Chapter 3
Phosphorylation State of HPr determines the Level of Expression and the Extent of Phosphorylation of the Lactose Transport Protein of *Streptococcus thermophilus*

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ABSTRACT

The lactose transport protein (LacS) of *Streptococcus thermophilus* is composed of a translocator domain and a regulatory domain that is phosphorylated by HPr(His~P), the general energy coupling protein of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). Lactose transport is affected by the phosphorylation state of HPr through changes in the activity of the LacS protein as well as expression of the *lacS* gene. To address whether or not CcpA-HPr(Ser-P)-mediated catabolite control is involved, the levels of LacS were determined under conditions that the cellular phosphorylation state of HPr differed greatly. It appears that HPr(Ser-P) is mainly present in the exponential phase of growth, whereas HPr(His~P) dominates in the stationary phase. The transition from HPr(Ser-P) to HPr(His~P) parallels an increase in LacS level, a drop in lactose and an increase in galactose concentration in the growth medium. Since the $K_m^{out}$ for lactose is higher than that for galactose, the lactose transport capacity decreases as lactose decreases and galactose accumulates in the medium. Our data indicate that *S. thermophilus* compensates for the diminished transport capacity by synthesizing more LacS and phosphorylation of the protein, which results in an increased transport activity. The link between transport capacity and lacS expression levels and LacS phosphorylation are discussed.

INTRODUCTION

The phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) catalyzes transport and concomitant phosphorylation of a number of carbohydrates in both Gram-negative and Gram-positive bacteria (Postma et al., 1993). The phosphorylation of carbohydrates is mediated by the general energy coupling proteins Enzyme I and HPr as well as carbohydrate-specific components (IIA and IIB), of which IIB transfers the phosphoryl group to the carbohydrate that is translocated via IIC. The PEP-dependent Enzyme I-mediated phosphorylation of HPr results in the modification of a histidine residue at position 15 ([HPr(His~P)]; Weigel et al., 1982a). In addition, to this type of phosphorylation, HPr of Gram-positive bacteria can also be phosphorylated on a serine residue [HPr(Ser-P)] in an ATP-dependent protein kinase catalyzed reaction (Deutscher and Saier, 1983; Reizer et al., 1984). The reverse reaction, the hydrolysis of HPr(Ser-P) is catalyzed by a cytosolic HPr phosphatase, which is stimulated by high concentrations of phosphate (Deutscher et al., 1985). HPr(Ser) kinases of several low-GC Gram-positive bacteria, including *Bacillus subtilis, Streptococcus pyogenes* and *Lactobacillus brevis* (Reizer et al., 1984; 1988; 1989b), are stimulated by early glycolytic intermediates, in particular fructose 1,6- bisphosphate (FBP), of which the concentra-
tions vary in response to the carbohydrate availability (Mason et al., 1981; Thompson et al., 1984). By contrast, the HPr kinases of Streptococcus salivarius and Streptococcus mutans Ingbritt are not stimulated by glycolytic intermediates (Thevenot et al., 1995; