0, 7.0 and 10.0 were recorded in the presence of hop compounds (figure 36). The respective hop compound concentration was obtained from the conductivity data in order to achieve comparable membrane conductance independent of pH value. The cyclic voltammograms for pH 7.0 and 10.0 were symmetric towards the origin and show the typical shape of cyclic voltammograms of ionophores (e.g. valinomycin) (Shirai et al. 2006). The forward and backward scans virtually coincide. The raise in conductivity in subsequent potential sweeps can be attributed to an increase in hop compound concentration in the membrane with time. In the contrast to this, the cyclic voltammogram from the pH 4.0 condition was highly asymmetric towards the origin. The anodic current through the BLM (cycle 1) at 100 mV of applied potential was more that ten times higher than that obtained at pH 7.0 and more than 20 times higher than that at pH 10.0. For the cathodic current at the opposite end of the potential sweep a nearly triple value (with regard to pH 7.0) and a fivefold value (at pH 10.0) was obtained. The positive forward scan at pH 4.0 led to a significant higher current through the BLM than the backward scan. In contrast to the cyclic voltammograms recorded at pH 7.0 and pH 10.0 the conductivity decreased in subsequent scans. To prove that a transmembrane redox reaction was responsible for the highly asymmetric I/U curve at low pH, membrane impermeable electron donors and acceptors ($K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ or ascorbic acid/$K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$) were added to the aqueous solutions on opposite sides of the membrane. As the choice of water-soluble

redox couple did not alter the measurement result, only data for the first redox couple is
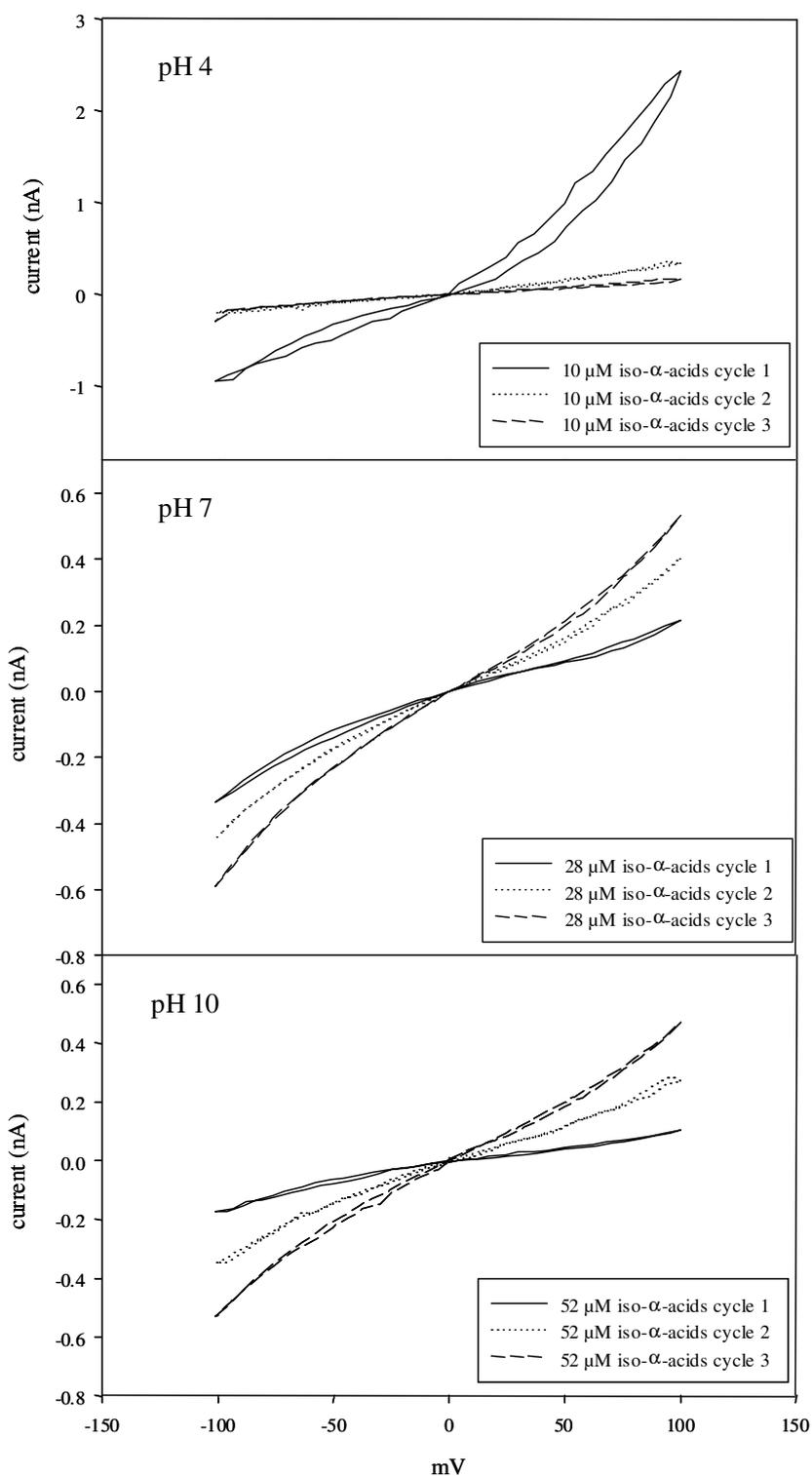
Fig. 36 Cyclic voltammograms for charge transfer through the BLM (PC) in the presence of iso-α-acids in both compartments of the electrolytic cell at pH indicated. Three subsequent scans are displayed. The scan rate was 0.3 mVs^{-1}. Composition of aqueous solutions: Tris, Mes, citrate, KCl at pH indicated.

shown. It was ascertained that the iron part of the redox couples did not act as di- or trivalent cation, which would form a complex with the hop compounds (Fe^{2+} and Fe^{3+} hop compound

complexes exhibit an intense orange color, which can be distinguished of the yellow-green
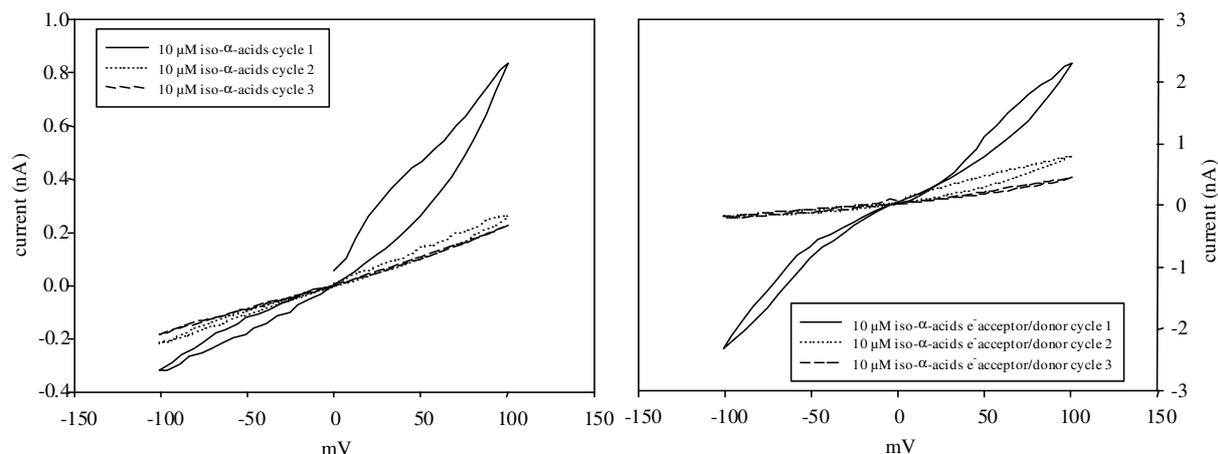
Fig. 37 Cyclic voltammograms for charge transfer through the BLM (PC) in the presence of 10 μM iso-α-acids in both compartments of the electrolytic cell with (right) or without (left) addition of 0.5 mM $K_3[Fe(CN)_6]$ (cis side) and $K_4[Fe(CN)_6]$ (trans side) at pH 4.0. Three subsequent scans are displayed. The scan rate was 0.3 mVs^{-1}. Composition of aqueous solutions: phosphate buffer, KCl.

color of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$). A comparison of cyclic voltammograms recorded with or without addition of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ is depicted in figure 37. The anodic current increased with the presence of an electron donor and acceptor couple in the aqueous solutions more than 2.5fold, the cathodic current more than sevenfold. The elevated cathodic
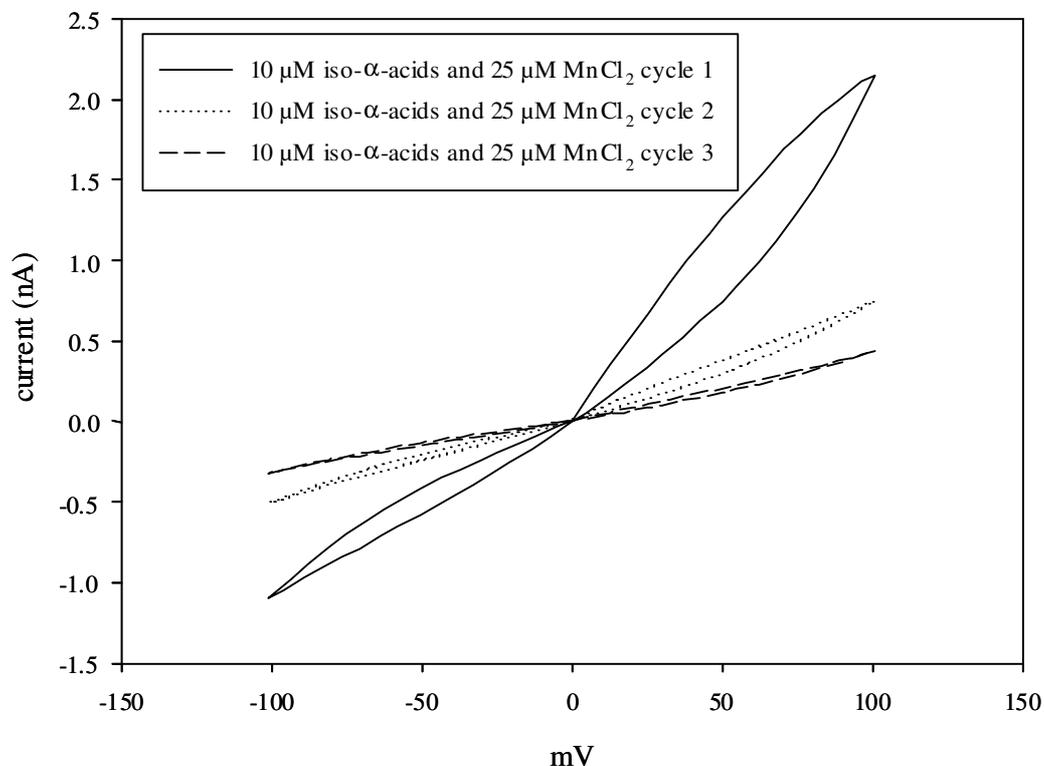
Fig. 38 Cyclic voltammogram for charge transfer through the BLM (PC) in the presence of 10 μM iso-α-acids and 25 μM MnCl$_2$ in both compartments of the electrolytic cell at pH 4.0. Three subsequent scans are displayed. The scan rate was 0.3 mVs^{-1}. Composition of aqueous solutions: phosphate buffer, KCl.
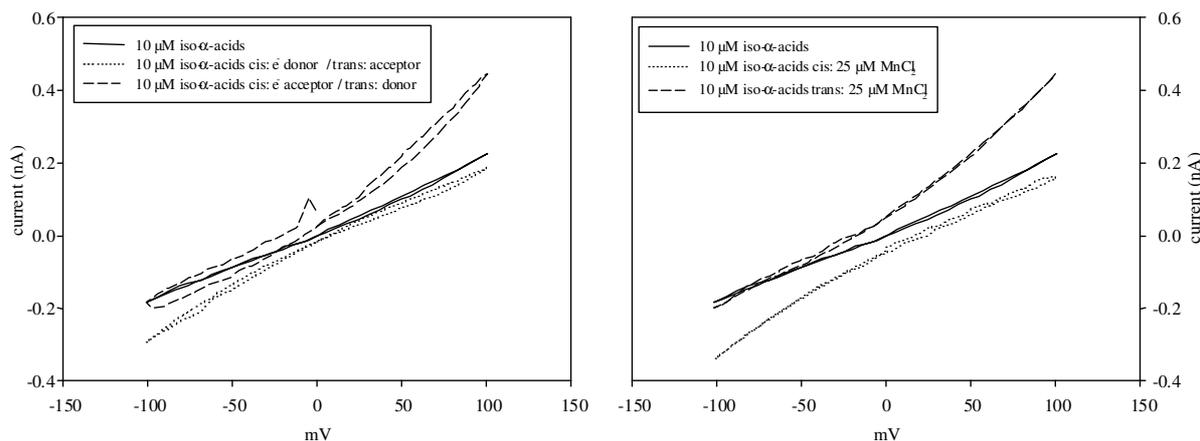
Fig. 39 Cyclic voltammograms for charge transfer through the BLM (PC) in the presence of 10 µM iso-α-acids in both compartments of the electrolytic cell in dependence of mode of 25 µM $MnCl_2$ (right) or 0.5 mM $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ addition (left) at pH 4.0. Scans 3 are displayed. The scan rate was 0.3 mVs^{-1}. Composition of aqueous solutions: phosphate buffer, KCl.

current can be attributed to the redox couples formed during the positive scan, which can undergo reversible reactions. If the redox couple in the aqueous solution was added in inverse compartments of the electrolytic cell (positive scan: $K_4[Fe(CN)_6]$ at the reducing side of the BLM and $K_3[Fe(CN)_6]$ at the oxidizing side of the BLM) the I/U curves were nearly identical to those recorded without the addition of the redox couple. The influence of $MnCl_2$ additions in both or one compartment of the electrolytic cell was recorded. Figure 38 shows the cyclic voltammogram obtained when $MnCl_2$ was added to both sides. The presence of $MnCl_2$ on both sides of the membrane resulted in a similar curve as in the presence of the $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ redox couple, however with a lesser markedness. The anodic current increased with the presence of $MnCl_2$ more than 2.5fold, the cathodic current more than threefold. Figure 39 displays a comparison of cycles 3 in dependence of mode of $MnCl_2$ addition with the cycles 3 of the data gained by the addition of electron donors and acceptors. Cycles 3 were chosen for the comparison, because manly reversible reactions were taking place (checked by running 6 additional cycles, that exhibited similar shapes), which are much easier to evaluate. A comparison of mode of $MnCl_2$ addition with data obtained by addition of $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ to the aqueous solutions for cycle 3 shows, that the Mn^{2+} hop compound complex can act as an electron donor. It is obvious that the two voltammograms are virtually identical. For example in the positive scan with $MnCl_2$ added at the oxidizing side of the BLM the current rose in comparison to that recorded without addition. The current in the negative cycle was not altered under this condition. The same is true, when the electron donor was added at the trans side (oxidizing in the positive voltage sweep) of the electrolytic

cell and the electron acceptor on the opposite. The offset measured at zero voltage for the
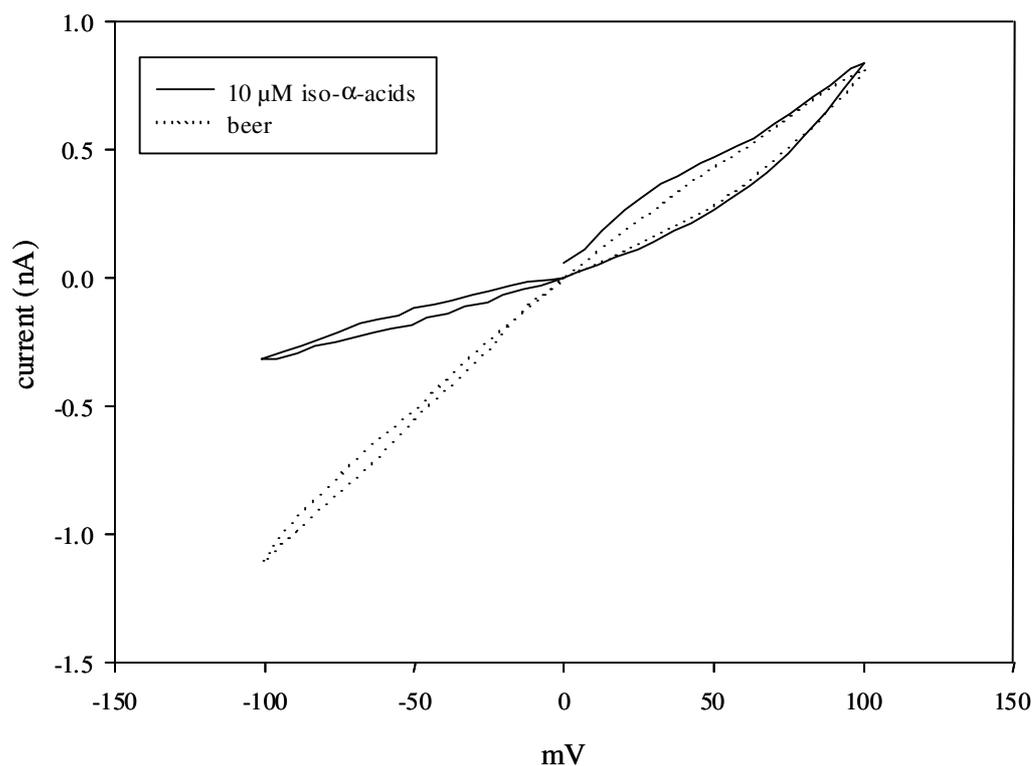
Fig. 40 Cyclic voltammograms for charge transfer through the BLM (PC) in the presence of 10 µM iso-α-acids or beer in both compartments of the electrolytic. Scans 1 are displayed. The scan rate was 0.3 mVs[-1]. Composition of aqueous solutions in case of iso-α-acids addition: phosphate buffer, KCl at pH 4.0.

voltammograms with $MnCl_2$ addition is attributed to the potential generated by a difference in $MnCl_2$ concentrations of the solutions separated by the membrane (see above 3.15.4). To assess the relevance of the model system for beer a cyclic voltammogram of beer separated by a BLM was measured. Only the first cycle could by recorded, because of the carbon dioxide in beer, which formed bubbles at the membrane. Figure 40 shows the comparison between the voltammogram of 10 µM of iso-α-acids in phosphate buffer at pH 4.0 and that of beer. Interestingly, the positive scans were nearly identical, the negative scan of beer led to a higher current through the membrane.

4   Discussion

The tolerance to hops is a prerequisite for the capability of lactic acid bacteria to grow in beer and thus cause beer spoilage. However, no quantitative definition of hop resistance exists. This is due to the fact, that the latter is affected by the composition of hop compounds and growth medium, as well as its pH value (Simpson and Smith 1992). Several studies on hop resistance in *L. brevis* report on a resistance to hop compounds at pH values (commonly pH 5.5) above 5.2 (Sami et al. 1998; Sakamoto et al. 2001; Sakamoto et al. 2002; Suzuki et al. 2002; Suzuki et al. 2004; Suzuki et al. 2004; Yansanjav et al. 2004; Suzuki et al. 2005). However, this pH value does not represent the pH of beer, which is in the range from 3.8-4.7. In this pH range minimum inhibitory concentrations (MIC) of trans-iso-humulone to non-adapted *L. brevis* strains were only determined by Simpson and Smith (1992) who found hop resistance at values of 4 µM at a pH of 4.0. For the beer spoiling *L. brevis* strain TMW 1.465 and its hop adapted variant TMW 1.465A, investigated in this thesis, MICs of 17.2 and > 100 µM (after 48 h at pH 4.0) were observed, respectively. Thus, the hop resistance of *L. brevis* TMW 1.465A goes far beyond those studied before, and was furthermore investigated in a pH range representative for beer. In this work it was demonstrated that in highly hop tolerant strains of *L. brevis* hop resistance mechanisms are not only associated with previously described pmf depletion, but imply mechanisms to cope with divalent cation limitation and oxidative stress.

Bactericidal hop compounds, mainly iso-α-acids, have been described as ionophores, which exchange H^+ for cellular divalent cations e.g. Mn^{2+} and thus dissipate ion gradients across the cytoplasmic membrane (Simpson 1993; Sakamoto and Konings 2003). The resistance of lactobacilli to hop compounds is recognized to require multiple interdependent resistance mechanisms. Mechanisms, involved in hop resistance have been described to include multidrug resistance transporters that excrete the hop compounds into the outer medium (Sakamoto et al. 2001; Suzuki et al. 2002; Suzuki et al. 2005), and proton export systems that maintain the intracellular pH (Sakamoto et al. 2002). HitA is a putative divalent cation transporter, whose gene is present in most beer spoiling lactobacilli (Hayashi et al. 2001). An alteration of teichoic acids in the cell wall (Yasui and Yoda 1997) and a changed lipid composition of the cytoplasmic membranes (Sakamoto and Konings 2003) have been suggested to additionally contribute to the hop resistance as barriers for iso-α-acids (cf. figure 41). However, the presence of unknown hop transport independent mechanisms of hop resistance has been proposed (Simpson 1993).
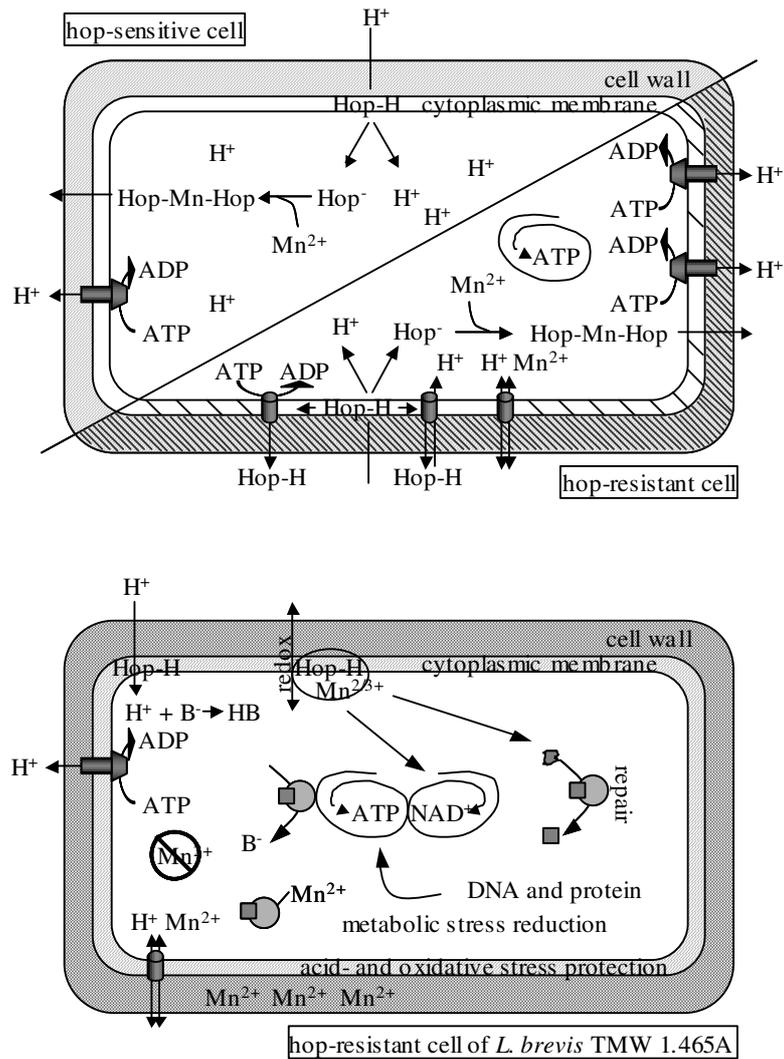
Fig. 41 left side: Hop resistance mechanisms in *Lactobacillus (L.) brevis* as described in literature. Hop compounds are described as ionophores, which dissipate the pH gradient across the cytoplasmic membrane and reduce the proton motive force (pmf). The low intracellular pH interferes with essential enzyme reactions and the pmf-dependent nutrient uptake is hampered, resulting in cell death of hop sensitive strains (Simpson 1993; Sakamoto and Konings 2003; Yansanjav, Siegumfeldt et al. 2004). The proton influx is mediated by a proton/divalent cation exchange upon the bacterial cytoplasmic membrane (Simpson 1993). Proteins contributing to hop resistance include pmf and ATP driven multi-drug-resistance (MDR) transporters that excrete the hop compounds into the outer medium (Sakamoto, Margolles et al. 2001; Suzuki, Sami et al. 2002), and proton export systems that maintain the intracellular pH. HitA is a putative divalent cation transporter present predominantly in beer spoiling lactobacilli (Hayashi, Ito et al. 2001). An alteration of teichoic acids in the cell wall (Yasui and Yoda 1997) and a changed lipid composition of the cytoplasmic membrane (Sakamoto and Konings 2003) might additionally contribute to the hop resistance. Adopted from Sakamoto et al. (Sakamoto and Konings 2003). Right side: New hop resistance mechanisms in *L. brevis* TMW 1.465A uncovered in this work. The described ionophore properties of hop compounds, as electroneutral H+/Mn2+ exchangers (see figure 41 left side), could not be reproduced. The ionophore action was characterized by a mixture of classes I and II uncoupler behaviour. The latter requires no cation counter-transport for the proton ionophore action. Mn^{2+}-hop compound complexes as well as the hop compounds itself take part in transmembrane redox reactions committing oxidative stress on the bacterium. The hop resistance of *L. brevis* TMW 1.465A could not be attributed to a MDR transport system or barriers in the cell walls or cytoplasmic membrane (cf. figure 41 left side). The mechanisms of *L. brevis* TMW 1.465A hop resistance implied significant changes in the metabolic products, resulting in minimized accompanying stresses (e.g. ethanol stress). Cell walls served as divalent cation sequestrant and an altered composition and fluidity of the cytoplasmic membrane protected from acid- and oxidative stress. Intracellular acidification was avoided by production of basic metabolites (e.g. arginine deiminase pathway, homocysteine desulfurase). Mechanisms to maintain redox balance coupled to effective energy generation and economy were upregulated. Oxidative damage was avoided by repair mechanisms, accounting for genetic information fidelity and enzyme functionality. If protein or DNA damage was

irreversible, both were degraded with concomitant energy gain. Manganese binding proteins were upregulated, while the overall manganese level was downregulated in order to avoid free intracellular manganese, which could be trapped by hop compounds. These strategies contribute to the maintenance of a metabolic active state, which ensures survival of lactic acid bacteria under growth limiting conditions (Poolman et al. 1987).

To delineate mechanisms of hop resistance in *L. brevis* TMW 1.465A and study their respective role in survival, cells from *L. brevis* TMW 1.465 were adapted to grow under 5fold iso-α-acids concentrations in comparison to the wild type. In order to differentiate between hop stress and accompanying acid stress, the experiments were carried out under hop stress, acid stress and reference (optimal growth) conditions. A variety of analytical methods were used to assess the hop resistance mechanisms of *L. brevis* as well as the origin of hop inhibition.

Fluorescence-based measurements indicated, that in contrast to hop resistance mechanisms described in literature (see above) the hop resistance of *L. brevis* TMW 1.465A could not be attributed to a MDR transport system or barriers in the cell walls or cytoplasmic membranes. Thus, the following mechanisms of hop tolerance and resistance can be assigned to mechanisms beyond those described in literature, as hop resistance in a range as high as that studied in this work has not been investigated until now. HPLC analysis demonstrated that upon hop-adaptation, significant changes appeared in the metabolic products, resulting in minimized accompanying stresses (e.g. acid- and ethanol stress), redox homeostasis, basic compounds production and thus elevation of the extra- and intracellular pH and an additional energy gain. Messenger RNA analysis confirmed the metabolic change to an enhanced production of basic compounds. Biochemical investigations of the cell walls and cytoplasmic membranes indicated higher levels of divalent cation binding lipoteichoic acids (LTA) incorporated into the cell wall, which function as reservoir of cations and enable a rapid intracellular divalent cation concentration regulation. An altered composition and fluidity of the cytoplasmic membrane is involved in acid- and oxidative stress protection in hop-adapted *L. brevis* TMW 1.465A. To identify the basis for these changes, a proteome approach was taken.

On the proteome level it was demonstrated that hop resistance in *L. brevis* TMW 1.465A implies mechanisms to cope with intracellular acidification, redox imbalance and oxidative damage and mechanisms for energy generation and economy, genetic information fidelity and enzyme functionality. Interestingly, the major part of hop-regulated enzymes is described to be manganese or divalent cation dependent. Manganese level regulation allows fine-tuning of the metabolism, which enables a rapid response to environmental (stress) conditions and is

considered to be a general lactic acid bacteria stress tolerance strategy (Kehres and Maguire 2003). Measurements of cellular manganese contents demonstrated that hop stress in bacteria obviously implies a low-level divalent manganese homeostasis, which limits the bacterial manganese-based regulatory potential. The regulation of several manganese dependent enzymes takes over the control of the bacterial metabolism and in consequence shifts the metabolism into an economic mode by effective substrate conversion. This strategy contributes to the maintenance of a metabolic active state, which is stringently required for survival of lactic acid bacteria under growth limiting conditions (Poolman et al. 1987). Effective DNA and protein repair and recycling mechanisms complete the hop defense mechanisms. In order to improve the understanding of the inherent complexity of the bacterial stress response to the action of hop compounds, the analytical method of bilayer lipid membrane measurements was used. The effect of hop compounds as proton ionophores could be confirmed. However, a revised proton ionophore mechanism, according to the class II uncoupler behavior of hops, including a proton transport via complex formation of neutral and dissociated hop compounds, is suggested. Nevertheless, maximum effects of hop compounds action were determined by measurements of hop compounds redox properties in the presence of divalent manganese. Accordingly, a new hop inhibitory mechanism was identified as a transmembrane redox reaction at low pH and in the presence of manganese. This precisely clarifies the role of the bacterial low-level manganese homeostasis, as normal intracellular levels of free manganese can be considered as bactericidal in the presence of hop compounds. On biological level, e.g. the expression of several hop resistance proteins (beyond those required to avoid intracellular acidification, cf. protonophore action of hops) involved in manganese binding and intracellular redox balance, as well as proteins of oxidative stress under highly reducing conditions (anaerobic cultivation, cysteine and "antioxidative" hop compounds in the growth medium) is comprehensible, now. For an overview, figure 41 depicts a comparison of hop resistance mechanisms described in literature and the hop resistance mechanisms of *L. brevis* TMW 1.465A. Beyond the specific model of hop tolerant bacteria, these investigations should provide general insight on the role of electrophysiology and ion homeostasis in bacterial stress responses and tolerance development to membrane active drugs. In the subsequent sections the mechanisms of hop inhibition, tolerance and adaptation are delineated at their various cellular levels in *L. brevis*.

4.1 Sugar metabolism

In heterofermentative lactic acid bacteria, like *L. brevis*, the phosphoketolase pathway generates 1 or 2/3 moles of ATP and two moles of NADH + H$^+$ from one mole glucose or fructose, respectively. The NADH + H$^+$ can be restored by the formation of ethanol from acetyl-P, to keep the cells redox balance. If acetate is formed from acetyl-P instead of ethanol, an additional mole of ATP is created by the acetate kinase. In this case the cellular redox balance has to be maintained by the use of external electron acceptors like fructose, which is converted to mannitol. The sugar metabolism in *L. brevis* TMW 1.465 changed from the reference conditions at pH 6.0 to the acid stress conditions at pH 4.0. With an increasing stress level (acid- and hop stress) the production of lactate and acetate was decreased and increased, respectively. This metabolic change was also reflected by an elevated mannitol production under acid- and hop stress conditions. This way, the formation of ethanol is avoided allowing the energetically favorable formation of acetate. At a high pH value the fructose acted more as C-source and only one third of the fructose was used as electron acceptor. Taken together *L. brevis* TMW 1.465A avoided the formation of ethanol and used the energetically favorable acetate kinase pathway. The remaining maltose and the decreased lactate production is caused by the earlier reached growth limiting environment, principally low pH, at acid stress- and hop stress conditions.

4.2 Amino acid metabolism

Hop adaptation of *L. brevis* TMW1.465A increased the conversion of arginine to ornithine via the ADI pathway (Suzuki et al. 2005). The observations on the level of metabolites were substantiated by quantification of the expression of the enzymes of the ADI pathway. A significant increase of *arcA*, *arcC* and even more of *arcB* under hop stress conditions was apparent, indicating that *arcB* could be the metabolic rate limiting step in the arginine deiminase pathway. The conversion of arginine to ornithine, ammonia, and CO_2 is coupled to production of ATP and furthermore contributes to the generation of the pmf and an increase of the extracellular pH. Accordingly, arginine metabolism is recognized as an important factor in bacterial acid resistance (Booth 1985). Glucose transport in *L. brevis* is pmf-dependent (Ye et al. 1994) and the pmf dissipation by hop compounds inhibits sugar uptake (Sakamoto and Konings 2003). An increase in the extracellular pH will reduce the activity of hop compounds, which is dependent on the pH (see below and (Simpson and Smith 1992).

L. brevis TMW 1.465 tolerated a challenge with hop stress (86 µM iso-α-acids) only in media supplemented with arginine. In contrast, *L. brevis* TMW 1.465A grew nearly independent from amino acid supplementations. This indicates, that *L brevis* TMW1.465A developed additional resistance mechanisms during adaptation, whereas the *L. brevis* TMW 1.465 requires the arginine deiminase pathway for survival. The utilization of this pathway thus appears to be an inducible early step for the adaptation to hop compounds, transiently conferring tolerance until full resistance is achieved.

4.3   Role of MDR transport and barrier functions

Whenever HorA activity was absent, MDR transport activity mediated by other transporters was observed in *L. brevis* TMW 1.465 and in *L. brevis* TMW 1.465A. Inactivation of membrane associated transport proteins via high pressure treatment and a subsequent growth challenge under hop stress conditions was used to further assess the role of MDR transport proteins in hop resistance (Sakamoto et al. 2001; Ulmer et al. 2002). Sublethal pressure treatment of lactic acid bacteria is known to inactivate the F_OF_1 ATPase and ATP-dependent as well as pmf-dependent MDR transport enzymes such as LmrP in *Lactococcus lactis* (Molina-Gutierrez et al. 2002). The glutamate-dependent acid resistance system of *E. coli* is pressure stable (Kilimann et al. 2005). The capability to grow in the presence of hops of *L. brevis* TMW 1.465A and TMW 1.465 was nearly unchanged after inactivation of the membrane-associated transporters. Measurements of the ionophore effect of iso-α-acids by monitoring the intracellular pH in energized cells indicate, that the efflux rate mediated by other possible transport mechanisms than HorA (e.g. HorC (Suzuki et al. 2005)) was far below the minimum iso-α-acid concentration (2 µM) tested. In hop adapted cells a change in cell envelope failed to protect against the drop of intracellular pH on account of hop compounds in the absence of a source of metabolic energy. As membrane-bound MDR transporters are easily inactivated by high pressure (data not shown) and hop transport activity or hop shielding was not detectable, the hop resistance of *L. brevis* TMW 1.465 and its variant TMW 1.465A appears to be mainly based on attributes beyond membrane associated transport proteins and barrier functions.

4.4   Role of membrane composition

Both acid stress and hop adaptation resulted in an altered fatty acid composition of the cytoplasmic membrane. Upon acid stress, the level of cyclopropane fatty acids increased at the expense of long chain unsaturated fatty acids. Methylation of unsaturated fatty acids to

cyclopropane fatty acids is catalzed by CFA synthase (Grogan and Cronan 1997). In *E. coli*, *cfa* expression is up-regulated as part of the stringent response and upon acid- and oxidative stress (Brown et al. 1997; Eichel et al. 1999). The substitution of mono-unsaturated fatty acids with cyclopropane fatty acids decreases membrane fluidity (Grogan and Cronan 1997) as observed with acid-stressed *L. brevis* TMW 1.465 and hop-stressed *L. brevis* TMW 1.465A. Hop adaptation of *L. brevis* TMW1.465A had a similar effect on membrane fluidity as acid stress but resulted in additional changes in the membrane composition. The most important change was the increased content of the 16/0 fatty acid at the expense of 16/1 and 18/1 monounsaturated fatty acids, resulting in a strongly elevated ratio of the 16/0 to 16/1 fatty acids. The 16/0 to 16/1 ratio was also strongly increased in proton ionophore resistant *Bacillus subtilis* strains (Guffanti et al. 1987; Krulwich et al. 1987). It was hypothesized that the increased 16/0 to 16/1 fatty acid content underlies other membrane-associated changes that are less obviously related to proton ionophore resistance (Krulwich et al. 1987).

4.5   Role of cell wall composition

With increasing stress level a higher content of lipoteichoic acids (LTA) was present in the cell walls of *L. brevis* TMW 1.465 and its hop-adapted variant. In cells grown at optimal growth conditions nearly no LTA could be detected. This inserted LTA creates a polyanionic matrix in the cell wall (Neuhaus and Baddiley 2003). Hughes et al. (Hughes et al. 1973) concluded that in whole cells the ordered array of anionic wall and membrane teichoic acids (LTA) provides a constant reservoir of bound divalent cations the membrane preferentially interacts with. To ensure a sufficient supply of divalent cations, the LTA plays an important role in "trafficking ions" (Neuhaus and Baddiley 2003). The LTA may therefore enable hop resistant cells to establish a bigger reservoir of divalent cations in their cell wall, than hop sensitive cells. This also could lead to a higher content of divalent cations near the membrane (Archibald and Duong 1984). The LTA-mediated acquisition of divalent cations could contribute to the altered decrease of membrane fluidity of *L. brevis*, which decreased upon acid stress and hop adaptation. Divalent cations interact with the negatively charged head groups of membrane phospholipids and lower the fluidity of the membrane (Asai et al. 2000; Riske et al. 2002). This effect cannot be assigned to the lipid part of the LTA, which is a very small fraction in comparison to the amount of membrane phospholipids.

4.6   Hop resistance on proteome level

For an overview, a short summary of the hop resistance on proteome level is given first. Hop stress resistance mechanisms detected on proteome level imply mechanisms to cope with

intracellular acidification, redox imbalance and oxidative damage and mechanisms for energy generation and economy, genetic information fidelity and enzyme functionality. The upregulation of several manganese dependent enzymes involved in energy metabolism is a triggering factor in hop resistance. This strategy shifts the metabolism into an economic mode by effective substrate conversion and thus contributes to the maintenance of a metabolic active state, which is stringently required for survival of lactic acid bacteria under growth limiting conditions (Poolman et al. 1987). Effective DNA and protein repair and recycling mechanisms complete the hop defense mechanisms.

To get a closer view of molecular mechanisms behind these metabolic and physiologic adaptations, were compared the proteomes of *L. brevis* TMW 1.465 cultured at reference conditions (pH 6.0) and acid stress conditions (pH 4.0) and of *L. brevis* TMW 1.465A grown under hop stress conditions (pH 4.0 and 86 µM iso-α-acids). The overexpression of several proteins directly supported the results of the biochemical characterization of *L. brevis* TMW 1.465A. Protein HI11, identified as ornithine carbamoyltransferase, is part of the arginine deiminase pathway, which contributes to pmf generation and elevates the extracellular pH to compensate for acid stress. The decrease in lactate production of hop grown *L. brevis* TMW 1.465A is accompanied by a decrease in HR1 lactate dehydrogenase expression to achieve the same result. The glycerol dehydrogenase (HO7) is part of the glycerolipid metabolism where membrane lipids as well as lipoteichoic acids (LTA) are generated. The generation of the latter is supported by the glucosamine-6-phosphate synthase (HI9), which provides amino sugars for incorporation in several macromolecules of the bacterial cell wall including peptidoglycan, lipopolysaccharides and teichoic acids (Milewski 2002). Changes in membrane lipids as well as cell wall LTA could be monitored in hop adapted *L. brevis* TMW 1.465A. The acid and oxidative stress inducible cyclopropane-fatty-acyl-phospholipid synthase (AI2) (Brown et al. 1997) expression could be verified also for *L. brevis* TMW 1.465 and its hop adapted variant on the level of protein expression and membrane fatty acid composition.

A cluster of enzymes involved in intermediate carbon metabolism was found to be overexpressed as a result of hop adaptation and could be assigned to previously unknown hop resistance mechanisms. The role of seven proteins, six of which were upregulated while Ldh was downregulated, is explained in figure 42. These enzymes are associated with bacterial primary energy generation and redox (NAD(P)H) homeostasis. Most enzymes converting phosphoglycerate, pyruvate and oxaloacetate intermediates are Mn^{2+}-dependent or highly

stimulated by Mn^{2+} (Kehres and Maguire 2003). The 3-hexulose-6-phosphate synthase (HPS) related protein (HI6), which is part of the ribulose monophosphate pathway and catalyzes the formaldehyde fixation and detoxification in bacteria, also contributes to an additional energy gain. Three moles of formaldehyde are converted to one mole of pyruvate with simultaneous formation of one mole ATP and NADH. HPS is Mg^{2+} or Mn^{2+} dependent and normally induced by formaldehyde. Among the genome sequenced lactobacilli, this protein can only be
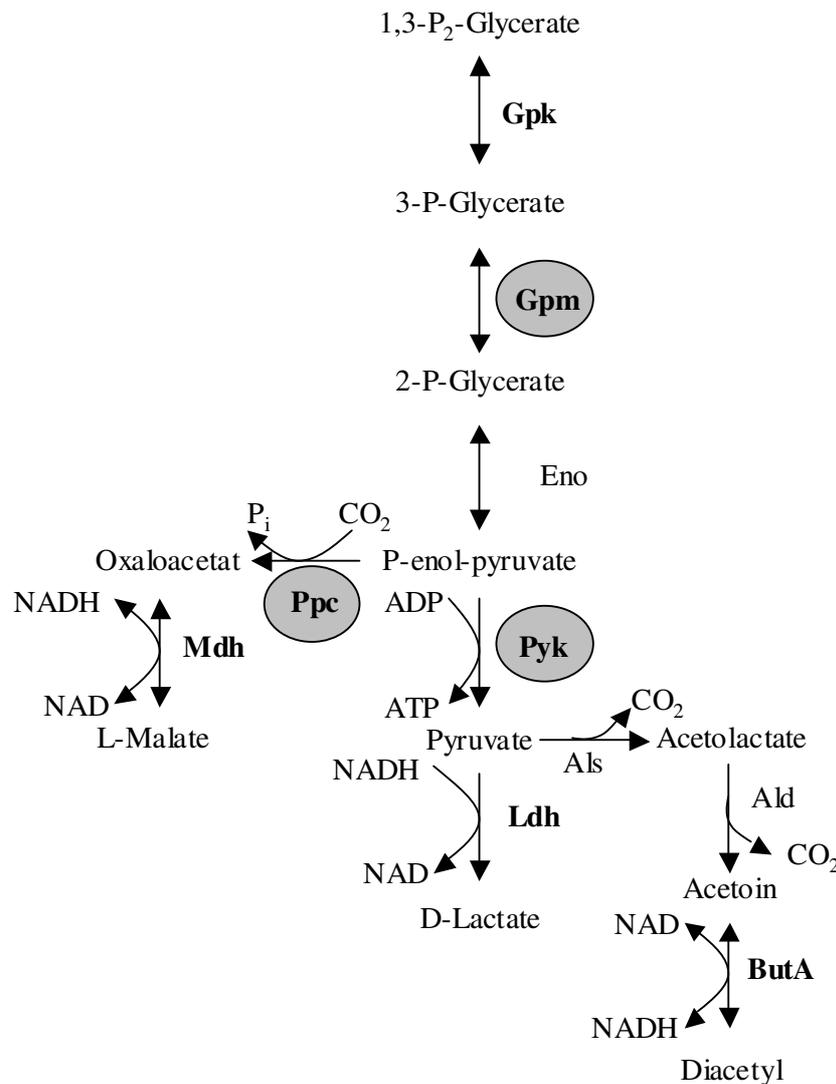
Fig. 42 Mn^{2+} stimulated enzymes in intermediate carbon metabolism are highlighted. Hop regulated enzymes are printed bold. Ggk, phosphoglycerate kinase; Gpm, phosphoglyceromutase; Eno, enolase; Pyk, pyruvate kinase; Ppc, phosphoenolpyruvate carboxylase; Mdh, malate dehydrogenase; Ldh lactate dehydrogenase; Als, acetolactate synthetase; Ald, acetolactate decarboxylase; ButA, acetoin dehydrogenase. Figure adapted from (Kehres and Maguire 2003).

found in *L. brevis* and *L. casei*, which are known as beer spoilage bacteria (Back 1994). This might be a possible additional target gene for discrimination of hop resistant and sensitive strains beyond previously described ones, including e. g. *hitA*, *horA* or *orf5* (Hayashi et al. 2001; Sakamoto et al. 2001; Suzuki et al. 2004). The use of the reductive TCA cycle and

concomitant formation of malate by HO11 contributes to manganese homeostasis based on the manganese complexing properties of malate (O'Sullivan et al. 1969). HO9, which is the 2-hydroxyacid dehydrogenase, may also catalyze the conversion from oxaloacetate to malate (Arai et al. 2001). The formation of neutral acetoin prevents acidification, and acetoin diacetyl interconversions are known to function as redox balancers (Speck and Freese 1973). In heterofermentative lactobacilli, NADH recycling doubles the ATP yield from hexoses and hence contributes to an enhanced energy yield despite a reduced metabolic flux in the central carbon metabolism. In agreement with the overexpression of several oxidoreductases involved in NADH recycling (table 4, figure 25), the hop adaptation induced a shift from fructose utilization as a carbon source to fructose utilization as an electron acceptor.

Another group of hop-regulated enzymes is involved in nucleotide metabolism. The phosphopentomutase DeoB (HO6) and the purine-nucleosid phosphorylase DeoD (HO15) are part of the ribonucleoside and desoxyribonucleoside catabolism. These enzymes can establish an additional source of energy, which exploits the carbon moiety of the degraded nucleotides. The co-expression of these two genes is consistent with their regulation in *Escherichia coli* (Albrechtsen and Ahmad 1980). Interestingly, phosphopentomutase as well as phosphoglycerate mutase (HO14, see above) are members of a superfamily of metalloenzymes and require two Mn atoms for activity (Galperin et al. 1998). For purine-nucleoside phosphorylase, a stimulatory effect of Co^{2+} in 1 mM concentration is reported, while Mg^{2+} had no effect at 100 µM level (Robertson and Hoffee 1973). This suggests stimulation by a high level of divalent cations, as described for the pyruvate converting enzymes of the intermediate carbon metabolism. However the influence of manganese was not yet tested.

RecR (HO12) is a DNA-binding protein, which is involved in DNA repair and recombination with binding affinity for damaged DNA. It does not recognize damaged bases, although unusual local deformability could be its target. It was concluded that maximal activity and binding requires ATP and divalent cations such as Mn^{2+}, and divalent cations may play a decisive role in the structure of the RecR protein (Alonso et al. 1993). Specific binding was only established in the presence of divalent cations. Thus, this enzyme can account for the fidelity of genetic information in bacteria. However, full functionality can only be achieved in the presence of mM (optimal 2 mM) levels of divalent cations (Alonso et al. 1993).

A known oxidative DNA damage is formed by imidazole ring-opening of adenine and guanine leading to the formation of formamidopyrimidines. The formamidopyrimidine-DNA

glycolase (HI1) excises formamidopyrimidine (Fapy) from DNA and contributes to DNA fidelity (Tudek 2003).

Enzymes involved in amino acid metabolism are a cysteine sulfinate desulfinase/cysteine desulfurase related enzyme (AI1) and a pyridoxal phosphate dependent decarboxylase (HI5). The former is known to be pyridoxal phosphate dependent and is associated with the mobilization of sulfur, required for metallocluster formation in proteins. In *Azotobacter vinelandii*, the cysteine desulfurase NIFS is involved in the activation of nitrogenase component proteins that have metallocluster prostetic groups (Zheng et al. 1993). As AI1 was found to be induced in response to acid stress, a function as a homocysteine desulfurase, which catalyses the reaction: L-homocysteine + H_2O <=> H_2S + NH_3 + 2-oxobutanoate, is also likely, due to the elevation of extracellular pH by ammonium production. A member of the pyridoxal phosphate dependent decarboxylase enzymes, notably the glutamate decarboxylase (Gad), confers the ability to survive in extremely acidic environments via production of γ-aminobutyrate (GABA) and NH_3, comparable to the arginine deiminase pathway (see above). These metabolites increase the intra- and extracellular pH (Tramonti et al. 2003).

The hop stress inducible peptidylpropyl isomerase (HI12) is involved in protein refolding and repair. Since hop stress conditions evoke a low intracellular pH and reduce the amount of intracellular divalent cations, it is likely that proteins could be rendered non-functional by conformational changes. The concomitant nutrient limitation during growth in hopped media (Sakamoto and Konings 2003) requires mechanisms to avoid exhaustive protein *de novo* synthesis by adequate protein repair mechanisms. If protein damage is irreversible, the cell's last-resort is degradation (Visick and Clarke 1995). ATP dependent proteases (HO17) can catalyze the recycling of proteins. Their activity is dependent on nucleotide or divalent cation binding. It is concluded that the rapid protein degeneration is achieved by ATP hydrolysis, however, $MgCl_2$, $MnCl_2$ and $CaCl_2$ allow some peptidase activity in the absence of any nucleotide. The absence of divalent cations abolishes all activity (Huang and Goldberg 1997).

Finally, a transcription regulation protein was identified. This might be an interesting target for detection of beer spoilage lactobacilli. Since hop stress results in a huge amount of regulated proteins, it is likely that exact regulation of all enzymes principally enables the adaptation to hop stress.

To verify, that a competition of hop compounds and divalent cation dependent enzymes for divalent cations, such as Mg^{2+} (Simpson 1993), can alter the enzyme activity, two types of Mg^{2+} dependent enzyme reactions were investigated. Both, an ATP dependent enzyme, which can only bind to ATP if it is in complex with a divalent cation, as well as an enzyme, which directly binds to divalent cations were obviously inhibited in the presence of hop compounds. The enzyme inhibition could be prevented by an addition of divalent cations or Mg^{2+}-ATP, confirming that the lack of divalent cations mediated by their complexation with hop compounds (Simpson and Hughes 1993) was responsible for the reduced enzyme activity. Further the ability of hop compounds to aggregate with divalent cation containing complexes is suggested.

4.7 Role of divalent cations

The role of divalent cations in the survival of hop stressed *L. brevis* TMW 1.465A beyond their regulation of enzyme functionality is described below. Lactic acid bacteria are known to have no requirement for iron (Archibald 1983; Pandey et al. 1994). Instead they contain very high intracellular Mn^{2+} levels (Archibald and Duong 1984). Like iron, Mn^{2+} can cycle in vivo between the oxidation states 2+ and 3+, whereas the ligand environment (cf. malate, see above) alters the redox potential. The latter determines whether a redox reaction between a redox couple can occur or not. E.g. in the presence of bicarbonate as ligand, Mn^{2+} shows a rapid catalase activity. Thus, activated Mn^{2+} can act as a scavenger of toxic oxygen species, via non-enzymatic detoxification mechanisms. It is not difficult to envision, that bacteria can or have "learned" to synthesize small liganding molecules, which adjust the Mn^{2+} redox reactivity (Kehres and Maguire 2003). As it is believed that the remarkable high concentrations of Mn^{2+} (35 mM) in lactic acid bacteria are "free" in a chemical sense (Kehres and Maguire 2003), it is evident to associate the complexation of intracellular manganese by hop compounds with a severe modification of the bacterial redox balance (cf. overexpression of proteins involved in redox homeostasis upon hop stress). Thus the impact of divalent cation supplementations on the growth of *L. brevis* TMW 1.465A under hop stress conditions was investigated. It was demonstrated that only Mn^{2+} enabled fast growth, indicating in first place the requirement of the redox reactivity of manganese, and subsequent the divalent cation. E.g. magnesium did not show such a growth supporting effect, although it can substitute manganese as "structural" cation (Kehres and Maguire 2003). The overexpression of *hitA* by *L. brevis* upon hop adaptation further supports the hypothesis that the manganese levels play an important role in hop resistance. Although the biochemical activity of HitA has not yet

been demonstrated, it is a homologue of the NRAMP proteins of *Salmonella enterica* and *E. coli,* constituting pmf dependent transport proteins that accumulate manganese in response to oxidative stress (Kehres et al. 2000). However, the cellular concentrations of Mn^{2+} were reduced to one tenth after exposure of *L. brevis* TMW 1.465A to hop compounds. The latter and the cross resistance to calcimycin, a proton/divalent cation exchanger with a high affinity for Mn^{2+} (Abbott et al. 1979) indicate the property of hop adapted *L. brevis* TMW 1.465A to grow under strict manganese limitation. This suggests a dynamic regulation of activation and distribution of intracellular and cell wall bound manganese (cf. LTA) in hop resistant cells, which enables its direct transfer to e.g. manganese dependent proteins or liganding molecules and diminishes the possibility of divalent cation complexation by hop compounds.

4.8   Mode of action of hop compounds in bilayer lipid membranes

In order to improve the understanding of the inherent complexity of the bacterial stress response to the action of hop compounds, a phenomenological investigation of the mode of action of hop compounds in bilayer lipid membranes (BLM) was realized. To give an overview, a brief summary of the interpretation of the results is given first. The reported ionophore properties of hop compounds, as electroneutral H^+/Mn^{2+} exchangers, could not be reproduced. The conductance experiments identified the hop compounds to act as a mixture between classes I and II uncouplers in dependence of hop concentration and pH value. The classes I and II indicate a linear or quadratic dependence of membrane conductance on uncoupler concentration, respectively. The latter is associated with a proton hopping or bimolecular process, where complexes of charged and uncharged uncouplers represent the transmembrane charge translocators. In particular a class II uncoupler behaviour means, that no cation exchange process is required for the proton transport. Potentiometric measurements confirmed the proton ionophore activity of hop compounds and indicated the manganese dependent enhancement of the resulting effect. However a cation/proton exchange processes could not explain the latter effect. Consequently, as class II uncoupler behavior of hop compounds was determined, a revised ionophore mechanism, including a proton transport via complex formation of neutral and dissociated hop compounds, is suggested. Cyclic voltammetry identified a new hop inhibitory mechanism as a transmembrane redox reaction at low pH and in the presence of manganese. In conclusion, the mode of antibacterial action of hop compounds can be extended from a proton ionophore to a redox reactive uncoupler. Subsequently, detailed interpretations of the particular BLM measurements are given.

The data published on the mode of antibacterial action of hop compounds is mainly derived from measurements in biological systems (Simpson and Fernandez 1992; Simpson and Smith 1992; Simpson 1993; Simpson and Fernandez 1994). Under these circumstances the influence of bacterial stress response or decay (e.g. hop transport by HorA, possible manganese transport by HitA or the loss of bacterial membrane integrity) on the results is outside of the control of the experimenter. The information provided by measurements in "simpler" membranes makes biological data more meaningful (Smejtek et al. 1976). Such measurements were published e.g. for ionophores like calcimycin, nigericin, CCCP and the ion channel gramicidin (Markin et al. 1975; Kasianowicz et al. 1984; Pohl et al. 1990; Kalinowski and Figaszewski 1995), which are commonly used for biological experiments. To investigate the mode of action of hop ionophores, BLM measurements were performed with regard to pH gradients or manganese gradients in solutions separated by the membrane. As initial experiment a comparison of membrane potential formation on account of a $MnCl_2$ gradient in the presence of hop compounds or A23187 (calcimycin) was chosen. As A23187 is a well described proton/divalent cation exchanger (Abbott et al. 1979; Antonenko and Yaguzhinsky 1983; Pohl et al. 1990; Jyothi et al. 1994), the comparison of mode of action of both ionophores will confirm or disproof the proton/divalent cation exchange properties of hop compounds. As depicted in figure 29, under the same experimental conditions, hop compounds cause a potential difference opposite in sign in comparison to calcimycin. Further, the potential difference of 12.3 mV in the presence of CCCP, a proton ionophore, which can cross the membrane in negative charged form (LeBlanc 1971; Kasianowicz et al. 1984) and thus compensates for potential differences beyond those caused by H^+, was much lower than that obtained in the experiments recorded without CCCP addition (65.1 mV). These observations clearly pointed to a mode of action of hop compounds, which is different to that of a nonelectrogenic proton/divalent cation exchanger. The higher potential difference in the absence of CCCP suggests an electrogenic charge transport process mediated by hop compounds in the presence of Mn^{2+}.

Membrane conductivity of BLMs recorded upon variation of ionophore concentration and $MnCl_2$ concentration will deliver information on the class of the ionophore. Uncouplers of class I exhibit a linear dependence of membrane conductance on the ionophore concentration. For uncouplers of class II the dependence is quadratic. The nature of the latter process is only partially understood. The transfer of charge is attributed to a proton hopping or a bimolecular process, where a complex is formed between a neutral molecule and an uncoupler anion (Smejtek et al. 1976). The conductance measurements in dependence of the hop compound

concentration indicated a class I uncoupler behavior at low iso-α-acids concentrations, which switches to a class II uncoupler characteristic above 60 µM at pH 7.0. The same characteristics were observed in the presence of additional $MnCl_2$ with a higher order of magnitude. The formation of such oligomer complexes is also known for nigericin, which forms a trimer at pH 4.0 (Toro et al. 1987). This suggests a charge transport, which is altered by aggregation of hop compounds within the membrane (Jyothi et al. 1994). Above a certain definite uncoupler concentration a self-exchange process of hop compounds in the membrane can mediate the charge transport (Shiba et al. 2003). The involvement of one Mn^{2+} per two charge transporting species suggests the manganese to function as coupler device (Simpson 1993) to form oligomer aggregates. The logarithm of concentration of iso-α-acids needed to raise the conductivity of the BLM by one order of magnitude was found to increase linearly with pH. A comparison of the degree of protonation (figure 15) and the conductance recorded in the presence of identical concentrations of iso-α-acids over pH showed an obvious coherence (figure 43). This very simple model is based on a correlation of the BLM conductance and the occurrence of protonated species of hop compounds at the respective pH ($G \sim A^{TOT} \bullet P$; G = conductance, A^{TOT} = total uncoupler concentration in aqueous solution, P = degree of protonation of uncoupler) (Foster and McLaughlin 1974), which are necessary to form the above mentioned bimolecular complexes of charged and uncharged uncouplers. Associated with this, the highest BLM conductivities are often found in the neighborhood of the pKa of the substance investigated (LeBlanc 1971; Smejtek et al. 1976). The known pKa values for hop compounds e.g. trans-iso-humulone (3.1), humulone (5.0) and colupulone (6.1) (Simpson and Smith 1992) cover a wide range. In addition to that, a second buffer range of hop compounds was detected between pH 9.7 and 11.4 (cf. figure 15). These observations clarify, why no bell shaped conductance/pH curve, which is characteristic for investigations of a single uncoupler (with one pKa), was obtained. The variation of degree of monomeric or dimeric charge transport processes upon pH can be attributed to the complex chemical composition of hop-derived extracts. Several dozens of articles titled "The chemistry of hop constituents" were published (not cited here), indicating the inherent complexity of the chemical composition of hop extracts. Accordingly, several charge transport mechanisms related to the chemistry of the various molecules are probable. As determined by the slopes of conductance versus hop compound concentration, a mixture between classes I and II uncouplers is more or less distinct at the respective pH. This complexity concerning the molecular composition of hop compounds used is also the reason for the omission of extensive electrochemical models as commonly calculated for uncouplers (Lieb and Stein

1974a; Lieb and Stein 1974b; Schmickler 1996). The influence of lipid composition with
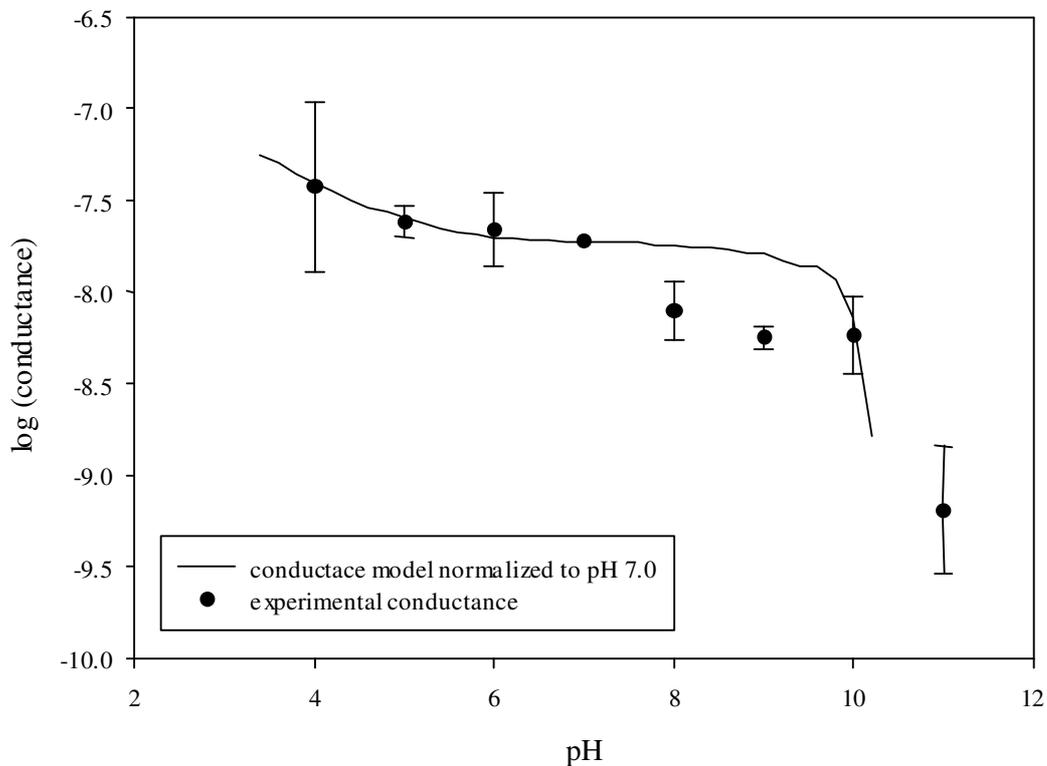
Fig. 43 pH dependence of the membrane (PC) conductance at 25 mV mediated by iso-α-acids added to both compartments of the electrolytic cell (full circles). The line represents a simplified model based on a correlation of the BLM conductance and the occurrence of protonated species of hop compounds at the respective pH (G ~ $A^{TOT} \cdot P$; G = conductance, A^{TOT} = total uncoupler concentration in aqueous solution; P = degree of protonation of uncoupler) (Foster and McLaughlin 1974). Composition of aqueous solutions: Tris, Mes, citrate, KCl at pH indicated.

respect to lipid charge on BLM conductance characterizes the charge of the translocated uncoupler species. For charged molecules their concentration at the charged membrane-solution interface, with regard to the bulk concentration, varies according to the Boltzmann expression (McLaughlin et al. 1970; Schmickler 1996). If it is assumed, that the mobility and the partition coefficient of the uncoupler are the same for charged and neutral membranes, a difference in the respective membrane conductance will allow to assess the charge of the translocator species (McLaughlin et al. 1971). Therefore, the measured decrease in membrane conductance in negative charged (GP containing) membranes points at negatively charged translocator species. The decrease in BLM conductance in pure *L. brevis* lipid BLMs in comparison to the PC doped *L. brevis* lipid BLMs, indicates that the *L. brevis* lipid contains negatively charged phospholipids (confirmed by electrostriction analysis in the presence of divalent cations (McLaughlin et al. 1971; Schoch et al. 1979), data not shown).

