Osmotic regulation of transport processes in Lactobacillus plantarum
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General Introduction
1. Introduction

Biological membranes are readily permeable for water molecules, but present an effective barrier for most other solutes. The total concentrations of osmotically active solutes (osmolytes) inside a cell are usually higher than those of the environment, resulting in diffusion of water from the extracellular medium to the cytoplasm. The flux of water down its concentration gradient increases the cellular volume, until the cytoplasmic membrane gets packed against the peptidoglycan of the cell wall. Additional influx of water results in turgor pressure that is exerted on the peptidoglycan layer. At equilibrium the difference in external and internal water concentration is balanced by the turgor pressure. Turgor pressure provides the mechanical force for the expansion of the cell wall and is critical for bacterial growth. In fact, optimal growth rates are only achieved when the cytoplasmic concentration of osmolytes is between certain limits that are well above the osmolarity of the extracellular medium.

Upon an increase of the medium osmolarity water diffuses out of the cells causing the turgor pressure and, ultimately, the volume of the cells to decrease, thereby altering the concentrations of all cytoplasmic solutes. Upon a decrease in the medium osmolarity water moves into the cells, which increases the high turgor pressure, through which the cells may eventually burst. The cells counteract those processes (maintain osmostasis) by the accumulation (uptake and/or synthesis) or efflux of specific solutes. These molecules have been termed compatible solutes, because they can be accumulated to high cytoplasmic concentrations without affecting life (i.e., vital cellular processes). According to this definition potassium ions are considered to be compatible solutes.

Osmoprotectants are solutes that increase the growth rate of osmotically stressed bacteria when they are added to the medium. This definition is rather vague, because the stimulatory effects of such solutes are not always purely osmotic. Alternatively, the addition of a solute to the medium may have no growth stimulating effect, because the medium already contains specific solutes, that are (preferentially) accumulated, thereby protecting the cells to osmotic stress. The confusion concerning osmoprotectants is complete when one takes into account that many researchers use this term for any solute that is accumulated by an organism. The definition of osmoprotectants also comprises exogenous solutes that might not be directly responsible for the protection. An example is choline, which is taken up from the medium and is internally converted into glycine betaine in a two-step oxidation reaction in many Gram-positive and Gram-negative bacteria, resulting in a stimulation of the growth rate.
2. Osmolarity of solutions

Osmolality describes the osmotic pressure (\(\Pi\)) of a solution in osmoles of osmolytes per kg solvent. One osmolal is defined as a solution having an osmotic pressure equal to \(RT\), where \(T\) is the temperature in Kelvin and \(R\) is the universal gas constant (Dick, 1959). In stead of osmolality, people often use the term *osmolarity* to describe the osmotic pressure of a solution. As the quotient \(\Pi/RT\) has units of moles of osmolytes per volume of solvent rather than volume of solution (after dissolving the osmolytes into the solvent), it is strictly speaking more appropriate to use the unit osmolal, and thus the term osmolality than osmolar (term osmolarity). Throughout this thesis we use both terms to describe the osmotic pressure of a solution.

In contrast to osmotic pressure, the definition of osmolality is independent of temperature. Note that the *osmole* is not a defined number of osmotically active particles, because the osmotic pressure is not solely determined by the particle number, but also by their size, shape and charge. Changes in osmotic pressure, boiling point, freezing point, and vapour pressure (the four colligative properties of a solution) are due to changes in the system’s chemical potential (defined as \(\Delta G = \Delta H - T \Delta S\); \(\Delta H\) is the change in enthalpy and \(\Delta S\) is the change in entropy). The same definition can be applied to the change in chemical potential of a single component of the system (in this case the symbol \(\Delta \mu\) is used in stead of \(\Delta G\), e.g. the solvent.

