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Transporters and their roles in LAB cell physiology

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Key words: phosphotransferase system, metabolic capacity, transport system

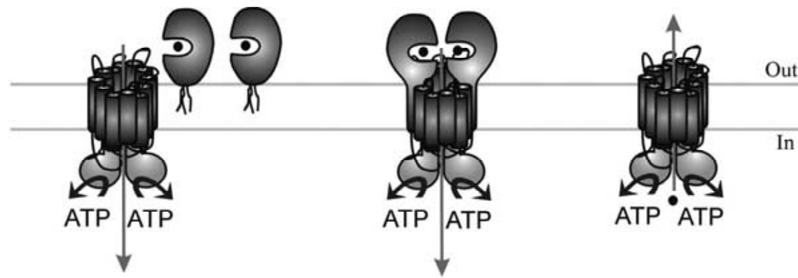
Abstract

For most metabolic pathways, the uptake of the substrate into the cell represents the first step. This transport reaction can exert a large control on the flux through the pathway, in particular when the substrate concentration becomes limiting. Besides serving a role in the uptake of nutrients and the excretion of metabolic (end)products or drugs, transport systems can have one or more other functions in the physiology of the cell. Two of these functions, control of carbohydrate utilization and regulation of cell volume, have been well established in lactic acid bacteria (LAB). The first example concerns the phospho*enol*pyruvate-dependent phosphotransferase system (PTS), which serves a role in the transport of sugars into the cell but also regulates the activity of metabolic pathways, either through regulation of transcription and/or (in)activation of transporters and key enzymes already present. The regulation by the PTS results in a hierarchy in the utilization of sugars and/or adjustment of the first step(s) of a metabolic pathway to the metabolic capacity of the cell and the availability of a particular substrate. The second example relates to the activation of transporters (and mechanosensitive channels), which represents the first mechanism of defence against osmotic stress. The activation by osmotic-upshift of the ATP-binding Cassette (ABC) transporter OpuA from *Lactococcus lactis* is compared with the activation by osmotic-downshift of mechanosensitive channels. The mechanosensitive channels have been best studied in organisms other than LAB, but the presence of similar systems in LAB, and their conservation of structure, suggest that the postulated functions and mechanisms generally hold.

Introduction

The first step in the metabolism of almost any substrate is the transport of the molecule into the cell. In bacteria substrates are taken up by *primary* or *secondary transport* systems or group translocation systems. Primary transporters are driven by ATP, whereas secondary transporters utilize the free energy difference stored in the electrochemical gradient(s) of the translocated solute(s) across the membrane (Poolman & Konings 1993). The by far most abundant class of primary transport systems in lactic acid bacteria (LAB) is that of the ATP-binding cassette transporters, and this type of mechanism is used to accumulate substrates and compatible solutes but also to excrete unwanted products (xenobiotics, drugs) (Figure 1A). Among the secondary transport systems one can distinguish *symporters* (cotransport of two or more solutes), *uniporters* (transport of one molecule) and *antiporters* (countertransport of two or more solutes) (Figure. 1B–D). Symporters usually couple the uphill movement of the substrate to the downhill movement of a proton (or sodium ion), i.e., the electrochemical proton (or sodium ion) gradient drives the accumulation of substrate. Antiporters use the electrochemical ion gradient to excrete a (end)-product, whereas uniporters do not use a coupling ion. Substrate transport by group translocation is restricted to carbohydrates and alditols and involves phospho*enol*pyruvate-dependent phosphotransferase systems (PTSs) (Postma et al. 1993). The PTS catalyzes the uptake of carbohydrate or alditol concomitant with its phosphorylation (Fig. 2).

The phosphoryl group is transferred from phospho*enol*pyruvate (PEP) via the general energy coupling proteins Enzyme I and HPr, and the substrate-



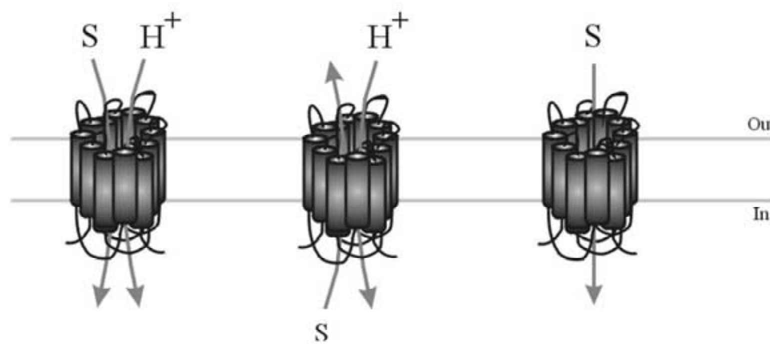


Figure 1. Transport mechanisms. (A) ATP-binding cassette (ABC) transporters for uptake and excretion of solutes. The domain organization of the first, second and third system are that of the oligopeptide transporter Opp, the osmoregulated glycine betaine transporter OpuA from *L. lactis*, and the drug efflux system LmrA, respectively. (B–D). Subdivision of secondary transport mechanisms into symporter (B), antiporter (C) and uniporter (D), respectively. S and H^+ refer to solute and proton, respetively. The coupling ion shown is H^+ (proton motive force-driven uptake or efflux) but in other systems this can be Na^+ (sodium motive force-driven).

specific phosphoryl transfer proteins/domains IIA and IIB. IIB∼P transfers the phosphoryl group to the sugar or alditol that is translocated via the substrate-specific IIC protein/domain. IIA, IIB and IIC can be separate proteins, domains in a single polypeptide or linked as pairs in any possible combination (Robillard & Lolkema 1988; Saier & Reizer 1992; Lengeler et al. 1994).

In this review, the regulatory roles of transporters in the cell physiology of LABs are discussed, with emphasis on the regulation of carbohydrate utilization (Section 2) and cell volume control (Section 3). Although the review focuses on lactic acid bacteria, key findings originally made in other organisms (e.g., *Bacillus subtilis* or *Escherichia coli*) but generally true for low-GC Gram-positive bacteria, or prokaryotes in general, are described for sake of completeness.

Regulation of carbohydrate utilization

Most bacterial cells have the capacity to utilize several carbohydrates as carbon and energy source and possess various transport proteins and catabolic enzymes for the metabolism of the different carbohydrates. In addition, different mechanisms that control the transport and the first steps of metabolism of a particular carbohydrate have evolved. These mechanisms generally result in sequential uptake and metabolism of the available carbohydrates and/or a tuning of the metabolic rate to the needs of the cell. These two regulatory phenomena, hereafter referred to as *hierarchical control* and *autoregulation*, are universal and have been reported for many bacteria. Hierarchical control of carbohydrates has been explained by (i) inhibition of expression of genes encoding enzymes that are in-