Membrane potentials at BLMs were recorded upon the setup of ionophore-, $MnCl_2$ and pH gradients. The potentials developed upon sole iso-α-acids gradients in the solutions separated by the BLM were small with respect to those measured in the presence of $MnCl_2$- or pH gradients. These results are consistent with data gained for other uncouplers (Foster and McLaughlin 1974; Smejtek et al. 1976). Still, this observation is of great importance for the understanding of the mode of action of hop compounds, since it rules out the possibility, that a removal of hop compounds by manganese complexation (and/or precipitation) on one side of the BLM is responsible for the potentials formed in the presence of $MnCl_2$ gradients. These potentials show a strict dependence on the $MnCl_2$ concentration gradient at pH 4 and pH 7 (cf. figures 34, 35). In the range from pH 5 to 8 the potentials generated by the same $MnCl_2$ gradient were nearly identical. Below pH 5 and above pH 8 an increase and decrease, respectively, of potential was monitored respectively. At high pH the potentials generated were low and exhibited a nonlinear shape, which might be attributed to the $Mn^{2+}/Mn(OH)_2$ conversions taking place above a pH of 8 (Shriver et al. 1997-99). An elevated hop concentration shifted the results towards that obtained at lower pH values. This indicates the reduced membrane solubility of iso-α-acids at high pH, as detected by the conductivity experiments. The major point of interest in this experiment is the sign of potential generated upon a setup of a $MnCl_2$ gradient. If Mn^{2+} is transferred through the membrane and released to the aqueous solution on the opposite side of the BLM, one would expect to receive a potential positive in sign on the side of lower (or zero) $MnCl_2$ concentration (Pohl et al. 1990). As the opposite was monitored in the presence of iso-α-acids and $MnCl_2$ gradients, a different origin of charge transfer is suggested (see below). The data gained in the presence of iso-α-acids and pH gradients were easier to evaluate. In the range from pH 4 to 9 the hop doped membrane worked nearly as a perfect pH electrode. This indicates that the hop compounds behaved as proton ionophores according to the Nernst expression. Only at high pH values, where the solubility of iso-α-acids in the membrane is reduced, the potentials were below the theoretic ones ($V = RT/F \ln\{[H^+]_{cis}/[H^+]_{trans}\}$; V = potential measured between the two aqueous solutions separated by the BLM, R = gas constant, F = Faraday, T = temperature) (Foster and McLaughlin 1974). A similar dependence is known for the proton ionophore CCCP (Kasianowicz et al. 1984). Normally, both pH gradients as well as manganese gradients are present at the same time *in vivo*. Measurements upon the set up of both manganese- and pH gradients resulted in potentials beyond the breakdown voltage of the BLMs. Accordingly, the potential formation upon a BLM in the presence of iso-α-acids and $MnCl_2$ and a pH gradient was recorded. Under these conditions the highest membrane

potentials were observed. From pH 4 to pH 6 the potentials formed by a gradient of 1 pH unit were approximately twice as high as compared to those formed without $MnCl_2$ in the solutions. From pH 8 to 10 the recorded potential coincided with the values from the sole pH gradient measurements. This indicates that the influence of the $MnCl_2$ addition on both sides of the BLM is restricted to the pH values below 7. The origin of these high membrane potentials in the presence of $MnCl_2$ caused by a pH gradient could be attributed to the redox properties of manganese and/or hop compounds. Such redox driven potential generation is e.g. known for electron conducting TCNQ (7,7′,8,8′-tetracyano-p-quinodimethane) doped BLMs in the presence of redox couples on opposite sides of the membrane (Tien 1984). As described above the ligand environment (e.g. iso-α-acids) alters the redox properties of manganese. Vice versa it can not be excluded, that the redox properties of hop compounds, which are known to be highly redox reactive substances (Belitz et al. 2001), are altered in the presence of manganese. Associated with this, the pH dependence of redox potentials has to be mentioned. For manganese, which has as complicated redox chemistry as any element known, this pH dependence can be obtained from the Frost diagrams (free energies versus oxidation state; cf. appendix) constructed for acidic and basic solutions. If we focus on Mn(II) and Mn(III) the Frost diagrams show a thermodynamic well for Mn(II) in acidic solution and for Mn(III) in base. Thus free Mn(II) can act as reducing agent in basic solutions, while the tendency to react in acidic solutions is low. On the other hand, Mn(III) will act as potent oxidizing agent at acidic pH (Shriver et al. 1997-99). In the same manner, the redox properties of iso-α-acids could be altered with pH as well. To support the above stated considerations the redox properties of hop compounds in aqueous solutions with regard to pH value and the presence of manganese were investigated via automated cyclic voltammetry and electrochemical ITT. The cyclic voltammetry data confirmed the influence of Mn^{2+} on the redox properties of iso-α-acids. An elevated reduction power of hop compounds in the presence of $MnCl_2$ was monitored. Further, an increase in oxidation power towards low pH values was monitored with a higher markedness for pure iso-α-acids in comparison to iso-α-acids in the presence of $MnCl_2$. However, the differences between controls and samples were small in this case. This can be attributed to the low iso-α-acids concentrations in the samples (100 µM), which could not be elevated due to the poor solubility of iso-α-acids in aqueous solutions at low pH in the presence of divalent cations. The ITT data showed that the reductone level of hop compounds was not altered by the presence of $MnCl_2$, indicating that the reducing power of hop derived enediol compounds will not be affected by $MnCl_2$. In conclusion, the cyclic voltammetry data supported the suggestion that differences in pH

and/or $MnCl_2$ concentrations upon a membrane can drive transmembrane redox reactions mediated by hop compounds.

In order to confirm or disproof this hypothesis and consider the influence of membrane participation, cyclic voltammetry for detection of redox reactions in BLMs (Tien 1984) was used. As initial experiment the pH dependence of cyclic voltammograms of iso-α-acids on both sides of the BLM was recorded (cf. figure 36). At higher pH values (pH 7. 0 and 10.0) the cyclic voltammograms showed a shape, which is typical for ionophores (e.g. valinomycin) (Shirai et al. 2006). At a low pH of 4.0 this behavior completely changed. The cyclic voltammogram got highly asymmetric towards the origin, the forward and backward scans differed significantly and high currents through the membrane were recorded. In contrast to the cyclic voltammograms recorded at high pH the conductivity of the BLM in the pH 4.0 condition decreased in subsequent scans. If redox reactions were taking place, the latter can be attributed to the fact that most redox reactive organic compounds undergo irreversible reactions (Sobiech et al. 1998). The asymmetry and shape of the cyclic voltammograms and the decreased conductance in subsequent potential sweeps recorded at low pH leave little doubt that transmembrane redox reactions are taking place with electrons moving through the BLM from one membrane-solution interface to the other. In this case the electron transport through the BLM requires both, oxidized and reduced form of the redox couple in the BLM as well as redox couples in the aqueous phase providing and accepting electrons at the membrane-solution interfaces (Shiba et al. 2003). Accordingly, the supply of additional membrane insoluble redox couples in the aqueous solutions will enhance the electron transfer through the BLM, as monitored by addition of $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ on opposite sides of the BLM in the presence of iso-α-acids (cf. figure 37). The role of manganese in this connection, which can cycle in vivo in the presence of an adequate ligand environment between the oxidation states II and III, will be clarified subsequently. A cyclic voltammogram with manganese and iso-α-acids on both sides of the membrane showed an elevated charge transport through the membrane as obtained by the measurements of membrane conductivity. To get insight in the mechanisms of charge transfer mediated by hop compounds in the presence of manganese gradients and their influence on transmembrane redox reactions, cyclic voltammograms were compared to those recorded in the presence of electron- donors and acceptors (cf. figure 39). It could be demonstrated, that the Mn^{2+}-hop compound complexes can act as electron donors, while the pure iso-α-acids can mediate the acceptor part. This data is consistent with the measurements of redox potential of hop compounds with and without $MnCl_2$ by automated cyclic voltammetry without membrane participation, which

determined the pure iso-α-acids as the more oxidizing condition (capable taking up electrons) and the manganese-hop complexes as the more reducing condition (providing those electrons). Thus, the mode of antibacterial action of hop compounds can be extended from a proton ionophore to a redox reactive uncoupler. In order to prove, that the above-described measurements represent an adequate model system for hop compounds in beer, a cyclic voltammogram of beer in both compartments of the electrolytic cell separated by the membrane was recorded. The positive scan was nearly identical to those obtained from the hop compounds in buffer system, while the negative sweep resulted in higher currents through the membrane. This indicates the presence of further redox reactive compounds in beer e.g. from malt and their ability to take part in reversible and irreversible redox reactions (Sobiech et al. 1998). Accordingly, the transmembrane redox activity can be found in beer as well as in the model system, underlining the suitability of the latter for investigations of the antibacterial activity of hop compounds and thus beer-spoilage.

4.9 Extended view of the antibacterial action of hop compounds

Antibacterial hop compounds, mainly iso-α-acids, are described as ionophores, which exchange H^+ for cellular divalent cations e.g. Mn^{2+} and thus dissipate ion gradients across the cytoplasmic membrane (Simpson 1993; Sakamoto and Konings 2003). In several studies it was demonstrated, that the main factors affecting the antibacterial activity of hop compounds are the pH value and their ability to bind to cations such as Mn^{2+} (Simpson and Smith 1992; Simpson 1993; Simpson and Fernandez 1994). A low pH value is suggested to contribute to an elevated amount of membrane soluble undissociated iso-α-acids, acting as weak acids and thus mediating the proton influx (Shimwell 1937; Simpson 1993). The function of manganese was assigned to enable the dissociated iso-α-acids to return from the inside of the bacterial membrane to the outside, release the divalent cation and transport another proton into the bacterial cell. Thus, the main antibacterial activity was dedicated to proton ionophore action of iso-α-acids, which was found to be stimulated by monovalent cations (Simpson and Smith 1992; Simpson and Hughes 1993). However, the therefore expected cross resistance to other proton ionophores (e.g. CCCP) could not be detected (Fernandez and Simpson 1993). In this study, the mode of action of iso-α-acids as proton ionophores could be confirmed by the BLM measurements. However, the proton ionophore action was not restricted to low pH values and distinct up to pH 9 and more. These findings are not in contradiction to the results of Simpson and colleagues, since it is known, that the cellular proton gradient is dispensable at pH values in the range of the normal intracellular pH of the bacterium (about 6.5 for *L. brevis*), if other

ion gradients, such as e.g. potassium or sodium take over its function as a driving force (Padan and Schuldiner 1987). In this context, the role of extracellular monovalent cations, beyond their cooperative binding to hops (cf. appendix; Simpson and Hughes 1993) can reside in the change in ion gradients upon the bacterial membrane, needed for survival under low proton gradients. The change from the proton gradient to e.g. sodium or potassium gradients as driving force under hop stress is supported by the fact, that the membrane potential ($\Delta \Psi$) in the presence of hop compounds (Simpson 1993) was altered to a lesser extent in comparison to the proton gradient. The proton ionophore mechanism itself can be described by a mixture of classes I and II uncoupler behavior, dependent on hop compound concentrations as well as on pH value. This can be attributed to the formation of charge transporting uncoupler complexes within the membrane (Foster and McLaughlin 1974). Consequently, a revised proton ionophore mechanism, according to the class II uncoupler behavior of hops, including a proton transport via complex formation of neutral and dissociated hop compounds, is suggested. The role of manganese in this mechanism was extensively investigated here. While the ionophore properties of hop compounds, which were described as electroneutral proton/Mn^{2+} exchangers (Sakamoto and Konings 2003), could not be reproduced, the participation of manganese in the mode of action of hop compounds was substantiated. As it is known for both, manganese and hop compounds, to be highly redox reactive substances (Shriver et al. 1997-99; Belitz et al. 2001), this fact was initially taken into consideration. With multiple experimental evidence it could be demonstrated, that Mn^{2+}-hop compound complexes as well as the hop compounds itself, take part in transmembrane redox reactions. In this context the high antibacterial activity can be linked to a redox reactivity of iso-α-acids at low pH values in addition to the revised proton ionophore effect. Such redox reaction based agents are known to be highly efficient in the µM range (Krämer 1997). Thus it can be understood, why as small changes as 0.2 in extracellular pH driving the proton ionophore action as well as shifting the redox potentials can result in a 50 % altered antibacterial effect of hop compounds (Simpson 1993). Unfortunately, transmembrane and intracellular redox reactions are hard to monitor *in vivo*. This is due to the fact, that the permeability of the cells for fluorescence based detectors of intracellular oxidative stress, like hydroxy-phenyl fluorescein, can be influenced by the addition of the stress committing antibiotic. Consequently, changing intracellular fluorescent dye levels and quenching effects complicate the interpretation of results. Accordingly, it is proposed, that a more reliable approach for detection of intracellular oxidative stress is the measurement of the expression levels of associated stress proteins in the bacterium (Hassett and Imlay 2007). These oxidative

stress associated proteins were recognized in hop stressed *L. brevis* via proteomics as CFA, formamidopyrimidine-DNA glycolase, HitA and several oxidoreductases (Brown et al. 1997; Kehres et al. 2000; Tudek 2003), indicating, that transmembrane redox reactions mediated by hop compounds and their manganese complexes cause intracellular oxidative stress. A proposal for the extended antibacterial mechanism of hop compounds is delineated subsequently.

As condition for the first contact of a bacterium to hops a low extracellular pH and manganese level, as common in beer, is assumed. Inside the bacteria a higher pH (normally about 0.5-1 pH unit, with respect to the outside) and high concentrations of Mn^{2+} are present. As hop compounds penetrate the cytoplasmic membrane, they get in contact with intracellular Mn^{2+} and form complexes (higher pH and Mn^{2+} concentration = reducing condition). Thus, an electron donor is formed at the inner membrane-solution interface. The electrons are transferred through the membrane to the iso-α-acids at the opposite membrane-solution interface, which act as electron acceptors (lower pH and Mn^{2+} concentration = oxidizing condition). As irreversible as well as reversible redox reactions were identified by BLM experiments, the remaining oxidized manganese-hop compound complexes inside the bacterium can now act as an electron acceptor, committing oxidative stress. It is known, that mainly metal ion redox couples such as Mn(II)/Mn(III), undergo reversible redox reactions (Sobiech et al. 1998). Thus, if we consider the manganese part of the manganese-hop compound complex as possible electron donor/acceptor and remember, that there is a thermodynamic well for Mn(II) at lower pH, the driving force for intracellular oxidation is taking shape. The preference of directing the reducing power of the Mn^{2+}-hop compound complexes through the membrane to the outside can reside in the proximity between the redox potentials of the redox species in the membrane and in the aqueous phase, which are stringently required for such reactions (Shiba et al. 2003). Even though a transfer of manganese through the membrane and its release at the opposite side mediated by hop compounds was not detected, it cannot be excluded, that neutral Mn^{2+}-hop compound complexes could leave the bacterial cell and thus lower the intracellular Mn^{2+} concentration as observed e.g. in the hop tolerant variant of *L. brevis* TMW 1.465 after an exposure to bactericidal hop concentrations. An alternative explanation is the induction of membrane leakage by high concentrations of hop compounds in hop tolerant *L. brevis* and thus the reduction of intracellular manganese as described for hop sensitive *Bacillus subtilis* (Teuber and Schmalreck 1973). In consequence, the lost or oxidized intracellular Mn^{2+} will further contribute to oxidative- and starvation stress, since the functions as a scavenger of toxic

oxygen species, via non-enzymatic detoxification mechanisms and as a metabolic regulator and enzyme activator (Kehres and Maguire 2003) are abolished. If no binding of hop compounds to Mn^{2+} occurs at the inner membrane-solution interface, a proton ionophore action takes place and protons are brought into the cell. According to the revised proton ionophore mechanism, hop compounds can return to extracellular space in form of a complex between charged and uncharged compounds and thus no Mn^{2+} is required to drive this part of the bactericidal effect.

4.10 Mechanism of hop adaptation

To delineate hop resistance mechanisms and study their respective role in adaptation, cells from *L. brevis* TMW 1.465 were adapted to increasing concentrations of iso-α-acids (Richards and Macrae 1964; Simpson and Fernandez 1992). Hop adaptation appears as a multifactorial process, which results in changes in metabolism, protein profile, membrane and cell wall composition and intracellular manganese levels. It involves mechanisms to cope with intracellular acidification, redox imbalance and oxidative damage and mechanisms for energy generation and economy, genetic information fidelity and enzyme functionality, and as a result enables beer spoilage. The ADI pathway contributes to hop resistance by generation of energy and elevation of intra- and extracellular pH and thus avoids large driving forces for the proton ionophore based antibacterial activity of hop compounds. It appears mainly to ensure the survival of *L. brevis* TMW 1.465 at the initial state of hop adaptation. In combination with effective NADH recycling a higher energy yield from hexose metabolism is achieved. In addition to that, the changed metabolism avoids concomitant stresses induced by acidic metabolites or the formation of ethanol. This strategy provides enough survival time to enable adaptation of a small fraction within the population to establish structural improvements to all the cellular defense mechanisms. These structural defense mechanisms imply an altered membrane composition, which accounts for maintaining the membrane integrity even at high iso-α-acids concentrations and protects from acid- and oxidative stress. The corresponding phospholipid head groups of *L. brevis* cytoplasmic membrane lipids exhibited negative charges. Although no barrier functions avoiding the drop of intracellular pH during an exposure to hop compounds at pH 4.0 (cf. figure 23) were detected in *L. brevis* TMW 1.465A, negative charged cytoplasmic membranes are clearly less permeable for negative charged hop compound complexes than neutral ones. However this resistance mechanism is only effective, when a class II uncoupler behavior of hop compounds dominates (pH range 5 to 8). It is suggested, that the charged membranes contribute to the initial step of

acquiring hop tolerance being effective at pH values > 5, e.g. pH 5.5, which is found in wort (Belitz et al. 2001). Structural changes in the cell walls were monitored as elevated lipoteichoic acid levels. The function of the cell wall lipoteichoic acids can be assigned to a divalent cation sequestrant, which contributes to a dynamic regulation of activation and distribution of intracellular and cell wall bound manganese in hop adapted cells. In contrast to MDR transport and proton-extrusion systems, which provide protection against hop compounds at the expense of ATP or the pmf, the alterations of the cell membrane and cell wall can be considered "passive" and operate largely independent from metabolic energy. In consequence, hop resistance of *L. brevis* TMW 1.465A was not eliminated by sublethal pressure treatment. Hop stress was associated with a low-level manganese homeostasis. It has been proposed that many bacteria can vary cytoplasmic manganese levels over several logs of concentration (Kehres and Maguire 2003). Fast growth and optimal conditions are normally associated with low intracellular manganese levels whereas slow growth or stress conditions lead to high intracellular manganese levels, implicating that low manganese levels contribute to large pools of intermediate metabolites and a more dissipative metabolism, whereas high manganese levels account for energy economy. Consequently, manganese level regulation may allow the bacteria to fine-tune metabolism as a rapid response to environmental insults (Kehres and Maguire 2003). As manganese levels were reduced to one tenth under hop stress conditions, this leads to a conflictive regulation of energy metabolism, which is rendered inefficient under environmental stress conditions (acidic pH, starvation and hop stress) despite the need of energy. The upregulation of manganese dependent enzymes of the intermediate carbon metabolism antagonizes the lowered enzyme activity and leads to an enhanced yield of energy production. Notably pronounced was the upregulation of several forms of the key glycolytic enzyme pyruvate kinase, which supports the theory that the lost enzyme activity has to be equalized by a strongly increased amount of enzyme. A second effect of this upregulation resulted in a shift in the rate of hop compounds and enzymes competing for divalent cation binding. Thus the lost manganese-based metabolic regulatory potential is taken over by a regulation on the level of protein expression. This results in a tolerable reduction of bacterial intracellular manganese levels, which will diminish the antibacterial manganese dependent redox reactivity of hop compounds. A similar low–level divalent cation regulatory mechanism is known for iron containing bacteria. In this case, the iron regulation ensures that there is no free intracellular iron accessible for superoxide, driving the Fenton reaction and thus causing oxidative damage (Touati 2000). If oxidative damage like DNA oxidation, which can be a lethal event (Hassett and Imlay 2007), occurs, overexpressed DNA

repair proteins (formamidopyrimidine-DNA glycolase, RecR), as recognized in hop-adapted *L. brevis*, take over. Accordingly, hop adapted cells can compensate for the deficit in intracellular manganese and thus regulatory potential by the means of a complex change in protein expression accounting for sufficient energy supply and stress minimization. These results explain previous findings, that beer isolated cells sometimes fail to grow upon reinoculation into hopped media unless they have first been exposed to subinhibitory concentrations of hop compounds (Simpson and Fernandez 1992), since the gain of resistance apparently requires an exact response. Additionally, the growth phase at the time of first contact with hop compounds could influence the acquisition of hop resistance, as cellular manganese levels are altered during the growth cycle. Once the culture has acquired hop resistance, protein expression was independent of growth phase (from early exponential to transient growth phase). This indicates that only a strict adherence to the hop resistance protein profile enables long-term survival. Such long-term adaptive response allows growth at hop concentrations even beyond those used in any beer and must be avoided by preventive measures.