When two compartments are separated by a semi-permeable membrane, e.g. the cytoplasmic membrane of a microorganism, the solvent (water) will move down the gradient in chemical potential. The osmotic pressure (\(\Pi\)) is defined as the hydrostatic pressure on the compartment with the lower chemical potential that exactly balances the difference in chemical potential such that no net movement of solvent occurs. In other words, the osmotic pressure corresponds to the hydrostatic pressure when the system reaches equilibrium. Turgor pressure is the actual pressure that is exerted on the peptidoglycan layer due to the differences in chemical potential between the cytoplasm and the external medium. The addition of solutes to a solution lowers the chemical potential of the solvent, mainly because the degree of randomness (\(\Delta S\)) of the solvent increases. Contributions from \(\Delta H\) (nonzero heat of mixing) occur when there are attractive or repulsive forces between solutes and solvent.

Osmolality can be determined via one of the four colligative properties of a solution: the freezing point, the vapour pressure, the boiling point, and the osmotic pressure.
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(Sweeney et al., 1993). Direct measurements of the osmotic pressure are not very practical, because a perfect semi-permeable membrane is difficult to obtain. Boiling point elevation is not suited for unstable solvents, and this property is therefore seldomly used to measure the osmotic pressure. It is best to measure osmolality by freezing point or vapor pressure depression assays, which are quick, easy, and well-suited for small samples. Vapor pressure depression is a technique that can be used for more viscous samples, but not for volatile solutes and samples with an osmolality below 100 mmol/kg. Freezing point depression can be used for aqueous solutions with a relatively low osmolality and viscosity (including volatile solutes). Unfortunately, the mathematical relationship between osmolality and freezing point depression is complex, and not the same for all solutes. It is often simplified to: osmolality (mol/kg) equals $\Delta T/-1.86$, in which $\Delta T$ is the freezing point depression, and the value –1.86 is the cryoscopic constant for water. The value for the cryoscopic constant is only true when the solution is dilute and ideal. Especially in solutions containing electrolytes, e.g. growth media and the cytoplasm, the cryoscopic constant changes in an unpredictable manner with the concentration of the different solutes, and is not a constant. A further complication arises from the fact that osmolality is not a linear function of concentration. Therefore, dilution of samples before measurement of the freezing point depression will result in unpredictable errors as well. Although one should realize the limitations of freezing point depression measurements to estimate osmolality, the overall effects of violation of the thermodynamic assumptions and other mathematical simplifications are usually below 15% for concentrations up to 2 molal. A practical caveat relates to the possible precipitation of solutes by decreasing temperatures.

The osmolality of the cytoplasm at a given external osmolality sets the turgor pressure of bacteria. Methods to estimate the turgor pressure are based upon measurements of the colligative properties of crude cellular extracts, the collapse of internal gas vesicles (via turbidity), or changes in cell volume (via turbidity, or flow cytometry) (Whatmore and Reed, 1990). These measurements indicate that the turgor pressure is usually in the range of 0.8-5 atm for Gram-negative and 15-25 atm for Gram-positive bacteria (Csonka, 1989; Whatmore and Reed, 1990).

3. Rationale for the accumulation of compatible solutes
After a hyper-osmotic shock, cells benefit from the accumulation of compatible solutes by balancing the osmotic disturbance via an increase in cytoplasmic osmolarity. High intracellular concentrations of compatible solutes are common under high osmolality conditions, but it is difficult to derive a quantitative
relationship between the internal concentrations of compatible solutes and the external osmolarity as multiple (macro)molecules are involved. The compatible solutes that are accumulated to high intracellular levels are restricted to a few categories (for reviews see Csonka, 1989; Csonka and Hanson, 1991; Galinski and Trüper, 1994), and they include (i) potassium ions, (ii) amino acids (glutamate, alanine), (iii) amino acid analogues (taurine, N-acetylglutaminylglutamine amide), (iv) methyl-amines and related compounds (glycine betaine, carnitine, ectoines), (v) polyols and sugars (glycerol, sucrose, lactose). In general, compatible solutes should not interact specifically with the (mostly negatively charged) cellular macromolecules, nor should they perturb cytoplasmic solutes via (de)hydratation, precipitation, or any other (charge) interaction. Therefore, in steady state situations most compatible solutes that are present in large amounts in the cytoplasm have no net charge. The accumulation of potassium ions in the initial stages of hyper-osmotic stress in enteric bacteria is usually only transient. Following the accumulation of potassium ions, other compatible solutes are synthesized (e.g. trehalose), and the uptake systems for glycine betaine and proline are induced. The accumulation of these solutes replaces potassium in time (see also Poolman and Glaasker, 1998). An exception to this rule may be the strategy that is used by thermophilic organisms (Bacteria and Archaea), in which negatively charged compounds seem to be accumulated in response to hyper-osmotic stress. These compounds include mannosylglycerate, glutamate, cyclic-2,3-bisphosphoglycerate, 1,3,4,6-tetracarboxyhexane, and myo-inositol-phosphate derivates (Martins et al., 1996; Lai et al., 1991; Martins and Santos, 1995). Although the experimental evidence is limited, it is tempting to speculate that negatively charged compounds play a role in thermostabilization as well (Rishi et al., 1998).