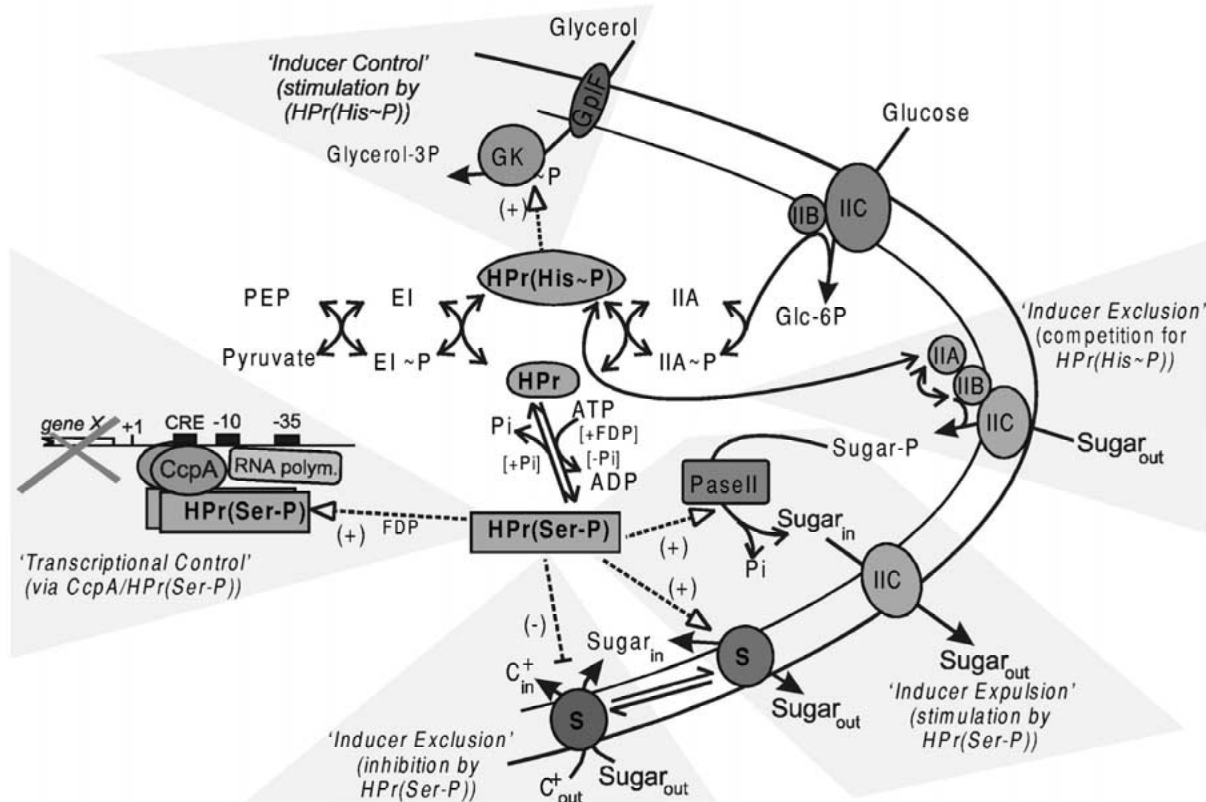


Figure 2. Schematic representation of the phospho*enol*pyruvate:glucose phosphotransferase system of Gram-positive bacteria. The central role of the HPr species in controlling transcription, inducer exclusion, inducer expulsion and inducer control is illustrated. EI, Enzyme I; Glc-6P, glucose-6P; Pase II, sugar phosphatase II CcpA, catabolite control protein A; RNA polym., RNA polymerase; C^{+}, cation; S, secondary transport protein; GK, glycerol kinase; GlpF, glycerol facilitator; CRE, catabolite responsive element; FDP, fructose-1,6-bisphosphate; and P_i, free phosphate.

volved in transport and metabolism of less preferred carbohydrates (Cohn & Horibata 1959); (ii) inhibition of activity of enzymes that effect the uptake or production of the transcriptional inducer (hereafter referred to as *inducer exclusion*; Mcginnis & Paigen 1969; Dills et al. 1980); and (iii) stimulation of efflux of intracellular inducer, that is, the carbohydrate or the phosphorylated derivative (this phenomenon is referred to as *inducer expulsion*, Reizer & Panos 1980; Thompson & Saier 1981; Romano et al. 1987). The inducer exclusion and expulsion mechanisms result in a lowering of the intracellular inducer concentration, and, thereby, indirectly affect gene expression. Autoregulatory control of carbohydrate utilization, on the other hand, occurs via adjustment of the rate of transport of a particular carbohydrate to the rate of its metabolism and the availability of the substrate, hereby providing a feedback or feedforward control to the pathway. Like with hierarchical control, autoregulation involves both control of gene transcription and control of enzyme/transporter activity. The two regulatory mechanisms differ in the sense that autoregulation of carbohydrate utilization controls the catabolic activities within a specific metabolic pathway, whereas hierarchical control involves the metabolic pathway of the preferred carbohydrate as well as that of the less preferred carbohydrate.

The mechanisms underlying the regulation of the initial steps of carbohydrate metabolism have been best studied in Gram-negative enteric bacteria, such as *Escherichia coli* and *Salmonella typhimurium*, and Gram-positive low-GC bacteria, such as *Bacillus subtilis* and several streptococcal, lactococcal and lactobacillus species. It has been established that the PTS system plays a crucial role both in the hierarchical control and auotoregulation of carbohydrate utilization.

How does the PTS exert all these regulatory functions? The PTS is able to sense the availability of

carbohydrates and the metabolic capacity of the cell to metabolize these carbohydrates via the phosphorylation state of the PTS components; key players are the IIA^{Glc} and HPr proteins. The regulatory role of IIA^{glc} is well established in enteric bacteria but this protein is not present in lactic acid bacteria and therefore not further discussed in this review (for a comparison of the regulatory roles of IIA^{glc} and HPr, one is referred to Gunnewijk et al. 2001). Instead, in lactic acid bacteria HPr is most relevant and this protein assumes a role similar to IIA^{Glc} in enteric bacteria. Since the phosphoryl transfer steps in the PTS system are reversible, the addition of a PTS sugar to the cell induces a dephosphorylating signal that is transmitted to the central regulatory protein HPr, either via the sugar-specific IICBA or by rerouting the phosphoryl transfer to other substrate-specific EII complexes, resulting in reduced ratios of HPr(His∼P)/HPr (Fig. 2). On the contrary, in the absence of a PTS substrate, a high [PEP]/[pyruvate] ratio will favor the histidine-phosphorylated state of HPr. Besides being phosphorylated at His-15, HPr in Gram-positive bacteria can also be phosphorylated at Ser-46 in an ATP-dependent protein kinase catalyzed reaction (Deutscher & Saier 1983; Reizer et al. 1984). The reverse reaction, the hydrolysis of HPr(Ser-P), is catalyzed by a cytosolic HPr(Ser-P)phosphatase, which is stimulated by high concentrations of phosphate (Deutscher et al. 1985). HPr(Ser)kinases from several Gram-positive bacteria, including *B. subtilis*, *Streptococcus pyogenes*, *Lactobacillus brevis* and *Lactococcus casei*, are stimulated by early glycolytic intermediates, in particular fructose 1,6-bisphosphate (FDP) (see references cited in Gunnewijk et al. 2001). The HPr kinases from *S. salivarius*, *S. mutans* Ingbritt and *E. faecalis* do not seem to be stimulated by FDP or other glycolytic intermediates, but instead, these enzymes are controlled by the cellular ATP and P_i levels (Brochu et al., 1999, Kravanja et al., 1999). Recent experiments on the HPr(Ser)kinase from *B. subtilis* showed that stimulation by FDP essentially occurred at low ATP and enzyme concentrations, and that positive cooperativity for FDP binding is related to oligomerization of the enzyme (Jault et al., 2000). Overall, the formation of HPr(Ser-P) is proposed to be governed by the relative cellular concentrations of ATP, P_i and/or FDP, which are indicators of the energy status of the cells (Mason et al., 1981; Thompson and Torchia, 1984). This suggestion is supported by measurements of the relative levels of HPr, HPr(Ser-P), HPr(His∼P) and the doubly phosphorylated species HPr(Ser-P/His∼P) in the cell. It has been shown that HPr(Ser-P) is the dominant phosphorylated form of HPr in rapidly growing streptococcal cells, whereas free HPr and HPr(His∼P) are the major species in slowly growing cells (Thevenot et al. 1995; Gunnewijk & Poolman 2000a). HPr(Ser-P/His∼P) is always a minor species (ranging from 5 to 30% of total HPr present in different streptococci) and only present in rapidly growing cells; the physiological function HPr(Ser-P/His∼P) is unknown.

Under *Hierarchical control of carbohydrate utilization*, the role(s) of the different phosphorylated HPr species in the hierarchical utilization of sugars is discussed. The recently postulated role of the PTS in the autoregulation of carbohydrate utilization is described under *Autoregulation of carbohydrate utilization*. The autoregulatory mechanism follows primarily from recent observations made for lactose transport and metabolism in *Streptococcus thermophilus*. This autoregulatory mechanism is unique for its involvement of a IIA-like protein, which is unusual for PTS-mediated regulation in Gram-positive bacteria.