5 Summary

The resistance to hop is a prerequisite for lactic acid bacteria to spoil beer. In this thesis were analyzed the mechanisms of hop inhibition, tolerance and adaptation in *Lactobacillus* (*L.*) *brevis* TMW 1.465*,* on the level of metabolism, transmembrane transport, membrane composition and fluidity, cell wall associated lipoteichoic acids, gene expression, proteome, cross resistance, cellular manganese levels, intracellular pH and mode of antibacterial action of hop compounds in artificial planar lipid bilayer systems (cf. figure) in order to establish a model on the interaction of the multiple resistance mechanisms and their respective role in bacterial hop tolerance and adaptation. *L. brevis* TMW 1.465 was selected as the most
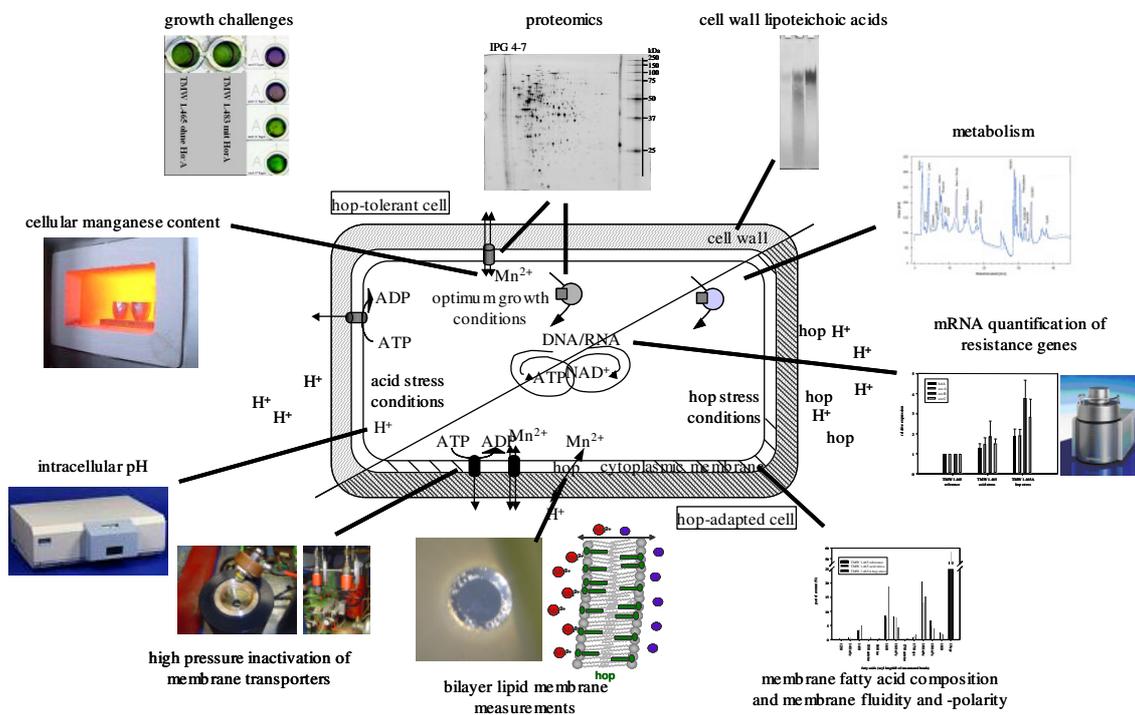
Methods and growth conditions to investigate hop inhibition, and –adaptation. The investigations comprised cell wall associated lipoteichoic acids, metabolism, gene expression, membrane composition and membrane fluidity and -polarity, mode of antibacterial action of hop compounds in artificial bilayer lipid membranes, transmembrane transport, intracellular pH, cellular manganese levels, growth and proteome of *L. brevis* and its adapted hop resistant variant.

potent beer-spoiling organism among 31 strains of lactobacilli and exhibited a 4fold hop resistance in comparison to published hop resistance levels of *L. brevis* strains (Simpson and Smith 1992). Previous studies on hop resistance in *L. brevis* report on a resistance to hop compounds at pH values above 5.2 (Sami et al. 1998; Sakamoto et al. 2001; Sakamoto et al. 2002; Suzuki et al. 2002; Suzuki et al. 2004; Suzuki et al. 2004; Yansanjav et al. 2004; Suzuki et al. 2005). However, this pH value does not represent the pH of beer, which is in the range from 3.8-4.7. Accordingly, the hop resistance of *L. brevis* TMW 1.465A goes far beyond

those studied before, and was furthermore investigated in a pH range representative for beer. In this work it was demonstrated that in highly hop tolerant strains of *L. brevis* hop resistance mechanisms are not only associated with previously described pmf depletion, but imply mechanisms to cope with divalent cation limitation and oxidative stress.

In literature bactericidal hop compounds, mainly iso-α-acids, have been described as ionophores, which exchange H^+ for cellular divalent cations e.g. Mn^{2+} and thus dissipate ion gradients across the cytoplasmic membrane resulting in cell death. While these reported properties of hop compounds, as electroneutral H^+/Mn^{2+} exchangers, could not be reproduced, the effect of hop compounds as proton ionophores could be confirmed and extended from a that one of redox reactive uncouplers. The ionophore action was characterized by a mixture of classes I and II uncoupler behavior, which was mainly typified by a class I and II behavior upon and between pH 5 and 10, respectively. Uncouplers of class I exhibit a linear dependence of transmembrane charge transport on the ionophore concentration, while it is quadratic for uncouplers of class II. In the later case the transfer of charge is attributed to a mechanism, where a complex is formed between a neutral molecule and an uncoupler anion. In particular a class II uncoupler behavior indicates, that no cation (Mn^{2+}) exchange process is required for the proton transport. Consequently, a revised proton ionophore mechanism, consisting of a proton transport via complex formation of neutral and dissociated hop compounds, is suggested. In addition to the ionophore properties, the redox properties of hop compounds were investigated. Maximum effects of hop compounds action were determined by measurements of the latter in the presence of divalent manganese. Accordingly, a new hop inhibitory mechanism was identified as a transmembrane redox reaction at low pH and in the presence of manganese. Thus the antibacterial action of hop compounds results from the revised proton ionophore activity, lowering the intracellular pH and pronounced redox reactivity, causing cellular oxidative damage.

To study hop adaptation and tolerance in beer spoiling *L. brevis* TMW 1.465, the strain was adapted to high concentrations of hop compounds (5fold of initial hop tolerance) and compared to the non-adapted strain. Hop resistance as described in literature, includes multidrug resistance transporters that excrete the hop compounds into the outer medium (Sakamoto et al. 2001; Suzuki et al. 2002; Suzuki et al. 2005), and proton export systems that maintain the intracellular pH (Sakamoto et al. 2002). HitA is a putative divalent cation transporter, whose gene is present in most beer spoiling lactobacilli (Hayashi et al. 2001). An alteration of teichoic acids in the cell wall (Yasui and Yoda 1997) and a changed lipid

composition of the cytoplasmic membranes (Sakamoto and Konings 2003) have been suggested to additionally contribute to the hop resistance as barriers for iso-α-acids.

In contrast to hop resistance mechanisms described in literature the hop resistance of *L. brevis* TMW 1.465A could not be attributed to a MDR transport system or barriers in the cell walls or cytoplasmic membranes. Upon hop adaptation significant changes appeared in the metabolic products, resulting in minimized accompanying stresses, redox homeostasis, basic compounds production and thus elevation of the extra- and intracellular pH and an additional energy gain. Hop resistance on proteome level reflected the major part of the metabolic shifts and additionally identified mechanisms to cope with oxidative damage and mechanisms for energy economy, genetic information fidelity and enzyme functionality. Structural cellular improvements, as higher levels of divalent cation binding lipoteichoic acids (LTA), incorporated into the cell wall and an altered composition and fluidity of the cytoplasmic membrane, were monitored. The latter is involved in acid- and oxidative stress protection, while the LTA functions as reservoir of cations and enables a rapid intracellular divalent cation concentration regulation. Such cation regulation was detected in *L. brevis* TMW 1.465A, as a low-level manganese homeostasis, in response to hop stress. As lactic acid bacteria are known to have no requirement for iron (Archibald 1983; Pandey et al. 1994) its essential function in bacterial life is taken over by very high intracellular Mn^{2+} levels in lactic acid bacteria (Archibald and Duong 1984). In this case, the crucial role of a low-level manganese homeostasis in hop resistance is explained by the hops redox reactivity, as according to the new hop inhibitory mechanism, normal intracellular levels of free manganese can be considered as bactericidal in connection with hop compounds. However, manganese level regulation, which is considered to be a general lactic acid bacteria stress tolerance strategy (Kehres and Maguire 2003) on the level of fine-tuning of the bacterial metabolism, is restricted under these conditions. The lost regulatory potential was taken over by a complex regulation of manganese dependent enzyme expression. Thus, the property of hop adapted *L. brevis* TMW 1.465A to grow under strict manganese limitation, suggests a dynamic regulation of activation and distribution of intracellular and cell wall bound manganese in hop adapted cells. This strategy shifts the metabolism into an economic mode by effective substrate conversion and thus contributes to the maintenance of a metabolic active state, which is stringently required for survival of lactic acid bacteria under growth limiting conditions (Poolman et al. 1987). Effective DNA and protein repair and recycling complete the hop defense mechanisms. In consequence a low-level manganese homeostasis avoids bactericidal oxidative stress and in combination with a complex change in protein expression

profile and adequate repair and recycling mechanisms enables log-term survival. Accordingly, hop resistance is a multifactorial dynamic property, which can develop during adaptation. The differences of hop resistance mechanisms described in literature and those uncovered in this study can be linked to the fact, that in contrast to earlier publications, in this study the mechanisms of hop inhibition and resistance, both highly affected by the pH value, were initially investigated at a given pH, which is representative for beer. Beyond the specific model of hop resistant bacteria, these investigations provide general insight on the role of electrophysiology and ion homeostasis in bacterial stress responses and tolerance development to membrane active drugs.

6   Zusammenfassung

Das Potential von Milchsäurebakterien zum Bierverderb korreliert stark mit der Resistenz gegenüber Hopfeninhaltsstoffen. In dieser Arbeit wurden die Mechanismen der Hopfenhemmung, -toleranz und –anpassung bei dem bierverderbenden *Lactobacillus* (*L.*) *brevis* TMW 1.465 auf Ebene des Metabolismus, des transmembranen Transports, der Membranzusammensetzung und –fluidität, der zellwandgebundenen Lipoteichonsäuren, der Genexpression, des Proteoms, der Kreuzresistenz, des zellulären Mangangehalts, des intrazellulären pH-Wertes, und der Wirkweise von Hopfeninhaltsstoffen in künstlichen planen Lipiddoppelschichtmembranen untersucht (vgl. Abbildung), um ein Model multipler Resistenzmechanismen zu erstellen und deren jeweilige Rolle in der bakteriellen Hopfenanpassung und -toleranz einzuschätzen. *L. brevis* TMW 1.465 wurde als potentester
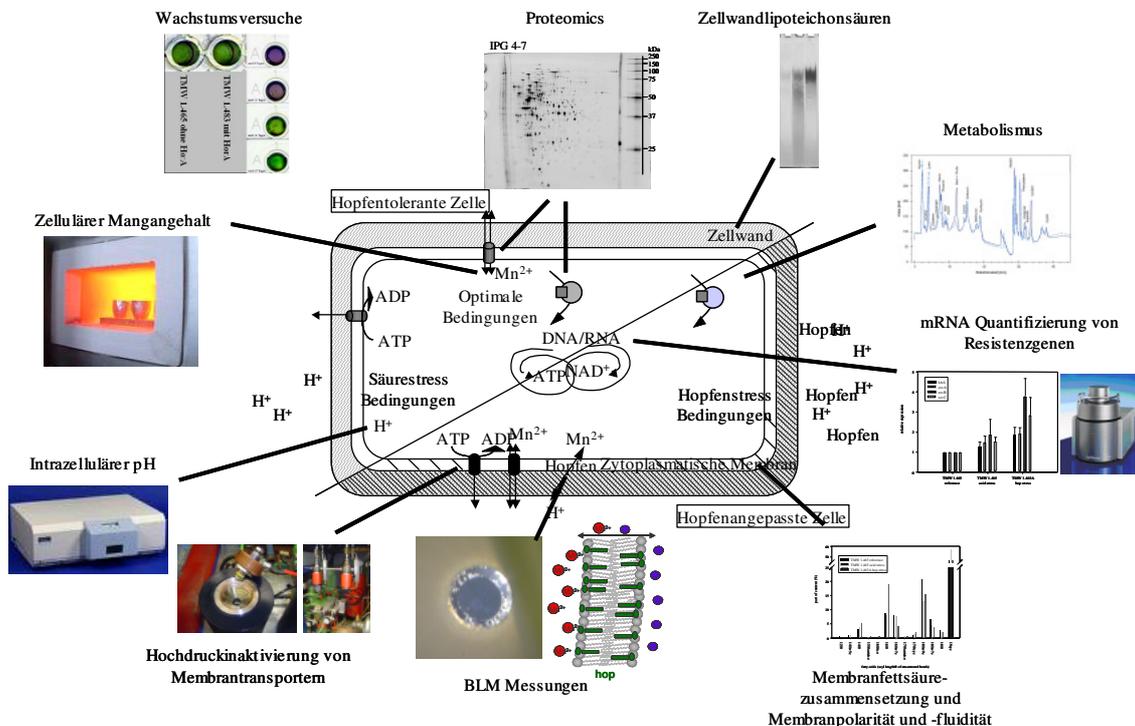
Methoden und Wachstumsbedingungen zur Untersuchung von Hopfenhemmung und -anpassung. Die Untersuchungen umfassten die zellwandgebundenen Lipoteichonsäuren, den Metabolismus, die Genexpression, die Membranzusammensetzung und Membranpolarität und –fluidität, die Wirkweise von Hopfeninhaltsstoffen in künstlichen planen Lipiddoppelschichtmembranen (BLMs), den transmembranen Transport, den intrazellulären pH-Wert, den zellulären Mangangehalt, das Wachstum und das Proteom des hopfentoleranten und des hopfenangepassten *L. brevis.*

Bierverderberstamm unter 31 Milchsäurebakterien ausgewählt und wies eine 4fach höhere Hopfentoleranz im Vergleich zu den publizierten Resistenzleveln von *L. brevis* Stämmen auf (Simpson and Smith 1992). Bisherig Studien zum Thema der Hopfenresistenz von *L. brevis* beschreiben die Resistenz gegenüber Hopfeninhaltsstoffen bei pH-Werten über 5.2 (Sami et al. 1998; Sakamoto et al. 2001; Sakamoto et al. 2002; Suzuki et al. 2002; Suzuki et al. 2004;

Suzuki et al. 2004; Yansanjav et al. 2004; Suzuki et al. 2005). Jedoch repräsentiert dieser pH-Wert nicht den pH von Bier, der zwischen 3.8-4.7 liegt. Demzufolge wurde in dieser Arbeit die Hopfenresistenz eines *L. brevis* Stammes, die weit über die bisher untersuchten Resistenzlevel hinausgeht, erstmalig in einem pH-Bereich untersucht, der für Bier repräsentativ ist. In dieser Arbeit wurde gezeigt, dass die Hopfenresistenzmechanismen in *L. brevis* Stämmen mit hoher Hopfentoleranz nicht nur der beschriebenen Zerstörung der protonenmotorischen Kraft entgegen wirken, sondern Mechanismen beinhalten um Bedingungen der zweiwertigen Kationen Limitierung und des oxidativen Stresses gewachsen zu sein.

Antibakteriell wirksame Hopfeninhaltsstoffe, hauptsächlich Iso-α-Säuren, werden in der Literatur als Ionophore beschrieben, die H^+ gegen intrazelluläre zweiwertige Kationen, wie z.B. Mn^{2+} tauschen, und somit Ionengradienten über der Zytoplasmamembran zerstören, was zum Zelltod führt. Obwohl diese Eigenschaft von Hopfenionophoren als elektroneutrale H^+/Mn^{2+} Tauscher in dieser Arbeit nicht bestätigt werden konnte, wurde die Wirkweise von Hopfen als Protonenionophore bestätigt und hinsichtlich einer Entkopplerfunktion erweitert. Die Ionophorwirkung war hierbei einer Mischung aus den Entkopplerklassen I und II, jeweils mehr oder weniger ausgeprägt in Abhängigkeit des pH-Wertes (Klasse I ausgeprägter unter pH 5 und über pH 10; Klasse II ausgeprägter zwischen pH 5 und 10), zuzuordnen. Entkoppler der Klasse I weisen eine lineare Abhängigkeit des transmembranen Ladungstransports von der Entkopplerkonzentration auf, während dieser Zusammenhang für Entkoppler der Klasse II quadratisch ist. Im letzteren Fall wird der Ladungstransfer einem Mechanismus zugeordnet, bei dem ein Komplex aus einem neutralen Entkopplermolekül und einem Entkoppleranion gebildet wird. Im Einzelnen bedeutet dies, dass kein Kationenaustausch (Mn^{2+}) für den Protonentransport bei letzterem Mechanismus nötig ist. Zusätzlich zu den Eigenschaften von Hopfeninhaltsstoffen als Ionophore wurden die Redoxeigenschaften untersucht. Die maximale Wirkung der Hopfeninhaltsstoffe wurde bei diesen Messungen in Gegenwart von zweiwertigem Mangan festgestellt. Somit wurde ein neuer inhibitorischer Hopfenwirkmechanismus als transmembrane Redoxreaktion bei niedrigem pH-Wert und in der Gegenwart von Mangan identifiziert. Folglich resultiert die antibakterielle Wirkung von Hopfeninhaltsstoffen aus der Wirkung von Hopfen als Protonenionophore, welche den intrazellulären pH –Wert senken, sowie in hohem Maße aus deren Redoxreaktivität, die eine oxidative Zellschädigung herbeiführt.

L. brevis TMW 1.465 wurde an hohe Hopfenkonzentrationen (das 5fache der anfänglichen Toleranz) adaptiert und mit der nicht adaptierten Variante verglichen, um die Aspekte der Hopfenanpassung und –toleranz im bierverderbenden untersuchen zu können.

Die Hopfenresistenz, die in der Literatur beschrieben ist, umfasst MDR (multidrug resistance) Transporter, die Hopfeninhaltsstoffe in den extrazellulären Raum zurückpumpen und Protonenpumpen (Sakamoto et al. 2001; Suzuki et al. 2002; Suzuki et al. 2005), die den intrazellulären pH-Wert aufrechterhalten (Sakamoto et al. 2002). HitA ist ein mutmaßlicher zweiwertiger Kationentransporter, dessen Gen hauptsächlich in bierverderbenden Laktobazillen vorkommt (Hayashi et al. 2001). Veränderungen der Zellwandteichonsäuren (Yasui and Yoda 1997) und eine veränderte Zellmembranzusammensetzung (Sakamoto and Konings 2003), die als Barriere für Hopfeninhaltsstoffe zur Hopfenresistenz beitragen, wurden vorgeschlagen.