The non-perturbing characteristics of uncharged compatible solutes from mesophilic organisms have been compared to a series of ions, empirically found by Hofmeister, that stabilize the native state of proteins (Yancey et al., 1982). Stabilizing anions are fluoride, phosphate, sulfate, and acetate, whereas stabilizing cations are tetra-methyl-ammonium, di-methyl-ammonium, and ammonium. Methylamines, like glycine betaine, resemble quaternary ammonium ions, the most stabilizing cations of the Hofmeister series. The destabilizing ions of the Hofmeister series are readily appreciated, at least for compounds such as urea, arginine, and guanidiniumchloride, as they are known to disrupt protein conformations and are often used to unfold proteins. Several in vitro studies have revealed the stabilizing effects of glycine betaine (up to 3 M) on proteins in solution (Arakawa and Timasheff, 1985; Winzor et al., 1992, Santorro et al., 1992). Also studies on the stability of membrane bilayers (Rudolph et al., 1986) and retention of enzymatic activity indicate that
glycine betaine has a stabilizing effect (Monteolivea-Sanchez et al., 1993; Petronini et al., 1993). In vivo studies have indicated that glycine betaine increases the survival of L. plantarum subjected to drying, and enhances the growth rate of cold-stressed Listeria monocytogenes (Kets and de Bont, 1994; Ko et al., 1994).

4. Osmosensors
Most osmotically controlled uptake systems are regulated both at the genetic (induction of the genes) and the enzymatic level (direct “activation” of the transport protein). The degree of induction can vary considerably (from 2 up to 500-fold), whereas activation of the carrier protein by an osmotic upshock is usually in the range of 5 to 35-fold. Both genetic and enzymatic regulation depend on some kind of osmotic sensor that is able to register (and propagate) changes in external osmolarity or osmotic imbalance. The signal needs to be transduced from this osmotic sensor and elicit a conformational change that (in)activates the target protein. In this section, osmosensing, a prerequisite for activation and induction of osmotically controlled transport proteins, is introduced.

Osmosensing is rather well-understood for the integral membrane protein KdpD of E. coli, a sensory kinase with four transmembrane segments that regulates transcription of the kdpFABC operon via the response regulator kdpE (Sugiura et al., 1994); kdpFABC encodes the high affinity potassium uptake system of E. coli. The integral membrane protein KdpD responds both to high internal concentrations of potassium (negative K\(^{+}\) signal), and to a hyperosmotic shock (positive osmotic signal). The latter results in autophosphorylation of KdpD and subsequent transfer of the phosphoryl group to the response regulator KdpE. Purified and reconstituted KdpD exhibited autophosphorylation and the phosphoryl group could be transferred to KdpE (Jung et al., 1997). To identify the regions involved in potassium sensing, a set of 23 independent KdpD mutants was isolated by random mutagenesis on a plasmid carrying the kdpD gene. Selection for mutants was performed on the basis of a high beta-galactosidase activity of the kdp-lacZ fusion gene in the presence of high concentrations of potassium. These mutants had single amino acid substitutions in transmembrane segments 3 and 4 (TMS 3 and 4), as well as in a positively charged internally located region that is flanking TMS4 at the carboxyl-terminus. All mutants still responded positively to a hyper-osmotic shock, but were defective in their response to the K\(^{+}\) signal. In both mutants and wild type, the transcription of the kdpFABC operon was increased by amphipaths, such as chlorpromazine and procaine that insert into the phospholipid bilayer. The responsiveness to amphipaths as well as osmolality changes suggests that alterations in membrane stretch or
tension form an important aspect of the osmosensing of KdpD (Sugiura et al., 1994). Changes in membrane stretch or tension could be transmitted to the TMSs and thereby alter the autophosphorylation or phosphoryl transfer activity of KdpD. Our knowledge of osmoregulated transport systems in bacteria and their mechanisms of osmosensing is described in chapter 2.