Hierarchical control of carbohydrate utilization

Hierarchical control of carbohydrate utilization was first described by Monod in 1942. Monod demonstrated that on a mixture of carbohydrates the growth of *E. coli* is biphasic as a result of the sequential use of the carbohydrates. For instance, glucose is used first when present in combination with lactose, melibiose, maltose and/or raffinose. The molecular basis for hierarchical carbohydrate utilization is well understood, in particular in enteric bacteria, and is generally referred to as catabolite repression. Catabolite repression is defined as the inhibitory effect of a preferred carbohydrate on the expression of other (catabolic) genes. Catabolite repression also includes inducer exclusion and inducer expulsion, regulatory mechanisms via which the cellular concentrations of inducer are reduced. The inducer exclusion and expulsion mechanisms thus indirectly affect gene expression. Most often the activity of the transport protein is modified such that the uptake of the inducer is prevented or the accumulated inducer is expelled from the cell. In some cases the first step(s) of the metabolism that produce(s) the transcriptional inducer is inhibited. The different mechanisms of inducer exclusion and inducer expulsion are described under *Catabolite repression by inducer exclusion or inducer expulsion*. In the control of gene expression not only 'inducer-specific'

transcription factors, but also 'general' ones are involved. In enteric bacteria, general transcriptional control is mediated by CRP (cAMP receptor protein), which requires cAMP as cofactor. The synthesis of cAMP is catalyzed by adenylate cyclase, which is activated by $IIA^{Glc} \sim P$ and thereby under the control of the PTS. In Gram-positive bacteria, cAMP is not present and CcpA, the equivalent of CRP, is regulated by HPr(Ser-P).

Catabolite repression by inducer exclusion or inducer expulsion

Inducer exclusion is established by different mechanisms in Gram-negative and Gram-positive bacteria, and involves different PTS proteins. In Gram-negative enteric bacteria, inducer exclusion is determined by the phosphorylation state of IIA^{Glc}, a mechanism not to be discussed here. In Gram-positive bacteria, on the other hand, inducer exclusion involves allosteric control of transporters by HPr(Ser-P) or control via HPr(His∼P)-dependent phosphorylation (Figure 2). In *L. brevis*, HPr(Ser-P) has been implicated in the control of uptake of non-PTS carbohydrates such as glucose, lactose and ribose (Ye et al. 1994a,b). Recent studies with *ptsH* and *hprK* mutants in *L. casei* showed that HPr(Ser-P) can also act in inducer exclusion by inhibiting the uptake of the non-PTS carbohydrate maltose (Dossonet et al. 2000; Viana et al. 2000). In some Gram-positive bacteria, like *E. faecalis, E. casseliflavus* and *B. subtilis*, the concentration of the transcriptional inducer of the *glp*-operon is controlled via the activity of glycerol kinase. The enzyme is stimulated via HPr(His∼P)-dependent phosphorylation (Charrier et al. 1997). The phosphorylation of glycerol kinase by HPr is reversible, and the dephosphorylated, less active form of glycerol kinase, is dominant when a PTS-substrate is present in the medium (Deutscher et al. 1993). The net result of this regulation is equivalent to the allosteric control of transporters by HPr(Ser-P), as in both cases the intracellular inducer concentration is lowered. HPr also affects the hierarchical utilization of PTS carbohydrates via competition for HPr(His∼P), a general mechanism that is operative in both Gram-negative and Gram-positive bacteria. As the affinity of HPr(His∼P) varies for the carbohydrate-specific IIA proteins/domains, competition for HPr(His∼P) leads to hierarchical uptake of PTS carbohydrates.

In some low-GC Gram-positive bacteria, catabolite repression is achieved by a mechanism in which the inducer is expelled from the cell. Two types of inducer expulsion mechanisms have been postulated (Figure 2). In homofermentative lactic acid bacteria like *E. faecalis, S. pyogenes, S. bovis* and *L. lactis*, lactose and glucose accumulate in the cytoplasm in their phosphorylated forms (Reizer & Panos 1980; Ye et al. 1996). Addition of a rapidly metabolizable sugar results in dephosphorylation of the accumulated sugar-P, which is followed by a rapid efflux of the free sugar from the cell (Reizer et al., 1983). A sugar-P phosphatase (Pase II) has been identified in *E. faecalis, S. pyogenes, S. bovis* and *L. lactis* and this enzyme seems to be absent in *S. aureus, S. mutans, S. salivarius* or *B. subtilis*, organisms that do not exhibit the sugar-P hydrolysis dependent expulsion phenomenon. Based on *in vitro* studies with toluenized vesicles or purified Pase II, it has been suggested that HPr(Ser-P) stimulates PaseII (Ye et al. 1994c). A second type of inducer expulsion has been observed in heterofermentative lactobacilli such as *L. brevis* and *L. buchneri* These bacteria transport lactose and glucose via proton symport mechanisms and accumulate these substrates as free (non-phosphorylated) sugars. Binding of HPr(Ser-P) to the glucose/H^+ and lactose/H^+ symporters is thought to alter the energy coupling mechanism, resulting in a conversion of the systems from carbohydrate-proton symport into carbohydrate uniport. Consequently, the accumulated sugars leave the cell down their concentration gradients, and thereby the inducer levels are lowered (Romano et al. 1987; Ye et al. 1994a,b).

CcpA-mediated catabolite repression

In many Gram-positive bacteria the general transcription factor, CcpA, mediates the repression of a range of catabolic genes (Hueck & Hillen 1995) (Figure 2). HPr(Ser-P) has been shown to interact with CcpA, allowing the latter protein to bind specifically to a *cis*-acting sequence. This sequence, named catabolite-responsive element (*cre*), is present in or near the promoter regions of many catabolite repression-sensitive operons (Weickert & Chambliss 1990). HPr(Ser-P)/CcpA forms a ternary complex with *cre*, consisting of two molecules of HPr(Ser-P), the CcpA dimer and the *cre* sequence (Jones et al. 1997). Both the formation of the HPr(Ser-P)/CcpA-complex and its binding to *cre* sequences is stimulated by FDP (Deutscher et al. 1995; Kim et al. 1998). The histidine residue at position 15 in the HPr protein, the active site for PEP-dependent Enzyme I phosphorylation, is important for CcpA-mediated repression, as mutation or phosphorylation of His-15 blocks the in-

teraction of HPr(Ser-P) with CcpA. This observation suggests a direct link between catabolite repression and PTS-mediated carbohydrate transport (Deutscher et al. 1995; Reizer et al. 1996). Indeed, the uptake of glucose or other rapid metabolizable PTS carbohydrates leads to dephosphorylation of the PTS proteins and to an increase of the concentrations of glycolytic intermediates that activate the HPr(Ser)kinase. As a result the levels of HPr(Ser-P) rise and CcpA-dependent genes become less efficiently transcribed. This regulatory mechanism leads to a hierarchy in the utilization of carbohydrates.

Catabolite control by CcpA not only involves repression but also activation of genes and operons. In *Lactococcus lactis*, CcpA was found to be a transcriptional activator of the *las* operon, thereby controlling the production of the three key glycolytic enzymes, that are, phosphofructokinase, pyruvate kinase and lactate dehydrogenase (Luesink et al., 1998).

Autoregulation of carbohydrate utilization

Besides hierarchical control, the PTS also mediates autoregulation of carbohydrate utilization. The mechanistic concepts of the autoregulatory control circuits are emerging and, in a few cases, it has been shown that the rate of carbohydrate uptake is tuned to the metabolic capacity of the cell and the carbohydrate availability in the medium. In *S. thermophilus* evidence was obtained for autoregulation of the transport of the non-PTS carbohydrate lactose. This involved the tuning of the uptake to the rate of sugar metabolism. In the following sections, the regulation of transport and metabolism of lactose at the level of protein activity (Section *Regulation of lactose transport in S. thermophilus*) and gene transcription (Section *Transcriptional control of the lac operon in S. thermophilus*) are described. The underlying mechanism involves HPr(His∼P)-mediated phosphorylation of the IIA-like domain of the non-PTS transport protein lactose transporter (LacS) from *S. thermophilus* and HPr(Ser-P)-dependent binding of CcpA to a *cre* site in the *lacS* promoter region.

S. thermophilus has a very limited capacity to utilize carbohydrates. Lactose and sucrose are fermented most rapidly, glucose is used very slowly, and only one or few other carbohydrates can be used by most strains. *S. thermophilus* co-metabolizes sucrose and lactose, a PTS and a non-PTS substrate, respectively, indicating that the utilization of these carbohydrates is not (strongly) hierarchically controlled. Instead, it has been proposed that HPr(His∼P)-mediated regulation of the lactose uptake rate serves to control the flux of glycolysis. This mechanism is based on studies of the kinetic properties of phosphorylated and unphosphorylated lactose transporter (LacS) from *S. thermophilus*, and on the LacS levels of the cell as a function of the phosphorylation state of HPr. The various species of HPr present in lactose-growing *S. thermophilus* cells have been quantified at different stages of growth (Gunnewijk & Poolman 2000). HPr(Ser-P) appears to be the dominant phosphorylated species in the exponentional phase of growth, whereas HPr(His∼P) dominates in the stationary phase. Similar results were obtained when *S. thermophilus* cells were grown on sucrose. The fact that the levels of HPr(Ser-P), HPr(His∼P) and HPr are similar in sucrose- and lactose-growing cells suggest that the rate of glycolysis of both carbohydrates is sufficiently high to keep Ser-46 phosphorylated and that the drain of phosphoryl transfer to sucrose is minor compared to the phosphorylation activity of Enzyme I. The similar HPr(His∼P)/HPr ratios also suggest that the PEP/pyruvate ratios are comparable in sucrose and lactose growing cells. Although PEP levels have not been measured in *S. thermophilus*, it has been firmly established for other lactic acid bacteria that concentrations of PEP are relatively low in rapidly metabolizing cells, whereas PEP concentrations increase under conditions of carbohydrate limitation (Mason et al. 1981; Thompson & Torchia, 1984; Konings et al. 1989). The increase in HPr(His∼P) in *S. thermophilus* at later stages of growth would thus correlate with increased PEP levels and a decreased metabolic activity.

Regulation of lactose transport in S. thermophilus

In *S. thermophilus*, lactose is taken up via the secondary transport protein LacS (Poolman et al., 1996). LacS catalyzes two modes of transport, solute-H^+ symport, driven by the proton motive force (Δp) and lactose/galactose exchange, which is driven by the concentration gradients of lactose and galactose across the membrane (Foucaud & Poolman 1992). The lactose/galactose exchange reaction via LacS is the most relevant transport mode *in vivo* as it is much faster than the lactose/H^+ symport reaction (Knol et al. 1996). In addition, the galactose moiety of lactose cannot be metabolized in most *S. thermophilus* strains, and therefore galactose has to be expelled from the cell. Kinetic studies have revealed that the affinity of LacS for galactose and lactose at the cytoplasmic

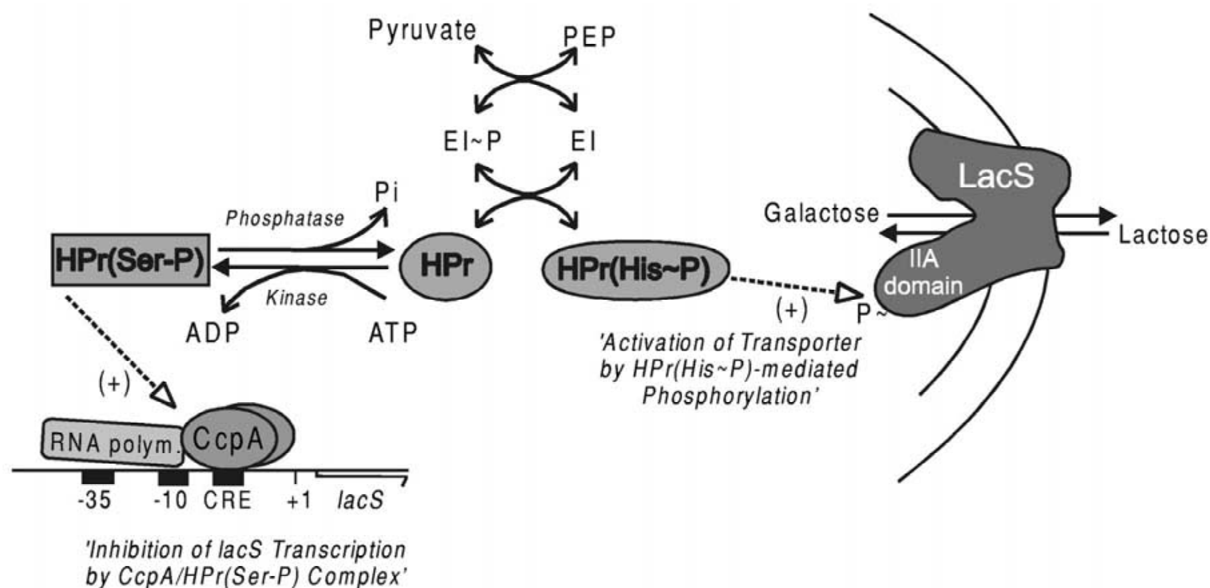


Figure 3. Regulation of lactose transport in *Streptococcus thermophilus*. Schematic representation of HPr(His∼P)-mediated phosphorylation of LacS (stimulation of lactose transport activity), and HPr(Ser-P)/CcpA-mediated regulation of lacS transcription. The depicted symbols are described in the legend of Figure 2.

binding site is 20-fold higher than at the extracellular binding site, and that, in this conformation, galactose is preferred over lactose (Veenhoff & Poolman 1999). These observations are consistent with the view that LacS has evolved into an efficient lactose/galactose exchanger.

Although LacS is not a PTS transport system, the protein has a carboxyl-terminal hydrophilic domain of about 160 amino acids (Figure 3), which is homologous to IIA proteins/domains of various PTS systems. This so-called IIA^{LacS} domain has evolved into a regulatory element, whose main function is not to transfer phosphoryl groups rapidly but rather to control the transport activity. The effect of HPr(His∼P)-mediated phosphorylation on lactose transport has been studied *in vitro* using an artificial membrane system, in which purified LacS protein was incorporated into liposomes with the IIA^{LacS} domain facing outwards (Gunnewijk & Poolman, 2000b). This system allowed phosphorylation and manipulation of LacS activity by adding PEP, Enzyme I and/or HPr to the outside medium. Upon phosphorylation of LacS the maximal rate of lactose exchange transport is increased, whereas the rate of Δp-driven lactose uptake is not affected. In line with a range of kinetic studies (Foucaud & Poolman 1992; Poolman et al., 1995b), it has been proposed that phosphorylation affects the rate constants for the reorientation of the ternary complex (LacS with bound lactose plus proton), which is rate-determining for exchange transport but not for Δp-driven uptake. Since the lactose/galactose exchange reaction and not the Δp-driven uptake is most relevant in lactose (glycolysing)-metabolizing cells of *S. thermophilus*, HPr(His∼P)-mediated phosphorylation of LacS evokes maximal activity of the lactose transport protein in vivo by increasing the V_{max} of the lactose/galactose exchange reaction. This condition is met in cells at the late-exponentional and stationary phase of growth, when HPr(His∼P) is the dominant species of HPr (Gunnewijk & Poolman 2000a).

The transition from HPr(Ser-P) to HPr(His∼P) at the late-exponentional phase of growth parallels an increase in the extent of LacS phosphorylation, a decrease in lactose and an increase in galactose concentration in the growth medium. Since both lactose and galactose are substrates of LacS (Veenhoff & Poolman 1999), the decrease in lactose/galactose ratio in the medium will reduce the lactose uptake (and galactose excretion) capacity as growth proceeds. This will at some point during growth be reflected in a reduced glycolytic activity, to which the HPr(Ser-P)/HPr(His∼P) ratio is very sensitive (Reizer et al. 1984,1989b; Deutscher et al. 1985; Deutscher & Engelman, 1984). By increasing the specific transport

activity via HPr(His~P)-mediated phosphorylation, *S. thermophilus* is able to partially compensate for the decrease in lactose concentration (and galactose accumulation) in the medium. Another, but slower, response involves adjustment of the LacS expression levels, which is described in the following section.

Transcriptional control of the lac operon in S. thermophilus

The observed transition from HPr(Ser-P) to HPr(His~P) at the late-exponential phase of growth parallels an increase in LacS level (Gunnewijk & Poolman 2000). At stationary phase, the expression level is about 10 times higher than the basal LacS level at early-exponential phase of growth, which is consistent with the idea that HPr(Ser-P) is a corepressor of the lac operon Figure 3.

Direct evidence for HPr(Ser-P)/CcpA-mediated regulation of the *lac* operon came from studies with a *ccpA* disruption mutant. Disruption of the *ccpA* gene impaired the growth of *S. thermophilus* on several sugars as has been observed for other Gram-positive bacteria (Hueck et al. 1995; Egeter & Brückner 1996; Monedero et al. 1997). The *lacS* promoter contains a *cre* site, overlapping the −10 box and the transcriptional start site (Poolman 1993), suggesting that expression of the *lacS-lacZ* operon is under control of CcpA (Henkin 1996). In accordance, disruption of the *ccpA* gene in *S. thermophilus* CNRZ302 resulted in derepression of *lacSZ* transcription during exponential growth on lactose (van den Bogaard et al. 2000). Moreover, the rates of lactose uptake and galactose excretion were at least 4-fold increased in the *ccpA* disruption strain relative to wild-type cells. The increased lactose uptake and hydrolysis does not result in an increased growth rate on lactose, but leads to massive expulsion of glucose into the fermentation medium. Apparently, loss of a functional CcpA in *S. thermophilus* uncouples the control of metabolism over transport and *vice versa*, as glycolysis can no longer keep up with the massive lactose intake. In other words, the *S. thermophilus ccpA* disruption mutant has a lactose transport capacity that exceeds the maximal glycolytic rate. The data indicate that the concerted activity of HPr(Ser-P) and CcpA results in fine-tuning of lactose transport and hydrolysis capacity in order to accommodate maximal glycolytic flux.