Im Gegensatz zu den in der Literatur beschriebenen Hopfenresistenzmechanismen konnte die Hopfenresistenz von *L. brevis* TMW 1.465A nicht auf MDR-Transportsysteme oder Barrieren in der Zellwand oder Zytoplasmamembran zurückgeführt werden. Durch die Anpassung an Hopfeninhaltsstoffe, veränderte sich der Metabolismus von *L. brevis* TMW 1.465A hin zu einer Vermeidung von zusätzlichen begleitenden Stressfaktoren, der Aufrechterhaltung der Redoxbalance, der Produktion von basischen Stoffwechselprodukten, die den intra- und extrazellulären pH-Wert erhöhen und einer effektiveren Energiegewinnung. Hopfenstressuntersuchungen auf Proteomebene, spiegelten einen großen Teil der Veränderungen im Metabolismus wider und identifizierten zusätzliche Mechanismen gegen oxidative Zellschädigung, Mechanismen der Energieökonomie, zum Schutz der genetischen Information und Enzymfunktionalität. Strukturelle Verbesserungen, wie höhere Gehalte an zweiwertigen Kationen bindenden Lipoteichonsäuren (LTA) in den Zellwänden, sowie eine veränderte Zusammensetzung und Fluidität der Zellmembran wurden festgestellt. Letztere ist am Schutz vor Säure- und oxidativem Stress beteiligt, wobei die LTA als Reservoir an Kationen fungiert und deren schnelle intrazelluläre Konzentrationsregulation erlaubt. Solch eine Kationenregulation wurde bei dem *L. brevis* TMW 1.465A, in Form einer Manganhomeostase auf niedrigem Niveau, als Hopfenstressantwort gefunden. Da Milchsäurebakterien kein Eisen benötigen (Archibald 1983; Pandey et al. 1994) wird die essentielle Funktion von Eisen im bakteriellen Leben durch sehr hohe intrazelluläre Mn^{2+}-Level in Milchsäurebakterien übernommen (Archibald and Duong 1984). Dabei wird die essentielle Rolle der bakteriellen Manganhomeostase auf niedrigem Niveau durch die Redoxreaktivität des Hopfens erklärt, da anhand des hier beschriebenen

Hopfenwirkmechanismus, normale intrazelluläre Konzentrationen von freiem Mangan als bakterizid in Verbindung mit Hopfeninhaltsstoffen einzustufen sind. Dadurch jedoch ist unter diesen Bedingungen die Mangankonzentrationsregulation, die als generelle Stresstoleranzstrategie in Milchsäurebakterien eingeschätzt wird, eingeschränkt. Das verlorene regulatorische Potenzial wurde in *L. brevis* TMW 1.465A durch eine komplexe Regulation der Expression von manganabhängigen Enzymen kompensiert. Somit deutet die Eigenschaft des angepassten *L. brevis* unter dieser strikten Manganlimitierung zu wachsen, auf eine dynamische Regulation der Aktivierung und Verteilung von intrazellulärem und zellwandgebundenem Mangan hin. Diese Strategie versetzt den Metabolismus in den Sparmodus, wodurch eine effektive Substratverwertung gewährleistet ist, und trägt somit zur Erhaltung eines aktiven Metabolismus bei, der zum Überleben von Milchsäurebakterien unter wachstumslimitierenden Faktoren, unumgänglich ist (Poolman et al. 1987). Eine effektive DNA- und Proteinreparatur und –recycling ergänzen die Hopfenresistenzmechanismen. Folglich verhindert die Manganhomeostase auf niedrigem Niveau oxidativen Stress und in Kombination mit einer komplexen Veränderung im Proteinprofil und adäquaten Reparatur- und Recyclingmechanismen, ermöglicht sie ein langfristiges Überleben. Dementsprechend stellt die Hopfenresistenz eine von vielen Faktoren abhängige dynamische Eigenschaft dar, die sich während der Hopfenadaptation entwickeln kann. Die Unterschiede der Hopfenresistenzmechanismen, die in der Literatur beschrieben sind, und denjenigen, die in dieser Arbeit aufgedeckt wurden, ist mit der Tatsache verbunden, dass im Gegensatz zu frühere Publikationen, die Mechanismen der Hopfenhemmung und –resistenz, welche beide stark durch den pH-Wert beeinflusst werden, erstmalig bei einem pH-Wert untersucht wurden, der den pH-Wert von Bier widerspiegelt. Über das spezifische Model der Hopfenresistenz hinaus, ermöglichen diese Untersuchungen einen generellen Einblick in die Rolle der Elektrophysiologie und Ionenhomeostase in der bakteriellen Stressantwort und der Entwicklung von Toleranzen gegenüber membranaktiven Wirkstoffen.

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8   Appendix
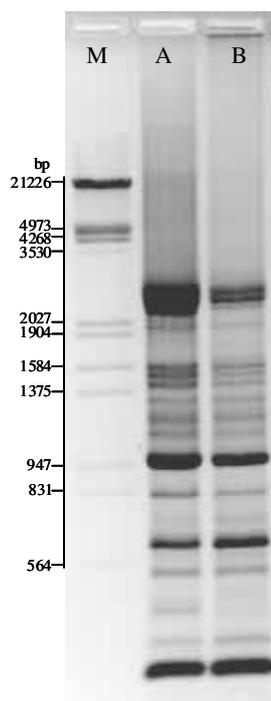
Fig. 44 RAPD patterns of *L. brevis* TMW 1.465A (A) and TMW 1.465 (B). To control for possible contaminations during the adaptation procedure a RAPD-PCR was performed as previously described (Behr 2002). M, molecular weight marker.
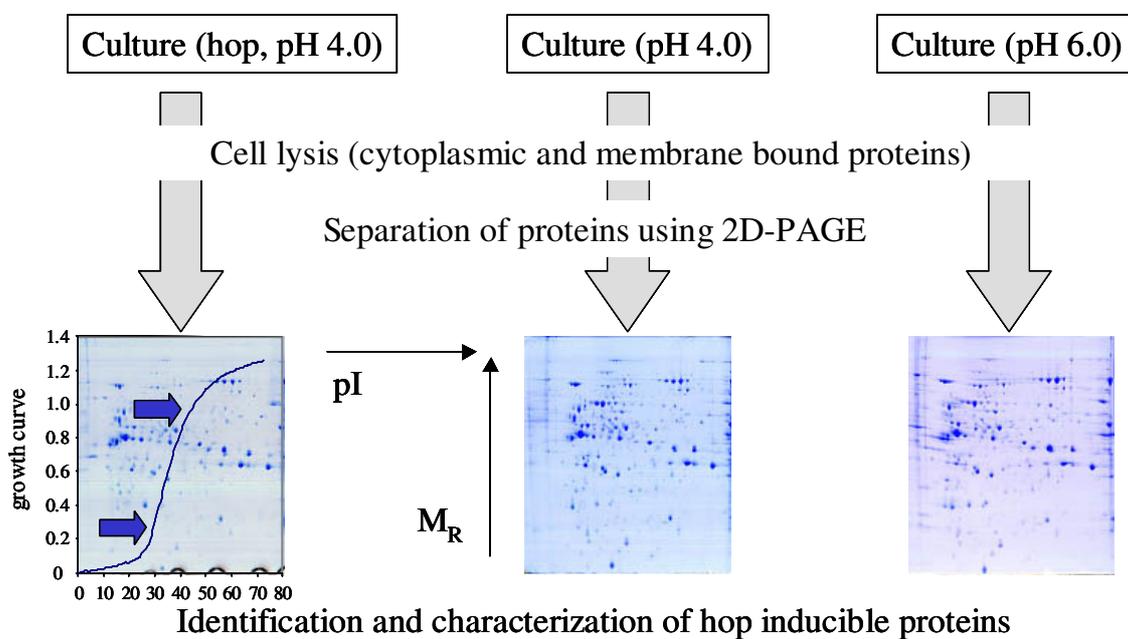
Fig. 45 Experimental procedure for identification of acid- and hop stress inducible proteins in *L. brevis* TMW 1.465 and TMW 1.465A. The dependence of protein expression on growth phase, was taken into consideration.
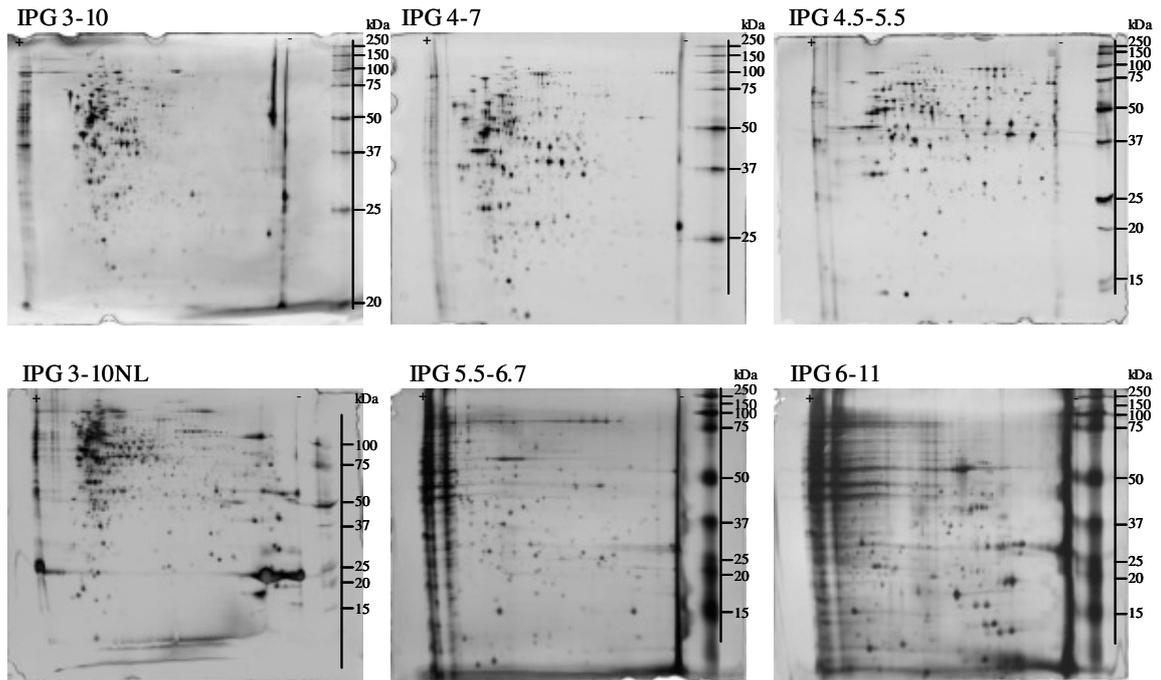
Fig. 46 Examples of IPGs (18 cm) used for detection of acid- and hop stress inducible proteins in *L. brevis* TMW 1.465 and TMW 1.465A. The molecular weight marker was precision plus protein unstained standards (10, 15, 20, 25, 37, 50, 75, 100, 150 and, 250 kDa; Bio-Rad, CA; USA).
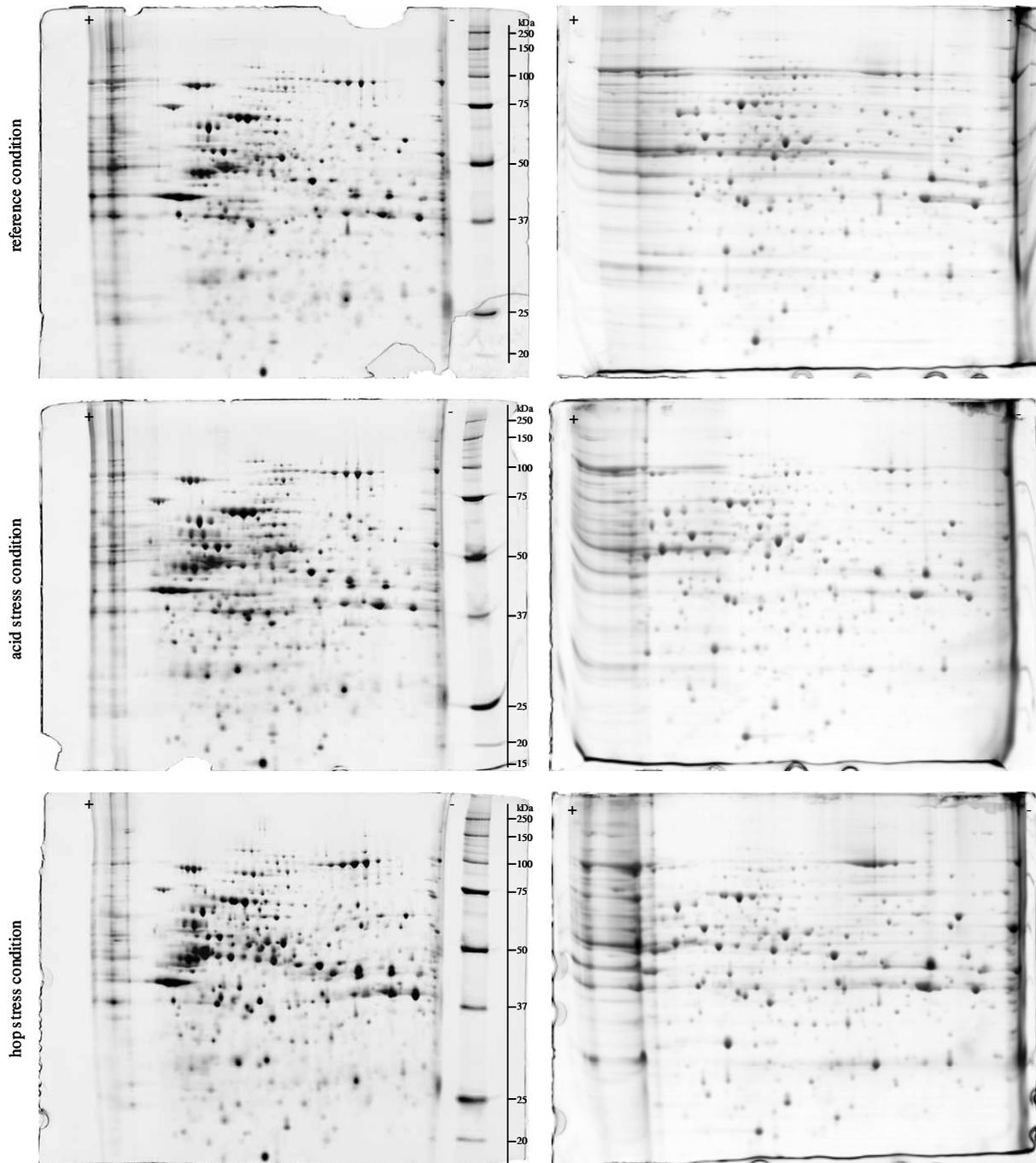
Fig. 47 Examples for 2D electrophoretic analysis of coomassie blue stained total protein from cells of *L. brevis* grown under reference, or acid stress conditions, and of hop-adapted *L. brevis* TMW 1.465A grown under hop stress conditions. 18 cm (left) and 24 cm (right) IPGs (4.5-5.5) are displayed. Molecular weight marker was precision plus protein unstained standards (Bio-Rad, CA, USA).