5. Osmotic regulation of gene expression
Osmoregulation, in particular with regard to the genetics, has been studied most thoroughly in E. coli, especially for the ProU glycine betaine and proline uptake system and the Kdp systems (see above). An osmotic upshift results in a transient, but instantaneous increase in transcription of the kdp operon. The expression returned to its basal level 30 min after the upshock. The phosphorylated form of the KdpE protein is postulated to interact with the promoter region of kdpFABC, thereby stimulating transcriptions from the kdp promoter (Epstein, 1986). In this perspective KdpE can be regarded as a specific transcription factor that increases transcription of the kdpFABC operon upon phosphorylation by KdpD. As the (auto)phosphorylation of KdpD increases in response to membrane stretch or tension, the system is affected directly by osmotic stress. In contrast to regulation of the kdp operon, no specific transcription factors are known for the regulation of transcription of the proU operon.

The kinetics of induction of the proU and proP operons were found to be similar; they involve a lag phase of 15 to 20 min, followed by a rapid increase in expression, and, subsequently, a slow decay in the expression rate (Jovanovich et al., 1988; Laimins et al., 1991). This genetic regulation is slow in comparison to the regulation of the kdp operon and the enzymatic activation of transport systems, which occur within seconds following a change in osmolality. Therefore, the increased expression of proU could be mediated by signaling molecules (second messengers) that have to build up in the cytoplasm, rather than by direct activation of signal transduction pathways, such as the osmotically regulated two-component system that affects the expression of the kdp operon. Indeed, some of the data on the expression regulation of proU point in this direction. Several earlier reports indicated that potassium-glutamate is (at least partially) responsible for the induction of proU (Ramirez et al., 1989; Booth 1992), but other studies rejected these claims (Csonka et al., 1994; Jovanovich et al., 1989). There are several mechanisms by which potassium is thought to affect the transcription of proU. The first mechanism involves de-repression of the inhibition of transcription by diminishing the interaction of the nucleoid-associated DNA-binding protein H-NS with regulatory
sites on the DNA sequence (Lucht et al., 1994). Other non-specific interactions involve the influence of cations on polyanionic macromolecules, such as DNA and RNA (Douzou, 1994). A third mechanism involves the activation by potassium of the RNA polymerase responsible for transcription of the proU gene, which is mediated by different sigma factors (Rajkumari et al., 1996; Mellies et al., 1995).

Transcription of proU is effected via the promoters P1 (sigma factor σS) and P2 (sigma factor σ70). During exponential growth, transcription from P2 contributes most to the expression by employing σ70. Sigma factor σS generally contributes to the expression of genes only in the stationary phase of growth, but the transcription of proU is not significantly increased under these conditions. The presence of potassium-glutamate enhances the transcription via σS and σ70, and increases the selectivity of σS for P1 in vitro (Rajkumari et al., 1996). Since specific transcription factors have never been found for proU, the activation of transcription by potassium-glutamate must be elicited by effects on the RNA polymerase, DNA supercoiling, general factors such as H-NS, etc.. Although these are general targets that play a role in the expression of many genes, in case of proU they elicit variations in the levels of expression of almost three-orders of magnitude (Csonka et al., 1994). Although the expression from other, non-osmotically regulated, promoters (lacUV5, glnA) is also increased by potassium, the stimulation of proU promoter activity is 30-fold larger than that of lacUV5 (Jovanovich et al., 1989). In conclusion, although potassium-glutamate seems to enhance the expression of various genes, several groups favour the hypothesis that potassium-glutamate has a special role in the transcription of proU, and it can be considered to be a relatively specific “second messenger” (Rajkumari et al., 1996; Sutherland et al., 1986).