Although most *S. thermophilus* strains cannot use galactose as a carbon source, galactose-fermenting mutants of strain CNRZ302 are readily obtained (Hutkins et al. 1985; Vaughan et al. 2001). In the Gal^+ variants of *S. thermophilus*, galactose is taken up by the LacS protein and fermented via the Leloir pathway (Poolman 1993; Vaughan et al. 2001), but growth is slower than with lactose. When *ccpA* is disrupted in the galactose-fermenting variants, derepression of *lacSZ* transcription is not observed during growth on galactose (van den Bogaard et al., 2000). This suggests that the glucose moiety derived from lactose induces repression of the *lacS* promoter. The repression is not observed when glucose is present in the growth medium, which is due to the low rate of uptake of glucose. The LacS transport protein of *S. thermophilus*, on the other hand, constitutes a fast and efficient system for lactose uptake, leading to high intake of glucose. The accompanying rapid glycolysis of glucose results in relatively high intracellular HPr(Ser-P) concentrations (Deutscher et al. 1995) and, consequently, repression of the promoter of the *lacS-lacZ* operon. Thus, repression of the lac operon in *S. thermophilus* is not carbon-source dependent but determined by the rate of glycolysis. Probably, FDP, PEP and/or ATP function as the intracellular indicators of the glycolytic flux, as has been suggested for other Gram-positive bacteria.

Model for autoregulation of lactose transport and metabolism in S. thermophilus

A model has been proposed for the control of lactose transport and metabolism in *S. thermophilus*, which accomodates the knowledge of the kinetic properties of the transporter, the regulation of transporter activity, the regulation of expression of the *lacS-lacZ* operon, and the metabolic status of the cells (Figure 3). The rate of lactose transport via LacS is susceptible to the lactose/galactose ratio in the growth-medium as the transporter has a higher affinity for galactose (end-product of the fermentation) than for the substrate lactose. This implies that the transport capacity will decrease when galactose accumulates in the medium even with millimolar concentrations of lactose available. At some point during growth this will be reflected in a reduced glycolytic activity, which affects the concentrations of different glycolytic intermediates to which the HPr(Ser-P)/HPr(His~P) ratio is very sensitive. ATP is an effector of HPr(Ser) kinase, whereas P_i is an inhibitor. In addition, the HPr(Ser-P)phosphatase is stimulated by P_i and inhibited by ATP (Deutscher & Saier 1983; Reizer et al. 1984,1989b; Deutscher et al. 1985). The intracellular concentrations of ATP, PEP and P_i vary in response to the carbohydrate availability as has been firmly established for other lactic acid bacteria. ATP levels are relatively high in rapidly

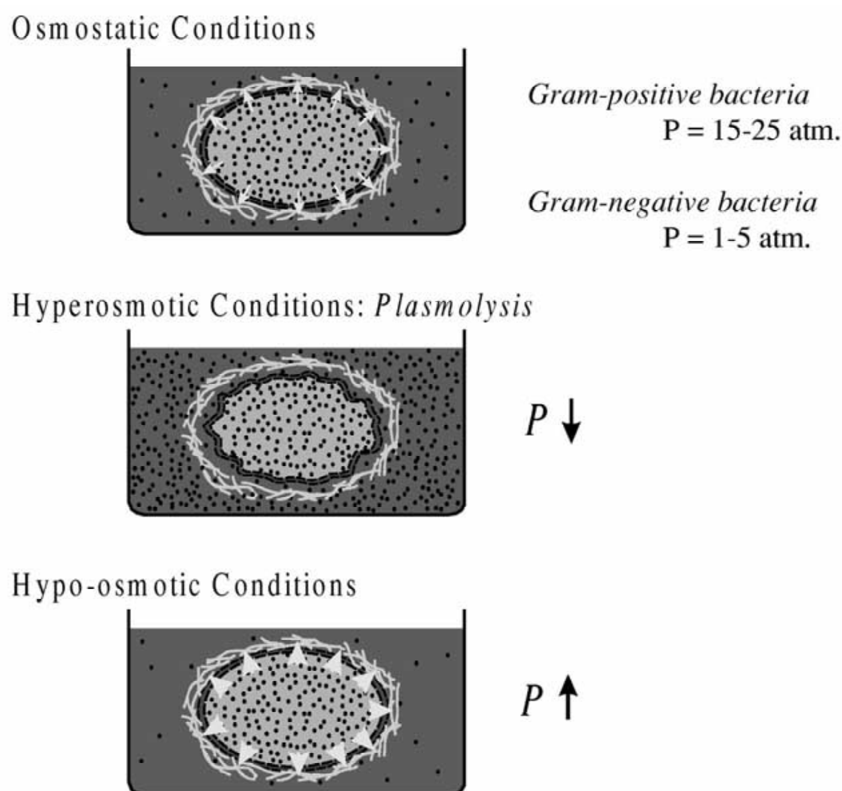


Figure 4. Schematic representation of the osmotic conditions experienced by a microbial cell. P, cell turgor.

metabolizing cells, whereas P_i and PEP are low under these conditions. These latter compounds become high at the end of the exponentional phase of growth and remain high in the stationary phase (Mason et al., 1981; Thompson & Torchia 1984; Konings et al. 1989).

The concentrations of the metabolites FDP, P_i, PEP and ATP reflect the metabolic status of the cell and determine the phophophyrylation state of HPr. Increasing PEP concentrations result in a rise in the HPr(His∼P) concentration due to PEP-dependent Enzyme I phosphorylation of HPr. HPr(His∼P) phosphorylates the LacS protein and this modification increases the maximal rate of transport rate. At the same time, the modified activities of HPr(Ser)kinase and HPr(Ser-P)phosphatase will decrease the HPr(Ser-P) concentration. Accordingly, a relief of the HPr(Ser-P)/CcpA-mediated repression of the *lacS* promoter will result in the synthesis of more LacS and β-galactosidase, which in turn will provide more glucose for glycolysis. In this way the uptake of lactose is tuned to the lactose/galactose ratio in the medium as well as the glycolytic capacity of the cell. The autoregulatory mechanism for the transport and metabolism of the non-PTS sugar lactose in *S. thermophilus* also allows the organism to co-metabolize lactose and sucrose.

Regulation of cell volume

Introduction

Enzymes and other macromolecules are not only sensitive to physical parameters such as pH, temperature and solute composition but also to water activity. This factor is often ignored in studies of enzyme activities and their regulation. Estimates of reaction rates and equilibria are generally made in dilute solutions with water activities close to one. The cytoplasm of a bacterial cell, however, is highly concentrated with 300–400 g/l of biological macromolecules (predominantly protein and RNA) and ∼100 g/l of low molecular weight osmolytes (incl. salts, amino acids, compatible solutes, etc.), resulting in low water activities. The fraction of macromolecules occupies 20–30% of the cellular volume, and, in this crowded and vis-

cous environment, the diffusion of solutes and macromolecules is impaired (Ellis 2001). The rate of any biochemical process that is diffusion-limited will be reduced in such a milieu and directly affected when the cytoplasmic volume changes upon osmotic up- or downshift. An increase in crowdedness will slow down diffusion and negatively effect rates. On the other hand, it increases the activity coefficients, i.e., the ratio of effective concentration to actual concentration, of enzymes by favoring the association of molecules. Knowledge of these omotic stress-related factors is not only crucial in understanding how an organism works, but also allows one to interfere with its metabolic activity. Osmotic challenge imposed to microorganisms forms the basis of food preservation strategies for several millennia, but, generally, little attention is paid to the actual consequences of this type of stress.