reference condition   acid stress condition   hop stress condition

13% SDS-PAG
silver stained

11% SDS-PAG
silver stained

11% SDS-PAG
colloidal coomassie sttained
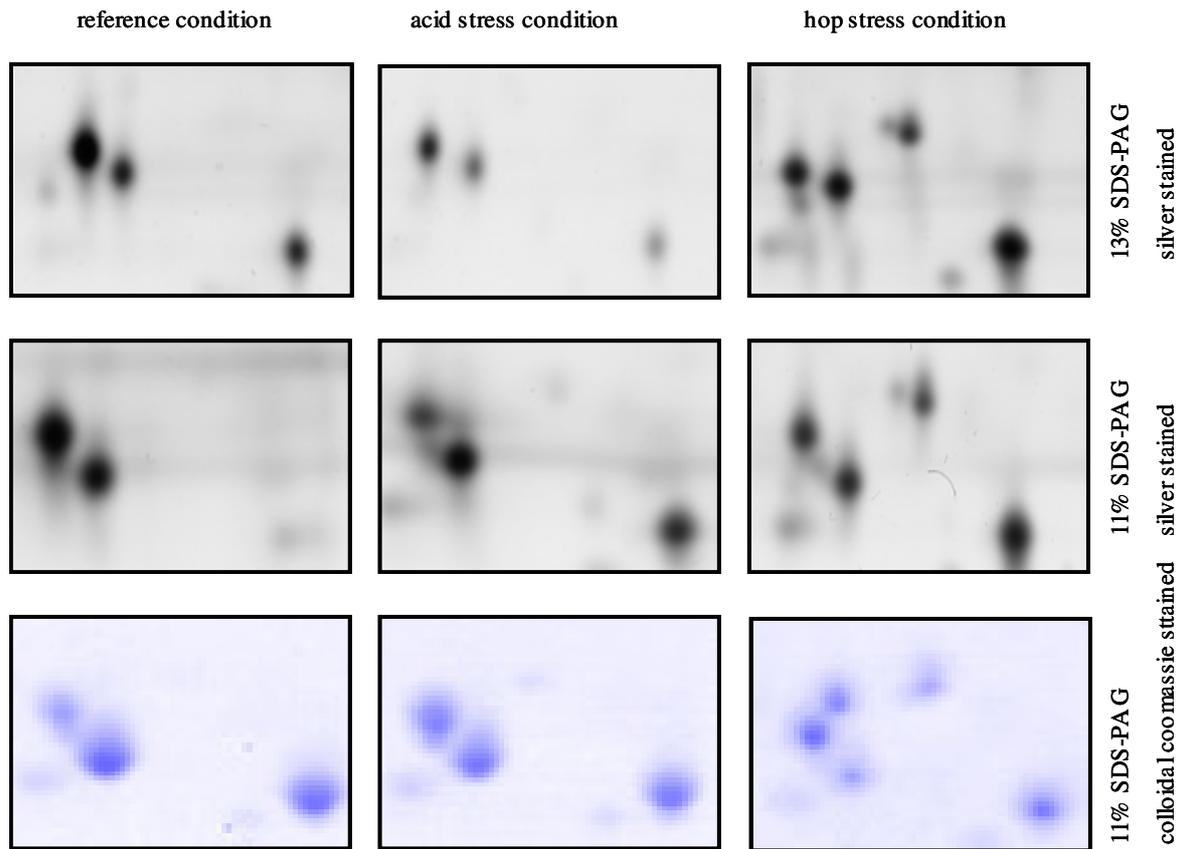
Fig. 48 Examples for the reproducibility of 2D electrophoretic patterns and a hop stress inducible protein (HI10). (18 cm IPGs 4.5-5.5).
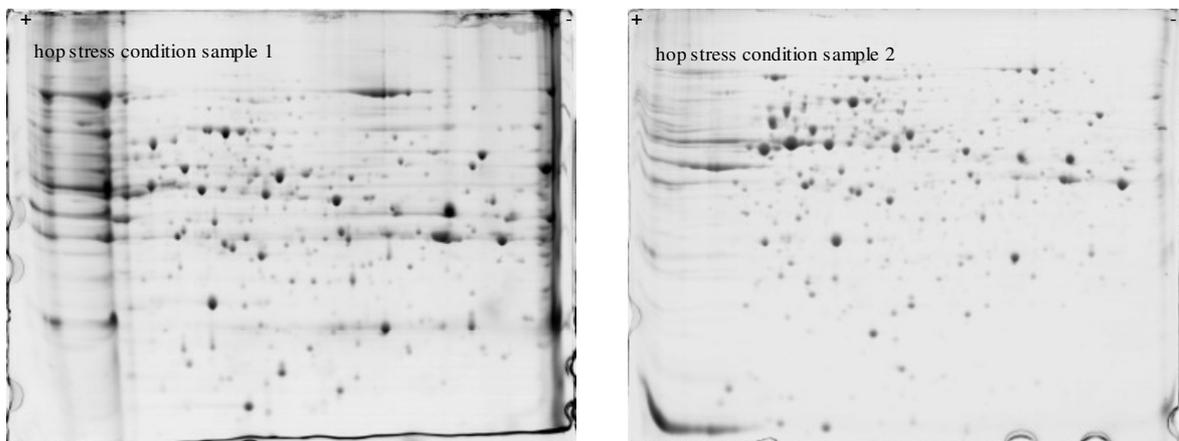
Fig. 49 Examples for the reproducibility of 2D electrophoretic patterns of coomassie blue stained total proteins from cells of hop-adapted *L. brevis* TMW 1.465A grown under hop stress condition (24 cm IPGs 4.5-5.5).
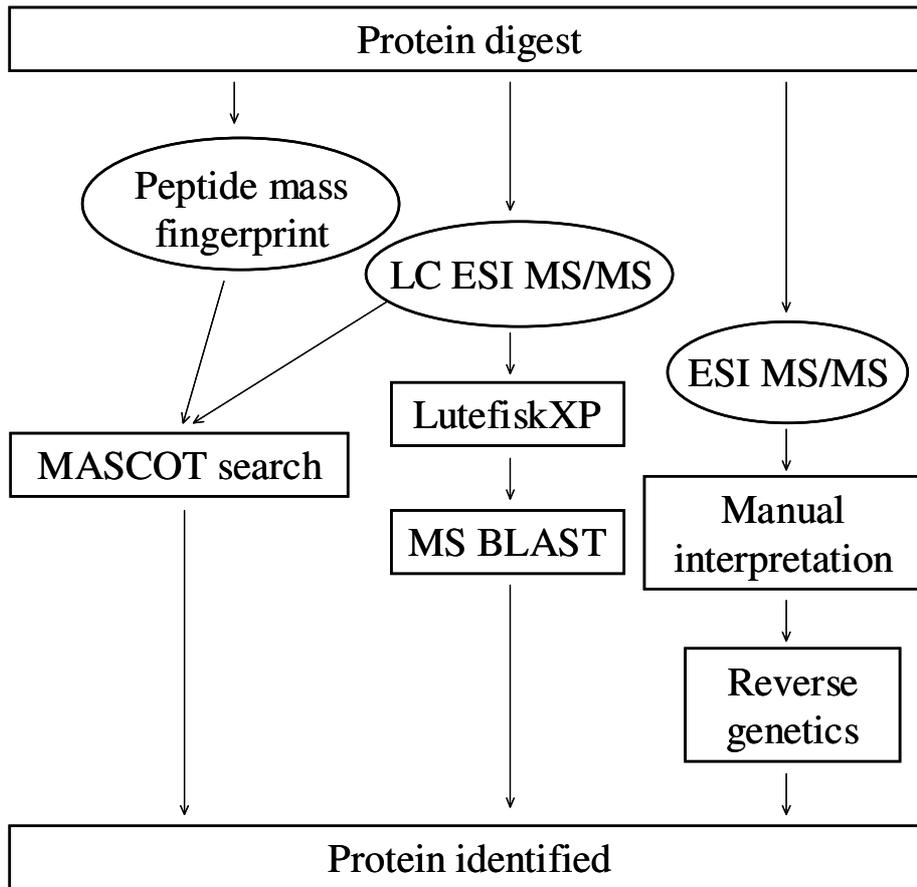
Fig. 50 The general strategy for identification of proteins from *L. brevis* TMW 1.465, whose genome is not sequenced.
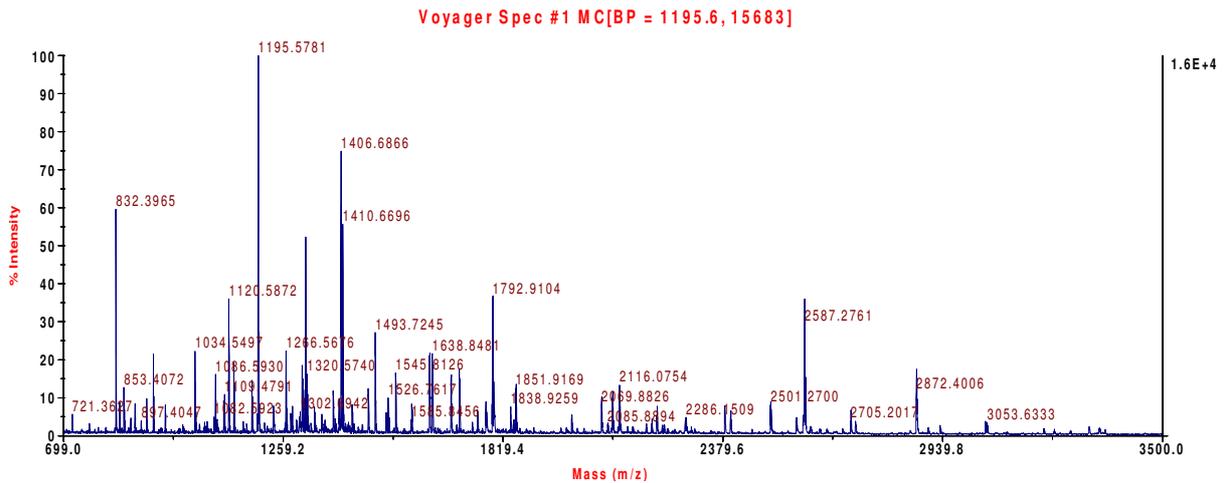
Fig. 51 Example of a peptide mass finger print of *L. brevis* TMW 1.465 phosphoketolase.
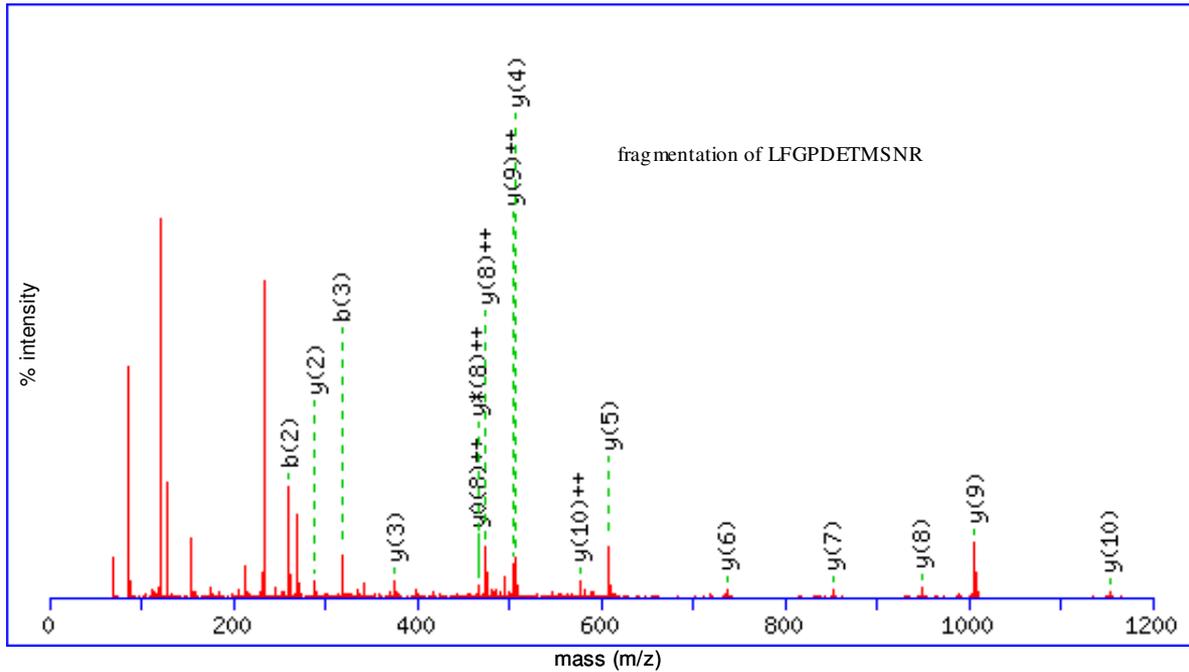
Fig. 52 LC-MS/MS spectrum (CID-spectrum; collision induced decay) of fragment 1265.55 from *L. brevis* TMW 1.465 phosphoketolase: LFGPDETMSNR was found in gi|15673484, phosphoketolase [*Lactococcus lactis* subsp. *lactis* Il1403].
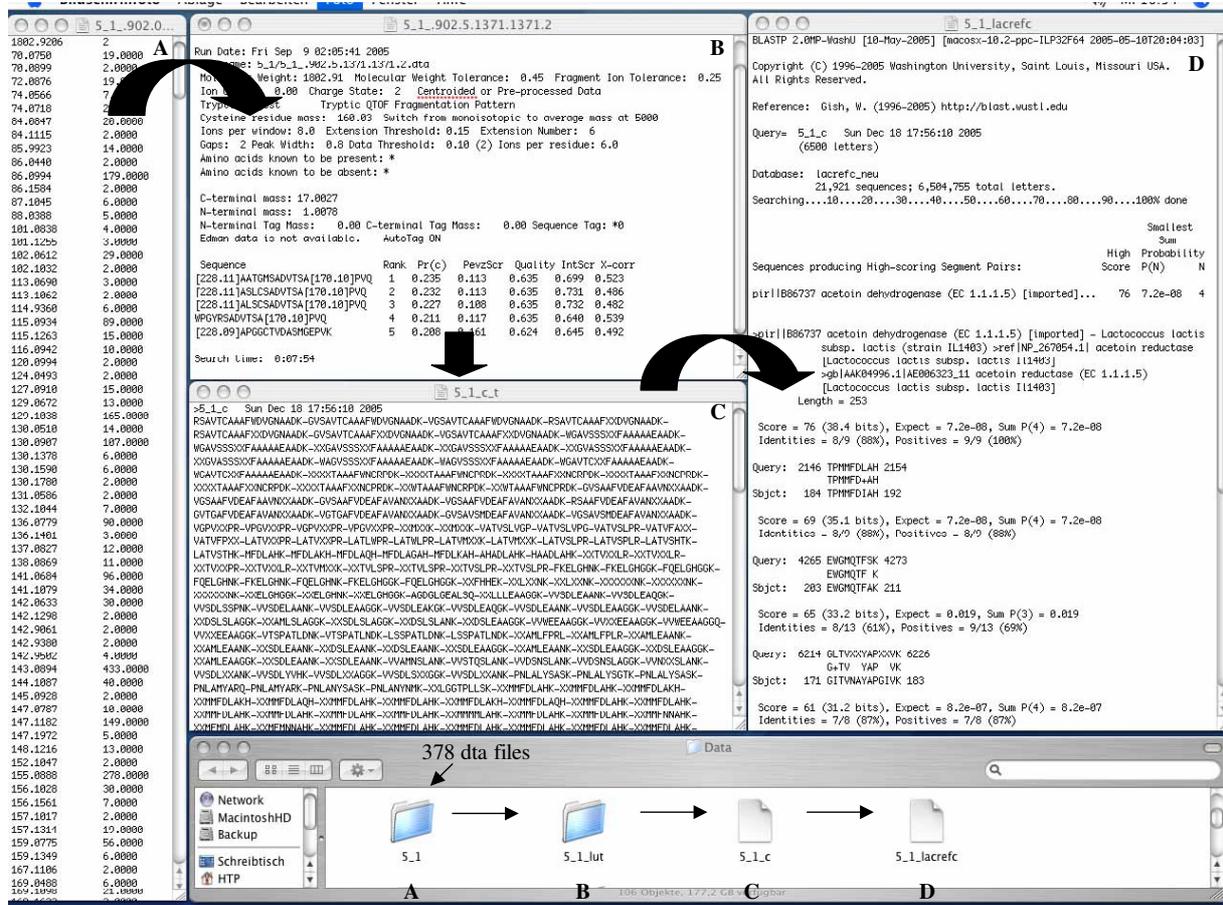
Fig. 53 Peptide sequence based protein identification. CID-spectra derived from LC-ESI MS/MS analysis were exported to Sequest dta files. dta formatted (Yates, Eng et al. 1995) files (A) were processed to peptide sequences (B) with LutefiskXP (Taylor and Johnson 1997). A program written in C (Lut2MSBLAST) generated

and submitted multiple permutations and catenations of peptide sequences (C) to MS-BLAST (D). As typically 300 to 500 dta files per sample were obtained from the LC-ESI MS/MS analysis, the workflow was completely automated and submitted to parallel machines.

```
>tr|Q74J38|Q74J38_LACJO phosphoenolpyruvate carboxylase – Lactobacillus johnsonii.
tr|Q74J38|Q74J38_LACJO      MSIKKLENSSDHAMVAEEVKILTNLLNESIRQLSGDVIFNKIQDLIKISA 50
HI10                        -------------------------------------------------
tr|Q74J38|Q74J38_LACJO      QKDYDALEEQIASLTNQEMIVVARYFATLPLLVNISEDVELASEVNLLNN 100
HI10                        -------------------------------------------------
tr|Q74J38|Q74J38_LACJO      TDQDYLGKLEDTIDLVSQKENASEILKHVNVVPVLTAHPTQVQRKTVLEL 150
HI10                        -------KLED----------------NVVP------------------
tr|Q74J38|Q74J38_LACJO      TDQIHGLLRSYREVKNGTINQAEWTEKLRAYIEILMQTDIIRSHKLQVSN 200
HI10                        ---------------------WTEK----DEVLMG--------------
tr|Q74J38|Q74J38_LACJO      EITNVLAYYPKALIPAITKFTARYQELAKKHNLNLSGATPITMGMWIGGD 250
HI10                        -----------------------ELA-KHNLNLQKAFV---------- 10
tr|Q74J38|Q74J38_LACJO      RDGNPYVTAETLKLSATLQSQVIFEYYIKQLNKLYRTISMSTSYMKPSSA 300
HI10                        ---------------------------------------TSYMK----
tr|Q74J38|Q74J38_LACJO      VEKLSDLSNDDSPFRTNEPYRRAFYYIESRLLHTEYTLLGTADKNSFVKK 350
HI10                        ----------------------FYY-E-R--------------------
tr|Q74J38|Q74J38_LACJO      RDLENLDKIPVYKNPQDFKADLITIKESLEEDHDQAVVRSFFTELLEAID 400
HI10                        --------LPVY-----------------------------ELLE---
tr|Q74J38|Q74J38_LACJO      IFGFHLATIDMRQDSSVNEACVAELLKSAGICDDYSDLPEKDKVKVLLNE 450
HI10                        -------------------------------------------------
tr|Q74J38|Q74J38_LACJO      LNNDPRNLHANNKPKSELLQKELNIYKTARQLKDCIGEDVIKQHIISHTE 500
HI10                        ------NLHA-----------------------EDVL----------
tr|Q74J38|Q74J38_LACJO      SVSDLLEQAIMLKEYDLLNNQGARI 525
HI10                        -------QALMLKSG---------- 8
```

Fig. 54 Identification of HI10, which was strongly induced by hop stress by *L. brevis* TMW 1.465A using nanospray sequencing ESI MS/MS. The spectra were manually interpreted by Lars Israel and led to the identification of HI10 as pyruvate carboxylase. The alignment with tr|Q74J38|Q74J38_LACJO phosphoenolpyruvate carboxylase (*L. johnsonii*) is shown.
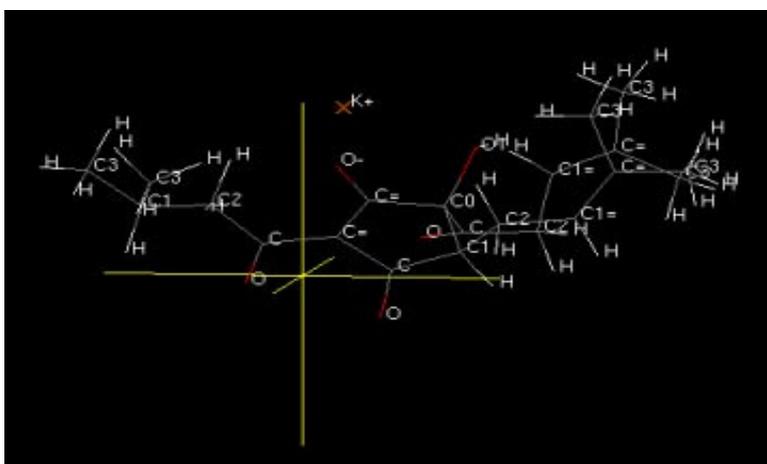
Fig. 55 Cooperative binding of transisohumulone to K^+ and Mn^{2+} (yellow cross) calculated with GRID (http://www.moldiscovery.com).
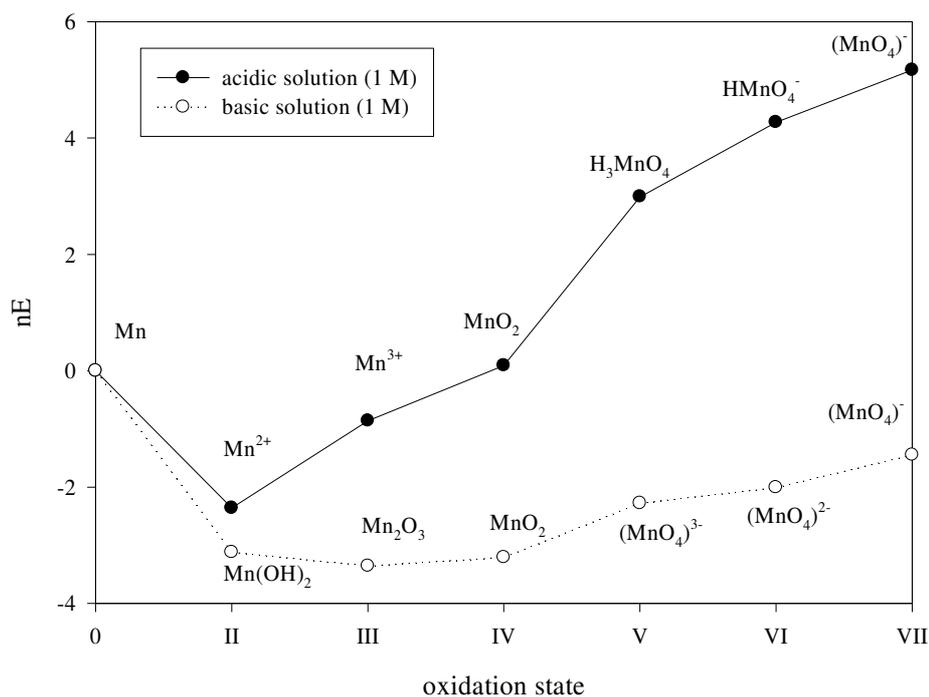
Fig. 56 Redox chemistry of manganese in dependence of pH value as obtained from the Frost diagrams (free energies (nE) versus oxidation state, Shriver, Atkins et al. 1997-09) constructed for acidic and basic solutions (1 M each).
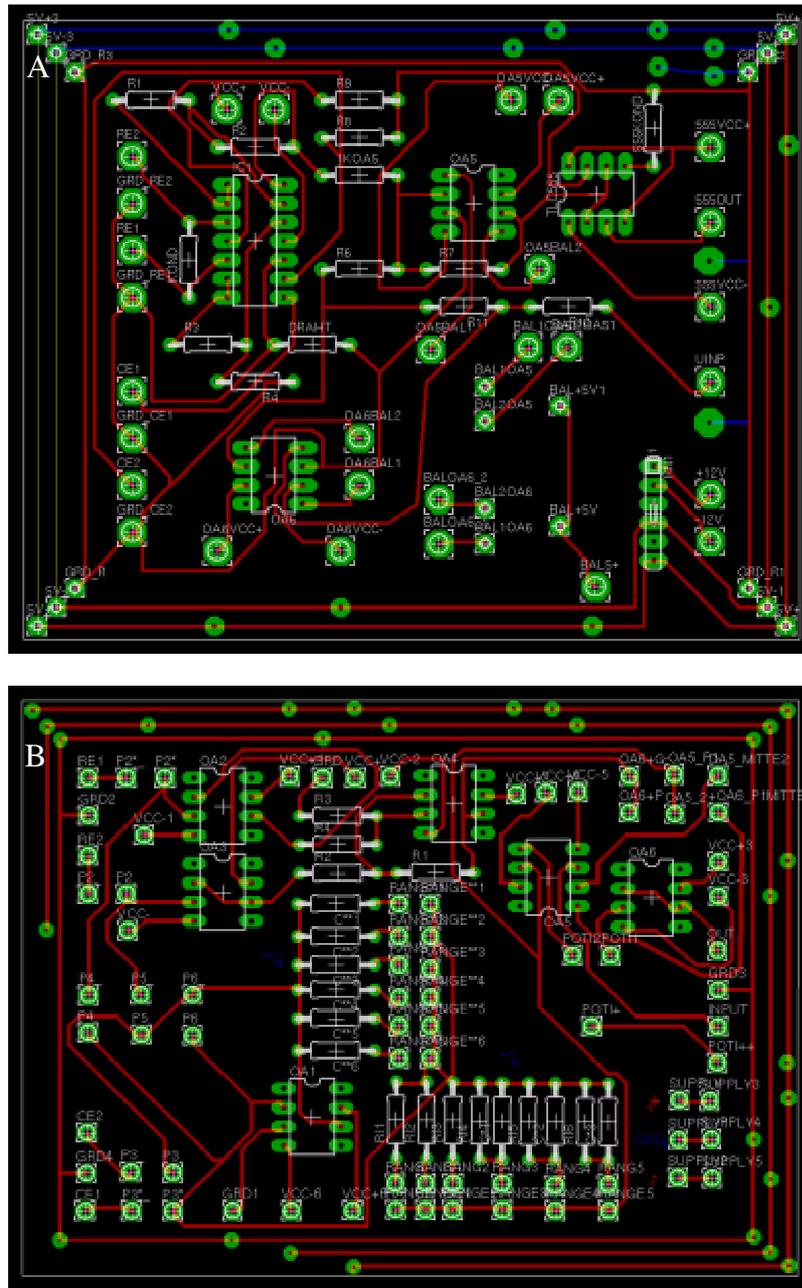
Fig. 57 Circuit board layouts for the capacitance measurement system (A) and potentiostat-galvanostat (B) were constructed with eagle (http://www.cadsoft.de). For choice of electronic parts consult Kalinowski et al. (Kalinowski and Figaszewski 1995; Kalinowski and Figaszewski 1995).
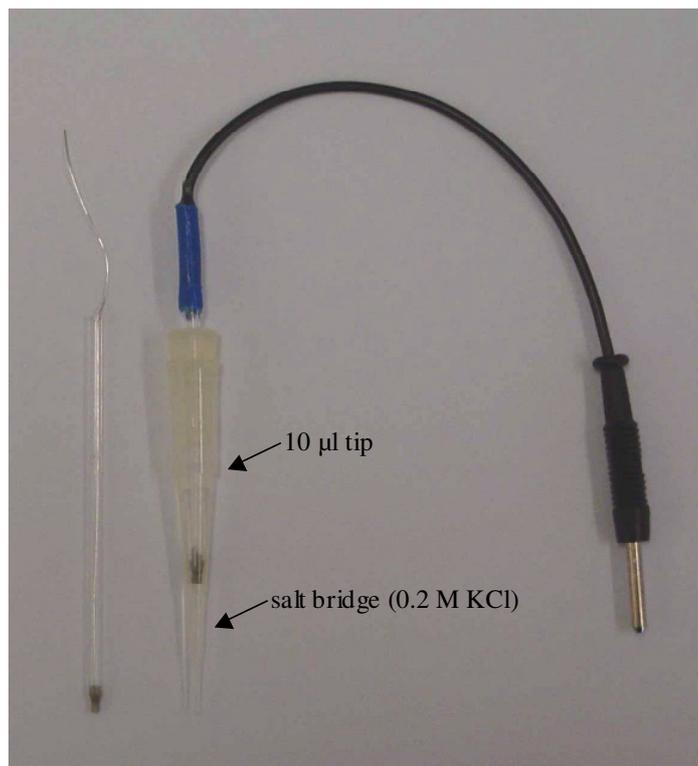
Fig. 58 Constructed Ag/AgCl electrodes for bilayer lipid membrane measurements. Sintered Ag/AgCl electrodes (In Vivo Metric, Healdsburg, CA, U.S.A.) were connected to the electrolyte solutions through 10 µl pipette tips filled with 2 % agarose in 0.2 M KCl.
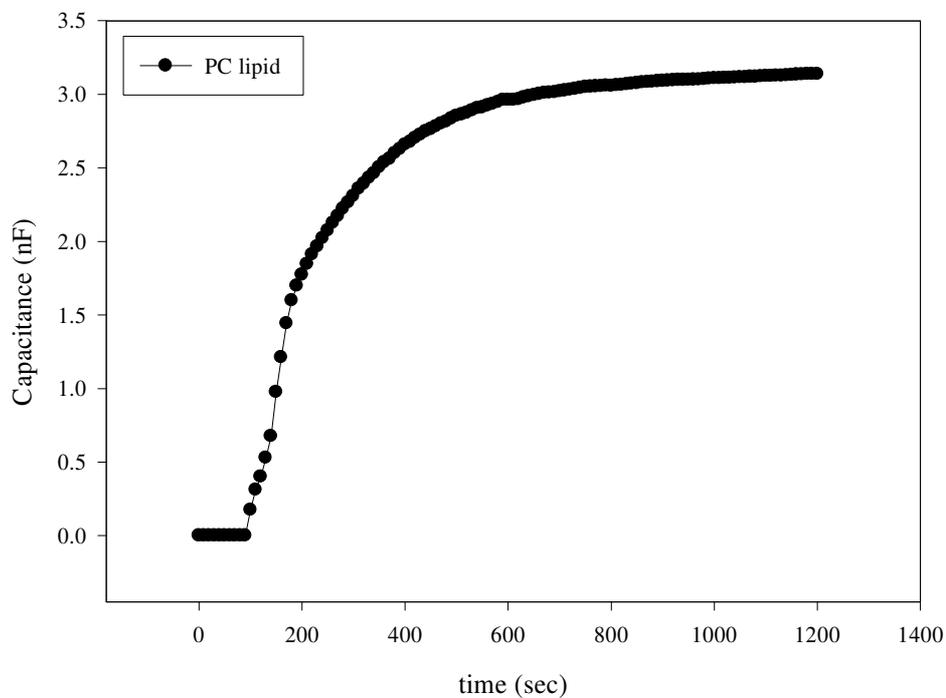
Fig. 59 Bilayer lipid membrane (PC) forming controlled by capacitance measurements. Composition of aqueous solutions: Tris, Mes, KCl (pH 7.0).