Transcription of proP is effected by two promoters P1 (sigma factor σ70; P1 of proP is homologous with P2 of proU) and P2 (sigma factor σS) in a similar manner as proU transcription. The physiological relevance of the σS-dependent P2 promoter of proP is greater than that of proU as transcription of proP is induced in the stationary phase. Many other osmotically regulated genes, e.g. otsAB, treA, osmB, osmY, and osmC, are also induced by high medium osmolality and stationary phase conditions. Since the promoter regions of proP and proU are similar, one would expect that also proU is able to respond to both growth-phase and osmotic signals, but this has never been observed. Another remarkable difference between proP and proU is that maximal induction levels of proP are approximately 15-fold, whereas proU can be induced almost 500-fold. The cause for this difference resides in the presence of sequences downstream of the proU promoter (cis-element) that prevent proU to be transcribed at low-osmolarity, and that might form target sites for H-NS.
The nucleoid-associated DNA-binding protein H-NS might be gradually released from the cis-element when the cytoplasmic osmolality or potassium-glutamate concentration increases. These regions are absent in the promoter region of proP, and the expression of proP is therefore semi-constitutive (Mellies et al., 1995).

6. Osmotic regulation of uptake systems
Regulation of existing enzymatic and/or transporter activity is rapid and occurs before the cells have adapted to the new situation by changing the composition of the membrane, peptidoglycan, capsule (or synthesize one), and/or cytoplasm. Osmotic regulation of activity has been observed for transport systems of compatible solutes, i.e. those for glycine betaine and related substrates, as well as for potassium uptake (Poolman and Glaasker, 1998). Regulation of activity of ProP of E. coli and a glycine betaine uptake system of Listeria monocytogenes was demonstrated in membrane vesicles (Milner et al., 1988; Gerhardt et al., 1996), which suggests that turgor pressure is not directly involved as membrane vesicles cannot withstand significant pressures. The osmotic activation in membrane vesicles also suggests that second messengers and/or the cell wall are not involved in the activation. Consequently, the most plausible explanation for the regulation is that alterations in external osmolality affect the activity.

The claims that some systems are not regulated at the activity level (Patchett et al., 1994; Gouesbet et al., 1996; Molenaar et al., 1993) should be treated with caution as osmotic regulation is sometimes not easily detected. For instance, activation of the carnitine uptake system of Listeria monocytogenes went unnoticed in the initial experiments. In fact, it is only observed when the cells contain large amounts of preaccumulated solutes such as carnitine and/or glycine betaine (Verheul et al., 1997). Activation is missed when an osmotic downshock is applied during the washing procedure, which releases accumulated solutes. In case of the carnitine uptake system of Listeria monocytogenes, the osmotic regulation involves changes in the degree of inhibition by trans substrate. Osmotic upshock increases the apparent Kₐ and thereby relieves the trans inhibition. This aspect is important in Listeria monocytogenes, Staphylococcus aureus, and Lactobacillus plantarum (Verheul et al., 1997; Pourkomaillian and Booth, 1994, Stimeling et al., 1994, this thesis).

Optimal activation of glycine betaine uptake is species dependent, i.e. activation of the E. coli proteins ProP and ProU is optimal around 0.2 M NaCl (Lucht and Bremer, 1994; Faatz et al., 1988). For more salt-tolerant species the optimal
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osmolarities for activation are considerably larger, i.e. 0.7-0.8 M NaCl for *L. monocytogenes* and *S. aureus* (Koo *et al.*, 1991; Pourkomailian and Booth, 1992). In *L. plantarum* the initial glycine betaine uptake rates increase up to 1.2 M of NaCl or KCl or the equivalent amount of non-ionic osmolytes (Glaasker *et al.*, 1996a).