In their natural environment, the majority of microbial cells experience from time to time changes in extracellular water activity, which has direct consequences for the water activity of the cytoplasm. Following an increase in external water activity (osmotic downshift), passive influx of water will increase the turgor and eventually the cells will lyse if there are no mechanisms to counteract the stress (Figure 4); for the definition of cell turgor see Section *Other parameters relevant for cell volume control and osmoregulation*. Similarly, upon osmotic upshift water will flow out of the cell, the turgor will decrease, and in the end the cells will plasmolyse. To keep turgor within a specific range, and to prevent cells from lysing or plasmolysing, microorganisms adjust their intracellular osmolyte concentration. Both Gram-positive and Gram-negative bacteria prefer particular zwitterionic organic co-solvents such as glycine betaine, carnitine or ectoine as osmoprotectants (Wood 1999). These compounds, generally referred to as compatible solutes, can be accumulated to molar levels without negative effects on macromolecular structure or function. In fact, several compatible solutes have been shown to stabilize enzyme structure (Arakawa & Timasheff, 1985). The stabilization of native protein structures by these compounds involves preferential exclusion of the compatible solutes from the protein's surface. The preferential exclusion implies that the interaction between protein and compatible solutes is thermodynamically unfavorable. Because more protein surface is exposed in the unfolded than in the native state, the free energy difference for the transfer from water to compatible solute solution is largest for unfolded protein. This large positive Gibbs energy effect renders proteins thermodynamically more stable in the presence of compatible solutes.

Because the *de novo* synthesis of additional transporters and biosynthetic enzymes would be too slow to be effective against a rapid external osmotic change, cells need transport systems for compatible solutes that are directly controlled by osmotic conditions. To accumulate compatible solutes upon osmotic upshift, *Lactococcus lactis* uses an ATP-binding cassette (ABC)-transporter for glycine betaine (OpuA); equivalent systems can be found in other lactic acid bacteria. To excrete compatible solutes, that is, in the event the turgor becomes too high, organisms activate mechanosensitive channels. The molecular identities of three proteins that contribute to these channel activities have been identified in *E. coli* (Sukharev et al. 1994; Levina et al. 1999). BLAST searches of the non-redundant database NCBI indicate that lactic acid bacterial species have homologues of one or more of these molecules. By far, best studied of the three is the protein responsible for the largest conducting activity, MscL, and a gene homologous to *mscL* is present in *L. lactis* (Bolotin et al. 2001). Our current knowledge of the mechanism of osmosensing and regulation of the osmotic upschift-activated OpuA and downshift-activated MscL is presented in the following sections. Other osmotic stress-related parameters, perhaps not directly relevant for these systems, but important for a complete understanding of cell volume control are summarized.

Mechanisms of Oosmosensing

Osmotic activation of membrane proteins may be signaled via: (i) a change in cell turgor; (ii) macroscopic change in membrane structure; (iii) mechanical stimulus originating within the exo- or cytoskeleton of the cell; (iv) a change in the hydration state of the protein (internal or external osmolality); (v) alterations in the physicochemistry of the membrane bilayer (protein–lipid interactions); (vi) a change in cytoplasmic ion concentration or ionic strength; (vii) specific molecule interacting directly with the protein. Of these physicochemical parameters, the ones relating to changes in cytoplasmic osmolality, ionic strength, specific molecular stimulus, and protein interactions with putative cytoskeleton, are also relevant for cytoplasmic enzymes and other macromolecules. The relevance of each of the signaling parameters for membrane-embedded osmoregulated proteins has re-

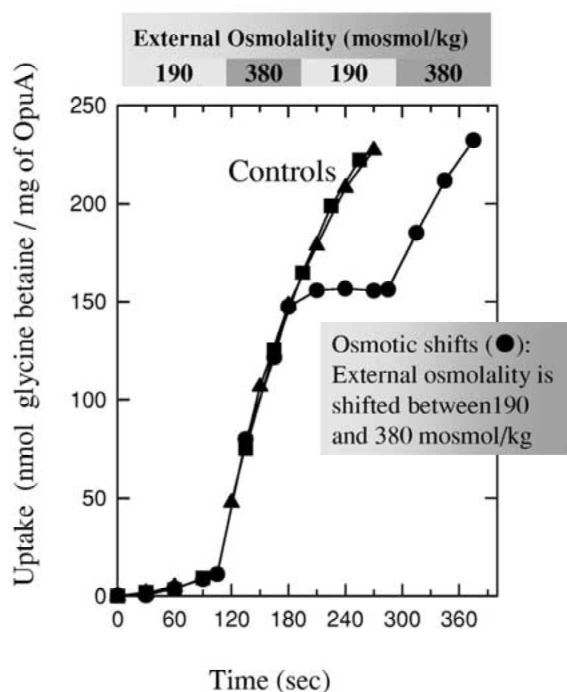


Figure 5. Kinetics and reversibility of the osmotic activation of OpuA. Uptake of [^{14}C]glycine betaine was assayed in 100 mM KP_i, pH 7.0, corresponding to 190 mosmol/kg. At 105 (●, ■, ▲) and 285 s (●) the proteoliposomes were subjected to hyperosmotic conditions by the addition of 100 mM KCl (final osmolality corresponding to 380 mosmol/kg). Isoosmotic conditions were restored at 180 s (●). Modified after van der Heide et al. 2001.

cently been evaluated (Poolman et al. 2002). In the *Molecular Microbiology* review, the focus is on transporters, channels and sensor kinases that have been well studied in intact cells and in vitro model systems, allowing detailed evaluation of the bacterial osmosensing mechanisms. Except for the ABC transporter OpuA from *L. lactis*, the systems are from organisms other than LABs, but the main conclusions may represent a firm basis for the situation in LABs, that is, if one believes in the conservation of biological structure and function.

To summarize the *Molecular Microbiology* review, the data from *in vivo* and *in vitro* experiments indicate that external ionic and non-ionic osmolytes activate a range of osmosensing devices, provided the compounds do not rapidly equilibrate (cross the membrane). Under these conditions, the osmotic stress causes the cytoplasmic or liposomal volume to decrease, resulting in an increased concentration of intracellular osmolytes, of which the ionic ones are critical for the activity of the OpuA transporter. *In vivo*, not only the decrease in cell volume, but also the accumulation of potassium ions in the initial response to osmotic upshift, may contribute to the increase in cytoplasmic ionic osmolyte concentration. The ionic osmolytes (or ionic strength) are sensed not only by the ABC-transporter OpuA from *L. lactis* but also by a number of other upshift-activated systems, among which sensor kinases of two-component regulatory systems (Poolman et al. 2002). Figure 5 shows that activation of OpuA by osmotic upshift is instantaneous and reversible upon returning to iso-osmotic conditions, which is a prerequisite for an effective response mechanism. Another important message here is that the effect of the osmotic upshift is indirect, that is, through the increase in concentration of ionic osmolytes on the inside (van der Heide et al. 2001). This condition is met with ionic (salts) and non-ionic (sugar, polyols) osmolytes added to the outside medium, provided the molecules do not equilibrate across the membrane on the time scale of the transport measurements (van der Heide & Poolman 2000b). Membrane permeant osmolytes such as glycerol cause a transient activation because these molecules diffuse across bacterial membranes with half times of seconds.

The equivalence of salts and sugars in the activation of an essential osmoresponsive system is important to emphasize as this point is not always clearly resolved in published literature. Salts and sugars do not always have the same osmotic effect, and often the stronger inhibition by salts is ascribed to additional 'electrolyte stress'. But what is actually meant by (additional) electrolyte stress? *Lb. plantarum* is dependent of an ATP-dependent glycine betaine transporter (QacT) for growth at high medium osmolalities. Like OpuA from *L. lactis*, *in vivo* the *Lb. plantarum* QacT system is activated by KCl, NaCl, sucrose and lactose (Glaasker et al., 1996). However, the growth defect elicited by the salts is much more severe than with the sugars (Glaasker et al. 1998a). It turns out that lactose and sucrose, although essentially membrane-impermeable in proteoliposomal systems, slowly enter the cell via some low-affinity sugar transport system. On the minute time-scale, the sugars cause osmotic stress, which can be observed as an activation of the QacT transporter. On the longer time-scale, the external and internal sugars equilibrate, that is, the carrier-mediated influx of sugar can keep up with the growth of the organism. Consequently, growth inhibition does not occur at medium osmolalities at which equiosmolar concentrations are already inhib-

itory. Thus, salts and sugars have the same 'osmotic effect' in terms of activation of the transporter but, for rather trivial reasons, the two types of osmolytes have different effects when growth is monitored. These observations, together with the transient activation of OpuA by glycerol, indicate that the increase in internal osmolality upon salt or sugar stress does not represent the signal for activation of these systems. What matters is the increase in intracellular ion concentration (ionic strength). In case of OpuA, there is strong evidence that the ionic signal is transduced to the protein complex via alterations in the protein–lipid interactions rather than direct sensing of the ion concentration or ionic strength by the protein (van der Heide et al. 2001).