Examples for programs created for enhanced protein identification (Lut2MSBLAST.c) and
BLM experiments (triangle.asm):

```
/*------------------------------------------------------------------------

     Filename:       Lut2MSBLAST.c
   Date:             14.02.2008
   File Version:     2.31

   Author:                 Jürgen Behr
   Company:        Technische Mikrobiologie, Technische Untversität München
                        Weihenstephaner Steig 16, D-85350 Freising
 -------------------------------------------------------------------------
   Notes:       Lut2MSBLAST is a program designed to aid in the interpretation
                of CID data of peptides by submitting the peptide sequence data,
                generated by LutefiskXP (Taylor and Johnson 1997) to a MSBLAST
                sequence database search. Up to 1024 LutefiskXP amino acid sequence
                files can be processed automatically. Permutations will be
                generated up to a limit of 8 per amino acid sequence string.
                No warranty is given for correct function of the software. You
                should assure yourself, that the software functions correct
                according to your own criteria.
 -------------------------------------------------------------------------*/


/* ANSI headers */
#include  <sys/dir.h>
#include  <unistd.h>
#include  <stdlib.h>
#include  <string.h>
#include  <stdio.h>
#include  <ctype.h>
#include  <getopt.h>
#include  <sys/types.h>
#include  <dirent.h>
#include  <sys/stat.h>
#include  <time.h>
#include  <math.h>


/* -------------------------------------------------------------------------
//  swap AA                          used to exchange amino acid (AA) positions
*/
void swap(char *a, char *b)
{
     char c;

      c=*a;
     *a=*b;
     *b=c;
}


/* -------------------------------------------------------------------------
//  my_fgets                    written by Taylor et al. (Taylor and Johnson 1997)
*/
char *my_fgets(char *s, long n, FILE *fp)
{
  register char *t = s;
  register long c;

  if (n < 1)
    return(NULL);

  while (--n) {
    if ((c = getc(fp)) < 0) {
      if (feof(fp) && t != s) break;
      return(NULL);
    }

    *t++ = c;
    if (c == '\n' || c == '\32') break;
    /* This is to handle stupid windows files that end each line with \r\n */
    else if (c == '\r') {
```

```c
    if ((c = getc(fp)) < 0) {
      if (feof(fp) && t != s) break;
      return(NULL);
    }
    if (c != '\n') {
      /* Roll back the file pointer or we will miss characters. */
      fseek(fp, -1, SEEK_CUR);
    }
    break;
    }
  }

  *t = '\0';

  return(s);
}

/* -------------------------------------------------------------------------
//  usage
*/
void *usage(void)
{
    printf("\nUSAGE: Lut2MSBLAST LutefiskXP output directory pathname [- options] \n\n");
    printf("       [-o]  Output file name\n");
    printf("       [-h]  prints the usage\n\n");
    fflush(stdout);
return(0);
}

/* -------------------------------------------------------------------------
//  main
*/

int main(int argc, char *argv[])
{
// variables
    register int option=0;                      // used to work through comand line options
    FILE *streami,*streamo;                      // input and output streams
    DIR  *dirp;                                      // pointer to LutefiskXP output
directory
    char lutdir[256];                            // LutefiskXP output directory name
    char output_filename[256];                   // output file name
    char name_extensions[256];                   // used to generate output file name

    char line_in[512];                           // AA sequence data from LutefiskXP output
from myfgets function
    int line_in_pos=0;                           // position in string line_in
    char line_proc[512];                         // intermediate (processed) AA sequence data
    int line_proc_pos=0;                         // position in string line_proc
    char line_out[512];                          // AA sequence output data
    int line_out_pos=0;                          // position in string line_out

    int swap_option=0;                           // swap_option = 1 if swaps are required
    int swaps=0;                                 // variable to hold the number of required
AA swaps
    int swap_pos[256]={0};                       // array to hold the position of the AA to
swap
    int swap_pos_nr=0;                           // counter to walk through this array
    int swap_nr=0;                               // counter to generate permutations

    char permutation[256][256]={""};        // array to hold permutations
    int permutation_nr=0;                        // counter to walk through this array

    int clear_array=0;                           // counter to clear arrays

    int o_option =0;                             // o_option = 1 if a output file name is
specified

    struct dirent *dir_entry, **namelist;   // used to access input files
    struct Argument                                 // used to hold input file names
    {
        char       arg_lut[256];


    }tParam[1024];
    int num_entries=0;                               // number of files in LutefiskXP output
folder
    int entry_nr=0;                                   // counter to walk through file names
```

```
    int b=0, j=0;                                 // counter to generate file paths


    int file_counter;                            // counter to walk through files in
LutefiskXP output folder
    int a;                                        // used to select a case
    const time_t thetime=(const time_t)time(NULL);


/* -----------------------------------------------------------------------
//  options                      get command line options
*/
    while((option=getopt(argc, argv,"ho:"))!= -1)
    {
    switch(option)
        {
            case'o':
                /*output file name*/
                strncpy(output_filename, optarg, sizeof(output_filename));
                o_option=1;
                break;
            case'h':
                /*usage*/
                usage();
                exit(1);
                break;
            default:
                usage();
                exit(1);
                break;
        }
    }

/* -----------------------------------------------------------------------
// organize input and output
*/
/*check number of command line arguments and get LutefiskXP output files directory*/
    if(argc<2)
    {
    usage();
    exit(1);
    }
    else
        strcpy(lutdir, argv[1]);

    if(o_option!=1)
    {
    strcpy(output_filename, lutdir);
    strcat(output_filename, "_out.fasta");
    }

/*transfer LutefiskXP output file names in array*/
    if ((dirp = opendir(lutdir)) == NULL)
    {
    printf("\nCould not find LutefiskXP output directory!\n");
    exit(1);
    }
    if ((num_entries=scandir(lutdir, &namelist, 0, 0)) < 0)
    {
    printf("\nScandir error!\n");
    exit(1);
    }

/*generate name_extensions to access LutefiskXP output files*/
    strcpy(name_extensions, lutdir);
    strcat(name_extensions, "/");
    for(entry_nr=0; entry_nr<num_entries-2; entry_nr++)
    {
    strcpy(tParam[entry_nr].arg_lut, name_extensions);
    }

    while ((dir_entry = readdir(dirp)) != NULL)
    {
    //printf("file: %s \n", dir_entry->d_name);
        if (b>=2)
        {
        strcat(tParam[j].arg_lut, dir_entry->d_name);
        //printf("\n%s\n", tParam[a].arg_lut);
```

```
            j++;
            }
      b++;
      }
      closedir(dirp);
      free(namelist);

/*generate output stream*/
      if((streamo = fopen(output_filename,"a")) == NULL) { /*9_open*/
                printf("\nCould not open output file!\n");
                exit(1);
                } /*9_close*/

/*generate unique fasta header*/
      fprintf(streamo,">%s    %20s",output_filename,ctime(&thetime));
      fflush(streamo);

/*generate input stream*/
      for(file_counter=0; file_counter<num_entries-2; file_counter++)
      { /*3_open*/
      /*printf("\nProcessing Lutefisk datafile: %s\n",tParam[file_counter].arg_lut);*/

          if((streami = fopen(tParam[file_counter].arg_lut,"r")) == NULL)
          { /*4_open*/
          printf("\nCould not open:%s!\n",tParam[file_counter].arg_lut);
          usage();
          exit(1);
          } /*4_close*/
/* next 10 lines were adopted from Taylor et al. (Taylor and Johnson 1997)*/
          while(my_fgets(line_in,sizeof(line_in),streami) != NULL)
          { /*5_open*/

              if (strstr(line_in,"Sequence") && strstr(line_in,"Rank"))
              { /*6_open*/

                  while(my_fgets(line_in,sizeof(line_in),streami) != NULL)
                  { /*7_open*/
    // Stop when we hit a blank line.
                      if (line_in[0] == ' ' || line_in[0] == '\n' || line_in[0] == '\r')
break;

    /* ----------------------------------------------------------------------
    /* process sequence              remove digits and replace by XX, remove "]" from AA
sequence and insert "-" after the AA sequence (tells MSBLAST where a sequence ends and a new
starts)*/
    */
      line_in_pos=0;
      line_proc_pos=0;
      while (line_in[line_in_pos])
      {/*8_open*/
      //printf("line_in_pos after while:%i\n",line_in_pos);
      //printf("line_proc_pos after while:%i\n",line_proc_pos);
          if ((line_in[line_in_pos] == '[' && isdigit(line_in[line_in_pos+1]) !=0) ||
line_in[line_in_pos] == ']' || isdigit(line_in[line_in_pos]))
          {
          a=1;
          }
          if (line_in[line_in_pos] == '.')
          {
          a=2;
          }
          if (line_in[line_in_pos] == ' ') {
          a=3;
          }
          switch(a)
          {
          case 1:
          line_in_pos++;
          a=0;
          //printf("Case 1 executed!\n");
          break;
          case 2:
          line_proc[line_proc_pos]='X';
          line_proc_pos++;
          line_proc[line_proc_pos]='X';
          line_proc_pos++;
          line_in_pos++;
          a=0;
```

```
            //printf("Case 2 executed!\n");
            break;
            case 3:
            line_proc[line_proc_pos]='-';
            line_proc_pos++;
            line_proc[line_proc_pos]='\0';
            line_in_pos++;
            line_in[line_in_pos]='\0';
            a=0;
            //printf("Case 3 executed!\n");
            break;
            default:
            line_proc[line_proc_pos]=line_in[line_in_pos];
            line_proc_pos++;
            line_in_pos++;
            //printf("Default executed!\n");
            break;
            }
        //printf("line_in_pos after switch:%i\n",line_in_pos);
        //printf("line_proc_pos after switch:%i\n",line_proc_pos);
        } /*8_close*/

/* ------------------------------------------------------------------
// prepare for sequence permutations
*/
/*check number and position of AA to swap*/
        for(line_proc_pos=0; line_proc_pos<((strlen(line_proc)-2)); line_proc_pos++)
        {
        if (line_proc[line_proc_pos] =='[' && isalpha(line_proc[line_proc_pos+1]) != 0)
            {
            swap_pos[swaps]=(line_proc_pos-swaps);
            swaps++;
            swap_option=1;
            }
        }
        /*fprintf(stdout, "\nline_proc:%s", line_proc);*/

/*remove remaining "[" from AA sequence*/
        line_proc_pos = 0;
        line_out_pos = 0;
        while (line_proc[line_proc_pos])
        {
            if (line_proc[line_proc_pos] == '[' )
            {
            line_proc_pos++;
            }
            else {
            line_out[line_out_pos]=line_proc[line_proc_pos];
            line_proc_pos++;
            line_out_pos++;
                }
        }

        line_out[line_out_pos]='\0';
        /*fprintf(stdout, "\nline_out befor permutation:%s", line_out);*/

/*swap AA if required and write AA sequences into output file*/
        if(swap_option==1 && swaps < 9)
        {
            strcpy(permutation[0], line_out);
            for (swap_nr=0; swap_nr<swaps; swap_nr++)
            {
                for (permutation_nr=0; permutation_nr<=(int)ldexp(1, swap_nr);
permutation_nr++)
                {
                strcpy(permutation[permutation_nr+(int)ldexp(1, swap_nr)],
permutation[permutation_nr]);
                swap(&(permutation[permutation_nr+(int)ldexp(1,
swap_nr)][swap_pos[swap_pos_nr]]), &(permutation[permutation_nr+(int)ldexp(1,
swap_nr)][swap_pos[swap_pos_nr]+1]));
                }
            swap_pos_nr++;
            permutation_nr=0;
            }
            for (permutation_nr=0; permutation_nr<(ldexp(1, swaps)); permutation_nr++)
            {
            fprintf(streamo, "%s", permutation[permutation_nr]);
            fflush(streamo);
```

```
            }
        }
        else
        {
        fprintf(streamo,"%s",line_out);
        }

/*clear variables and arrays for next sequence:*/
        strcpy(line_in,"");
        strcpy(line_proc,"");
        strcpy(line_out,"");
        line_in_pos=0;
        line_proc_pos=0;
        line_out_pos=0;

        for (clear_array=0; clear_array<256; clear_array++)
        {
        strcpy(permutation[clear_array], "");
        swap_pos[clear_array]=0;
        }
        permutation_nr=0;
        swap_pos_nr=0;

        swap_option=0;
        swaps=0;
        clear_array=0;

                        } /*7_close*/
                    } /*6_close*/
            } /*5_close*/
            fclose(streami);
        } /*3_close*/
        fflush(streamo);
        fclose(streamo);
        printf("\nLut2MSBLAST finished!\n\n");
/*everything done*/
        exit(0);
}




;***********************************************************************
;
;   Filename:       triangle.asm
;   Date:           09.03.2007
;   File Version:   1
;
;   Author:             Jürgen Behr
;   Company:        Technische Mikrobiologie, Technische Universität München
;                           Weihenstephaner Steig 16, D-85350 Freising
;
;
;***********************************************************************
;
;   Files required:   p16F690.inc
;                     myheader.inc
;                     16f690.lkr
;
;***********************************************************************
;
;   Notes:      Controller: PIC 16F690, Microchip Technology Inc., Chandler,
;               Arizona, USA. Counts up to 255 and down to 0 and displays on
;               PortC. Use with R/2R converter as 8-bit resolution DAC
;               generates a triangular voltage sweep.
;
;***********************************************************************
;
;
;

        list      p=16f690      ; list directive to define processor
        #include <C:\MPprojects\Linker_Header\p16F690.inc>
        #include <C:\MPprojects\Linker_Header\myheader.inc>

        __config (_INTRC_OSC_NOCLKOUT & _WDT_OFF & _PWRTE_OFF & _MCLRE_OFF & _CP_OFF & _BOR_OFF &
_IESO_OFF & _FCMEN_OFF)
;***********************************************************************
        cblock 0x20
```

```
Delay1          ; assign an address to label Delay1
Delay2          ; assign an address to label Delay2
Delay3          ; assign an address to label Delay3
Display         ; define a variable to hold the display
    endc
;***********************************************************************
    org 0x00            ; processor reset vector
    goto    Start       ; go to beginning of program
    org  0x04           ; interrupt vector location
    ; context saving and isr code can go here or be located as a call
    subroutine elsewhere
;***********************************************************************
    org 0x15
Start
    Init_PortC_D_O      ; ADC PORTC off, make IO PortC all output
InitLoop_up
    movlw    .128       ; WREG = 128
    movwf    Display        ; Display = 0
    movwf    PORTC          ; copy the display PORTC
;***********************************************************************
Startdelay
    call DelayLoop ; waste time.
    decfsz   Delay3,f  ; waste time.
    goto     Startdelay    ; waste time.
;***********************************************************************
Loop_up                     ; DAC output voltage increases from 0 to 5 V
    movf Display,w ; copy the display WREG
    movwf    PORTC          ; copy the display PORTC
    call     DelayLoop ; wait
    incf Display       ; increment display for the next cycle
    btfss    STATUS,Z  ; test if display is .0 (avoid voltage jump)
    goto     Loop_up       ; next cycle
;***********************************************************************
InitLoop_down
    movlw    0xFF
    movwf    Display        ; copy 255 to the display (avoid voltage jump)
;***********************************************************************
Loop_down               ; DAC output voltage decreases from 5 to 0 V
    movf Display,w ; copy the display WREG
    movwf    PORTC          ; copy the display PORTC
    call     DelayLoop ; wait
    decf Display       ; decrement display for the next cycle
    btfss    STATUS,Z  ; test if display is .0 (avoid voltage jump)
    goto     Loop_down ; next cycle if display =! .0
    goto     Loop_up       ; else Loop_up again

DelayLoop
    decfsz   Delay1,f  ; waste time.
    goto DelayLoop ; the Inner loop takes 3 instructions per loop * 256
                            loops = 768 instructions
    decfsz   Delay2,f  ; the outer loop takes and additional 3 instructions
                            per lap * 256 loops
    goto DelayLoop ; (768+3) * 256 = 197376 instructions / 1M
                            instructions per second = 0.197 sec.
    return              ; call it a two-tenths of a second.
    end
;***********************************************************************
```

List of publications that resulted from this dissertation:

Original papers

Behr, J., Gänzle, M.G., and Vogel, R.F. 2006. Characterization of a highly hop-resistant *Lactobacillus brevis* strain lacking hop transport. Appl Environ Microbiol **72**: 6483-6492.

Behr, J., Israel, L., Gänzle, M.G., and Vogel, R.F. 2007. A proteome approach for characterization of hop inducible proteins in *Lactobacillus brevis*. Appl Environ Microbiol. **73** : 3300-3306.

Oral presentations

Behr, J., Gänzle, M.G., and Vogel, R.F. 2003. A proteome approach for detection of hop inducible proteins involved in hop resistance of beer spoiling lactobacilli. In European Brewery Convention. Dublin.

Behr, J., Gänzle, M.G., and Vogel, R.F. 2004. A Proteome Approach for Detection and Characterization of Hop Inducible Proteins Involved in Hop Resistance of Beer-Spoiling Lactobacilli. In World Brewing Congress. San Diego.

Behr, J., and Vogel, R.F. 2007. Role of manganese in beer spoilage - a revised view of hop resistance. In European Brewery Convention. Venice.

Poster Presentations

Behr, J., Gänzle, M.G., and Vogel, R.F. 2003. Nachweis bierverderbender Milchsäurebakterien: spezifische, PCR-gestützte Detektion auf Basis der Hopfenresistenz. In Technologisches Seminar für das Brauwesen. Weihenstephan, Freising.

Behr, J., Gänzle, M.G., and Vogel, R.F. 2004. High-Pressure Inactivation of Beer-Spoiling Lactobacilli. In World Brewing Congress. San Diego.

Behr, J., Israel, L., and Vogel, R.F. 2005. A proteome approach for characterisation of bacterial resistance to hop ionophores in *Lactobacillus brevis*. HUPO 4th Annual World Congress. Munich.

Jürgen Behr

Geburtsdatum:	25. April1974
Geburtsort:	Kulmbach
Aktuelle Adresse:	Hittostr. 14, 85354 Freising
Heimatanschrift:	Hutschwinkel 13, 95326 Kulmbach

15.09.80 - 30.06.84	Volksschule, Kulmbach
15.09.84 - 30.06.93	Markgraf-Georg-Friedrich-Gymnasium, Kulmbach
	Abschluß : Abitur, erreichen der Hochschulreife
1.07.93 - 31.08.94	Zivildienst, Bayerisches Rotes Kreuz, Kulmbach
ab 1.11.94	Weihenstephan, Technische Universität München,
	Studiengang Technologie und Biotechnologie der Lebensmittel
	Betriebspraktika: Brauerei Haberstumpf, Molkerei Bayreuth,
	Brauerei Kulmbacher
	Studentische Hilfskraft, Lehrstuhl für Lebensmittelverfahrens-
	technik und Molkereitechnologie
10.11.98	Diplom-Vorprüfung im Studiengang Technologie und Bio-
	technologie der Lebensmittel
	Diplomarbeit: Hochdruckinaktivierung bierschädlicher
	Milchsäurebakterien, Lehrstuhl für Technische Mikrobiologie
24.07.02	Studienabschluß als Dipl.-Ing. Technologie und Biotechnologie
	der Lebensmittel, (Note: 1,9)
ab 1.09.02	Beginn der Dissertation am Lehrstuhl für Technische
	Mikrobiologie, Technische Universität München