The glycine betaine uptake systems that have been studied in most detail at the activity level are ProP and ProU of *E. coli* and QacT of *Lactobacillus plantarum*; the properties of QacT are described in the following parts of this thesis. For *Bacillus subtilis* it is known that three systems are present: OpuA, OpuC, and OpuD. Their contributions to glycine betaine uptake in media of high osmolality has been determined (Kempf and Bremer, 1995; Kappes *et al.*, 1996; Kempf *et al.*, 1997). The growth inhibition of the triple mutant (OpuA, OpuC, and OpuD negative) at high osmolarity conditions could not be alleviated by the addition of glycine betaine, and the uptake was completely abolished, suggesting that these systems represent the complete set of glycine betaine uptake systems. The OpuA transport system is semi-constitutive, whereas the OpuC and OpuD systems are induced by osmotic stress. In addition, OpuD is regulated at the activity level. The OpuA and OpuC (sometimes called ProU) uptake systems also transport other quaternary ammonium compounds (carnitine and choline), and belong to the ABC-superfamily. They consist of an ATP binding protein (OpuAA and OpuCA), an integral membrane protein (OpuAB and OpuCB), and an external substrate binding protein (OpuAC and OpuCC). The latter is anchored in the cytoplasmic membrane via a lipid modification. OpuD is a highly specific ion motive force-driven uptake system for glycine betaine. Each glycine betaine uptake system has a substrate affinity in the range of 2 to 13 μM both under high and low osmolality conditions. The most important contribution to glycine betaine accumulation (at high and low osmolality) is made by OpuA, which has the highest transport capacity and the best affinity for glycine betaine.

7. Osmotic regulation of efflux systems

Efflux of compatible solutes in response to an osmotic downshock has been observed for the following substrates and organisms: (i) glycine betaine in *Salmonella typhimurium* (Koo *et al.*, 1991), *E. coli* (Lamark *et al.*, 1992), *L. monocytogenes* (Verheul *et al.*, 1997), and *L. plantarum* (Glaasker *et al.*, 1996a), (ii) proline in *S. typhimurium*, *L. plantarum*, and *B. subtilis* (Koo *et al.*, 1991; Glaasker *et al.*, 1996a; Wong *et al.*, 1995), (iii) choline in *E. coli* (Lamark *et al.*, 1992), (iv) carnitine in *L. monocytogenes* (Verheul *et al.*, 1997), (v) glutamate in *Corynebacterium glutamicum* and *E. coli* (Ruffert *et al.*, 1997; Lambert *et al.*, 1997).
1995; Schleyer et al., 1993); a temporary release of glutamate was observed in *L. plantarum* (Glaasker et al., 1996a), (vi) trehalose in *E. coli* (Stryrvold and Strøm, 1991; Schleyer et al., 1993), (vii) potassium in *E. coli* (Meury et al., 1985; Schleyer et al., 1993). Upon osmotic downshock a specific release of several amino acids, that are generally not considered to be compatible solutes, was observed in *B. subtilis* for methionine, histidine, and lysine (Wong et al., 1995) and in *L. plantarum* for alanine (Glaasker et al., 1996a).

The recently described mechanosensitive channels (Sukharev et al., 1994) might play a role in the osmotically regulated exit of compatible solutes, since the efflux is fast, independent of metabolic energy, inhibited by the unspecific channel blocker gadolinium, and triggered by amphipaths (Berrier et al., 1992, Glaasker et al., 1996b, Lambert et al., 1995, Martinac et al., 1990; Ruffert et al., 1997). The best-studied bacterial mechanosensitive channel is MscL from *E. coli*, which has been purified and functionally reconstituted into artificial liposomes indicating that MscL responds to the mechanical strain in the membrane (Häse et al., 1995). Membrane tension increased the open probability of MscL by several orders of magnitude (Sukharev et al., 1994). The 136 amino acid MscL protein contains two transmembrane domains with both the carboxyl- and amino-termini at the cytoplasmic side of the membrane, and it has an open pore diameter of approximately 4 nm (Cruickshank et al., 1997). The nature of the functional MscL complex is most likely homohexameric (Sukharev et al., 1997); however, some studies have indicated that the majority (75%) of the MscL protein has a monomeric organization (Häse et al., 1997). Multimerization of MscL might be the underlying mechanism for the increased open probability of the channel protein in response to membrane strain. The physiological importance of MscL and other stretch-activated channel proteins in *E. coli* has not yet been fully established, but the presence of highly conserved homologues in various Gram-positive and Gram-negative bacteria (Sukharev et al., 1997) suggests that it plays an important role in diminishing turgor pressure upon osmotic downshock (see also chapter 2).