The osmotic signal and regulation of downshift-activated mechanosensitive channels, including MscL of *L. lactis*, is different from that of OpuA, but the primary activation signal is also transduced via the membrane. The MscL channel opens, and jettisons solutes with little discrimination (except for size), when the tension in the membrane reaches a certain threshold value. The critical value for MscL gating is close to the tension at which the membrane ruptures and the cell lyses. The system thus serves as pressure valve that opens when the difference in internal and external osmolality becomes too high. An analogy with a balloon under high pressure may be useful to explain this concept. Upon further inflation with air, the balloon breaks since it does not have a means (equivalent of MscL) to release the pressure. Too high membrane tension may occur in nature when, for instance, a soil bacterium, after a period of drought, is suddenly confronted with rainfall. Such osmotic downshift conditions lead to an increase in turgor and tension in the membrane, and, depending on the mechanic and elastic properties of the cytoplasmic membrane and peptidoglycan layer, the cell may lyse.

Other parameters relevant for cell volume control and osmoregulation

Cell turgor

Cell turgor (ΔP) is the hydrostatic pressure difference that balances the difference in internal and external osmolyte concentration. In equation:

$$\Delta P = (RT/V_w)\ln(a_o/a_i) \sim RT(c_i - c_o)$$

in which V_w is the partial molal volume of water, 'a' is the water activity, 'c' is the total osmolyte concentration, and the subscripts 'i' and 'o' refer inside and outside, respectively. A cell plasmolyses when ΔP becomes negative. Although cell turgor is required for the expansion of the cell wall, there is little information on what the lower limit of turgor should be before cell growth ceases. Membrane vesicles and liposomal systems can only withstand low pressures as compared to cells with a peptidoglycan layer (Csonka & Hansen 1991), and thus functional incorporation into such artificial membranes of sensors that respond to low turgors should lead to constitutive activity. Several classes of osmoregulated systems, however, including the ABC transporter OpuA from *L. lactis*, show normal functional regulation when incorporated into proteoliposomes, suggesting that turgor is not the salient stimulus. In fact, contrary to the impression one may get from published literature on bacterial osmoregulation, there is little or no evidence that osmosensors are directly responding to changes in cell turgor (Poolman et al. 2002). A change in turgor does lead to a change in intracellular water activity, ionic strength and crowdedness (depending on the elasticity of the cell envelope) and membrane properties, and one or more of these parameters are more likely candidates to which osmoregulated systems respond.

Hydration state

Substrate and ligand binding to enzymes and transporters is typically associated with changes in the conformation of the proteins. Since it is likely that different protein conformations sequester different amounts of water, osmotic stress could potentially affect a system's activity through a change in the hydration state of the protein. Hexokinase is a classic example of water activity as regulator of enzyme activity (Parsegian et al. 1995). The dissociation constant (Kd) for glucose binding to hexokinase decreases with increasing osmotic pressure of the assay medium, when varied with either low or high molecular weight polyethylene glycol (PEG) in the solution (Reid & Rand 1997). The smaller effects of the low molecular weight PEGs are explained by their less effective steric exclusion from a cleft in the surface of the enzyme. Similarly for the channel-forming peptide alamethicin, it has been shown that the open probability decreases with increasing concentrations of high molecular weight PEGs (Vodyanoy et al. 1993). Parsegian and colleagues (1995) have formulated a thermodynamic hypothesis for these observations. In the transition from the closed to the open state, the channel will require additional water as the open state is most likely more

hydrated. Solutes too big to enter the channel such as high molecular weight PEGs will compete with the protein for water. Consequently, the excluded solute will cause the channel to perform extra osmotic work, which will lower the probability of the open conformation. The extra amount of work is less with solutes that are able to enter the channel, e.g., low molecular weight PEGs. The low molecular weight PEGs give rise to a smaller excluded volume and are thermodynamically less unfavorable than high molecular weight PEGs. In other words, the low molecular weight PEGs have a smaller dehydrating effect on the protein than high molecular weight ones. These two examples illustrate how osmotic stress could affect cytosolic and membrane-bound enzymes (and other macromolecules, e.g., protein-DNA interactions), but for LABs this is largely an unexplored area of research.

Macromolecular crowding

Cellular volume changes as a result of osmotic stress will result in changes in cytoplasmic protein concentration (macromolecular crowding), which affect the equilibrium of oligomeric enzymes and thereby their function. Although macromolecular crowding may not directly affect the function of systems embedded in the cytoplasmic membrane, membrane proteins that have a tendency to associate with soluble macromolecules may be influenced (Minton et al. 1992). As stated before, the crowdedness of the cytoplasm influences the diffusion of molecules. Three independently acting factors have been identified that account for a slowed diffusion (Verkman 2002): (i) the viscosity of the fluid-phase; (ii) the binding of molecules to other components; (iii) the collision of molecules with other cell components. The larger a molecule, the greater the contribution from viscosity and collision is. For a typical enzyme with a mass of 50 kDa, the diffusion may be slowed 10-fold relative to a dilute aqueous medium. With regard to factor (ii), it is worth noting that rod shaped bacteria possess genes coding for actin-like filaments, and in case of *Bacillus subtilis* it has been shown that these filamentous helical structures (‘bacterial equivalent of a cytoskeleton’) have a actin-like role in cell morphogenesis (Jones et al., 2001). Although homologues of these actin-like proteins are absent in round-shaped organisms (*Streptococcus* and *Enterococcus* sp.), they are likely to be present in lactobacilli. Whether or not these proteins affect the diffusion of cytoplasmic or membrane-bound proteins, the elasticity of the cell envelope, etc., is unknown, but, certainly, it represents an area of research that is relevant for a basic understanding of the stress response of (lactic acid) bacteria.

Physicochemical properties of the membrane

The role of the membrane as signal transducer of ionic osmolyte- (ionic strength) or bilayer tension-stress has already been mentioned in the context of the osmotic regulation of the OpuA transporter and the MscL channel. Ionic strength and bilayer tension, however, are not the only osmotic signals to be transduced via the membrane. On the assumption that the lipid bilayer behaves as an elastic solid, the intrinsic mechanical properties of the membrane can be described by four elasticity moduli that describe the response of a unit area of bilayer to volume compression, area expansion, bending/curvature and extension/shear (Evans & Skalak 1980). The responses of membranes to these elastic deformations have been recently reviewed by Hamill & Martinac (2001), and their main conclusions relevant for this paper are summarized here. Firstly, the bilayer is at least 10-fold more compressible in area than in volume during mechanical deformations encountered under physiological conditions. Osmotic downshifts will thus lead to relative increases in membrane area and concomitant decreases in membrane thickness. Secondly, the bending rigidity of the bilayer is dependent upon the lipid composition and area of each monolayer, and this parameter determines, amongst others, the shape of lipid vesicles. Thirdly, above the phase transition temperature, the membrane behaves like a fluid in response to extension. Of the elastic deformations, the ones that lead to thinning of the membrane are thought to have major impact on protein conformations and may thus signal activity changes. For the mechanosensitive channel MscL, it is thought that thinning of the membrane upon osmotic downshifts contributes to the ability of the protein to sense membrane tension (Hamill & Martinac 2001).

Bilayer thickness is obviously important for any membrane protein, as mismatch would result in exposure of hydrophobic surfaces of either the protein or lipid to an aqueous environment. The membrane–water interface of the bilayer comprises a chemically complex environment, which offers multiple possibilities for interactions with protein side-chains (Killian & von Heijne, 2000). If the bilayer thickness is suboptimal for these interactions, the protein or lipid may react to prevent hydrophobic mismatch that may lead to alterations in protein conformation and activity. When OpuA from *Lactococcus lactis* was incorporated in membranes in which 50% of the lipid fraction

was varied in terms of acyl chain length (from C14 to C22), the protein displayed a clear optimum in maximal activity at C18 (dioleoyl lipids), but the threshold for osmotic activation was the same in all cases (van der Heide et al., 2001). Thus, changes in bilayer thickness upon osmotic up- or downshift are not of prime importance for the regulation of OpuA activity. On the other hand, in one model for gating the mechanosensitive channel MscL, the tilts of the transmembrane α-helices are predicted to increase as they move away from the axis of the pore, that is, when the membrane expands (Sukharev et al. 2001ab). The osmotic downshift-induced thinning of the membrane and the resulting hydrophobic mismatch could thus provide at least part of the energy required for the transit from the closed to the open state.