Release of potassium in *E. coli* is mediated by the Kef potassium efflux systems. The KefB and KefC systems are potassium efflux systems gated by glutathione metabolites, but these are not osmotically regulated (Elmore et al., 1990; Meury and Robin, 1990; Ferguson et al., 1995). The KefA protein is thought to be involved in modulation of the activity of osmotically regulated mechanosensitive channels in *E. coli* by prolonging their open time (Cui et al., 1995; Cui and Adler, 1996). One of these channel proteins could be MscL as mutants in this protein exhibit increased loss of potassium and open more easily upon membrane tension elicited by osmotic
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downshifts (Blount et al., 1997).
8. Aim and outline of the thesis
Understanding the fundamental processes of osmotic adaptation in bacteria is of utmost importance, not only from a scientific, but also from an industrial point of view. Bacterial osmoregulatory mechanisms are key targets in the control of the growth of pathogens and spoilage bacteria in foods. In this study, the Gram-positive lactic acid bacterium *Lactobacillus plantarum*, isolated from spoiled dressings, was used as model organism to study osmoregulation. The aim of this thesis was to identify the osmolytes that are accumulated and those that are released as a primary response towards changes in medium osmolality. We also focussed on the elucidation of the mechanisms of osmotic regulation (activation) of uptake and efflux of glycine betaine, the major compatible solute in *Lactobacillus plantarum*.

Chapter 2 gives an overview of bacterial uptake and efflux systems for compatible solutes that are osmotically regulated. Different mechanisms for osmosensing are discussed and, when appropriate, compared with the mechanisms of induction of the corresponding genes. The molecular basis for osmosensing is evaluated. In chapter 3, the osmolytes accumulating in *Lactobacillus plantarum* under salt-stress are identified. The role of glycine betaine uptake and efflux in maintaining the osmotic balance is described. It was also observed that sugar stress elicits a different response than salt stress, i.e. growth of *L. plantarum* was impaired to a lesser extent by sugars than by KCl (or NaCl) when added to equivalent osmolality. Moreover, glycine betaine alleviated growth inhibition caused by salt stress, but did not overcome growth inhibition caused by sugar stress. In chapter 4 it is shown that sugars (lactose and sucrose) only impose a transient osmotic stress, due to the presence of semi-constitutive facilitated diffusion systems with a very high capacity (maximal activity) and unusually high affinity constants for transport. Initially, glycine betaine is accumulated in sugar-stressed cells, but in time internal glycine betaine is replaced by sugar. The osmotic regulation of glycine betaine transport was studied further in chapter 5. The overall and unidirectional rates of glycine betaine uptake and exit were measured at osmostasis, and after osmotic upshock or downshock. A model for the positive and negative regulation of glycine betaine uptake and efflux by changes in osmolality is proposed. In chapter 6 we have begun to explore the molecular basis of the osmotic regulation of glycine betaine uptake in *L. plantarum*. Dehydroproline-resistant mutants defective in glycine betaine uptake (at high and low osmolarity) were isolated, and a kinetic analysis of glycine betaine uptake in wild type and mutant strains is described. Chapter 7 is partly methodological, and deals with the development of new pH sensitive fluorescent dyes to measure the cytoplasmic pH of bacteria under various stress conditions. It also describes the process of excretion of these dyes, following conjugation to
endogenous cellular compounds. Parallels between those excretion mechanisms and
the well-known drug excretion systems in mammalian cells are discussed. Chapter
8 summarizes the most crucial findings of this thesis and their implications. The
perspectives of future studies on osmoregulation are discussed.

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