The build up of membrane tension upon osmotic downshift has been compared with the increase in tension in a balloon upon inflation, but the biological equivalent of the balloon is clearly more complex and can be described in further detail. The different local intermolecular forces between lipid molecules in a fluid membrane originate from steric hindrance, hydration, electrostatic charge and/or hydrogen bonding in the headgroup region, interfacial tension and acyl chain pressure. The differences in the components of the interactions as a function of membrane depth lead to enormous local transverse pressures that correspond to bulk pressures of several hundreds of atmospheres (Marsh 1996; Cantor 1999). A protein embedded in such membrane will thus not experience uniform tension as a function of membrane depth. The local pressure as a function of membrane depth is referred to as the lateral pressure profile.

Statistical thermodynamic calculations of the equilibrium pressure profiles of membranes predict large redistributions of lateral pressure when the acyl chain length, the degree, position and configuration of unsaturation, or headgroup repulsion are varied (Cantor 1999). Similarly, the incorporation into a lipid membrane of cholesterol or interfacial active solutes such as anesthetics are predicted to increase the lateral pressure selectively near the aqueous interfaces, resulting in a compensating decrease in lateral pressure near the center of the bilayer. Also an osmotic up- or downshift or the application of food preservatives with amphipatic properties (e.g., parabens) will affect the lateral pressure and such changes may influence protein conformation and activity. Because the osmotic activation profile of the OpuA transporter from *L. lactis* is not affected by variations in acyl chain length, position and configuration of the unsaturation, and/or headgroup repulsion as long as the charge of the lipid headgroups is kept constant (van der Heide et al. 2001), it seems unlikely that osmotic stress is detected as a perturbation of the lateral pressure profile. On the other hand, given the membrane tension-dependent gating of MscL, it is likely that changes in the lateral pressure profile influence the opening and closing of this and related mechanosensitive channel proteins.

Specific stimulus

A shift in medium osmolality causes a change in the cytoplasmic volume. In principle, the change in concentration of one particular molecule could be sensed and serve as a specific stimulus to switch on or off a particular system. A few such examples are described in the literature on osmotic regulation of transporters and sensor kinases (reviewed in Poolman et al. 2002). A special case of such a regulatory mechanism concerns the *trans*-inhibition of transport proteins, which is the equivalent of product-inhibition in metabolic pathways. For *Listeria monocytogenes* and *Lactobacillus plantarum*, it has been shown that pre-accumulated (*trans*) substrate inhibits the corresponding osmoregulated transport systems (Verheul et al., 1996; Glaasker et al., 1996b). The *trans*-inhibition may serve as a control mechanism to prevent the accumulation of these compatible solutes to too high levels and thereby the turgor pressure from becoming too high.

In the case of *L. monocytogenes*, carnitine is taken up via an ABC transporter that is specific for this substrate but is inhibited by high intracellular concentrations of both carnitine and glycine betaine (and perhaps other compatible solutes) depending on the osmotic status of the cells. In kinetic terms, the osmotic activation of the system parallels an increase in apparent inhibition constant (K_I) for glycine betaine and carnitine at the inner surface of the membrane. Apparently, as a consequence of the water efflux following an osmotic upshift, the internal binding site for glycine betaine and carnitine is altered. Binding of compatible solutes to an internal site thus seems to represent a key step in the activation–inactivation mechanism of some, but not all, osmoregulated transporters. We have recently shown that the *trans*-inhibition mechanism does not play a role in the osmotic regulation of the OpuA transporter from *L. lactis* (J. Patzlaff, unpublished).

Response of cells to osmotic up- and downshift

In the previous sections, the various osmotic stress-related signals to which a system, and thus a bacterial cell, could respond have been summarized. Despite the variety of possible signals and osmosensing mechanisms, we propose that not all of these are used as primary mechanism. In line with a strong believe in the conservation of biological mechanisms, the majority of osmotic upshift-activated systems (transporters and sensor kinases) may respond to changes in cytoplasmic concentrations of ionic osmolytes, whereas the downshift-activated channels sense tension in the membrane (Poolman et al. 2002).

Why would the cell use ionic osmolytes rather than intracellular osmolality (affecting protein hydration) or a specific signaling molecule (specific regulatory site on the protein)? When the medium osmolality is raised, the initial change in cytoplasmic water activity depends on the elasticity of the cell wall. Contrary to what is often thought, the cell wall of bacteria is not rigid but actually quite elastic (Csonka & Hansen 1991; Doyle & Marquis 1994). Consequently, even at turgors above zero, the cytoplasmic volume decreases with increasing external osmolality, and the ion (osmolyte) concentrations increase accordingly. The increase in ionic strength accompanying the volume decrease is undesirable as too high concentrations of electrolytes interfere with macromolecular functioning in eubacteria as well as higher organisms (Yancey et al. 1982). Most eubacteria expel ionic compounds in the event the electrolyte concentration becomes too high and replace these molecules with neutral ones such as glycine betaine to balance the cellular osmolality. The increase in electrolyte concentration (or ionic strength) upon a modest decrease in turgor pressure would thus represent an excellent trigger ('osmotic signal') for the activation of any osmoregulated transporter of neutral compatible solutes. Actually, it would prevent the osmotic stress from turning into 'cytoplasmic electrolyte stress'. Why, then, is the increase in intracellular osmolality less suitable as osmotic signal? In order to maintain a relatively constant turgor at different external osmolalties, the cell must be able to switch on transporters and take up organic compatible osmolytes with maximal activity at different internal osmolalities. In other words, the ability of (the majority of) microorganisms to grow at their maximal rate over a wide range of medium osmolalities implies that cellular processes function optimally over a wide range of intracellular osmolalities. Finally, the cell could use the osmotic upshift-dependent change in concentration of a specific molecule as signal, but ionic strength or collective ion concentration seems to be a more general signal for osmoresponsive systems. Specific signals may be used to tune the activity of a system as examplified by the *trans*-inhibition mechanisms in *L. monocytogenes* and *Lb. plantarum*.

Why does membrane tension, or more specifically a change in the lateral pressure profile of the lipid bilayer, represent a sensible mechanism to respond to osmotic downshifts? Following an osmotic downshift, the increase in turgor can be to some extent sustained by the cell envelope. The elastic properties of the cell membrane and peptidoglycan layer are such that some expansion is tolerated, but at some point the membrane or wall will rupture and the cell lyses. In this regard, it is worth noting that the cell turgor of a Gram-positive bacterium is in the range of 20–30 atm, which is more than 10 times the pressure a car tire has to resist. We propose that the decrease in cytoplasmic ion concentration, upon a severe osmotic downshift is much less detrimental to the cell than the accompanying increase turgor. Given the elasticity of the cell envelope, the increase in turgor will result in an increase in membrane tension. This tension within the membrane will be experienced by any membrane-embedded protein, and, for pressure valves such as MscL, this parameter represents an ideal gating signal. At present, we cannot exclude the possibility that the decrease in cytoplasmic ionic strenth or osmolality has some modulatory role in the gating of the mechanosensitive channels.

Concluding remarks

The emerging picture is that intracellular ionic solutes (or ionic strength) serve as a signal for the activation of the upshift-activated bacterial transporters and sensor kinases. For at least one system from a lactic acid bacterium, there is strong evidence that the signal is transduced to the protein complex via alterations in the protein-lipid interactions rather than direct sensing of ion concentration or ionic strength by the protein(s). The osmotic downshift-activated mechanosensitive channels, on the other hand, sense tension in the membrane but also here protein–lipid interactions serve a direct role in the transduction of the osmotic signal. This membrane-mediated transduction of the osmotic signal offers, in principle, additional means of control via alterations in lipid composition in the process of osmotic adaptation. Such potential regula-

tion is suggested by the *in vitro* studies with the OpuA ABC transporter from *L. lactis*, for which the ionic set point for activation could be shifted to higher concentrations of intracellular ionic osmolytes by increasing the fraction of ionic lipids in the membrane.

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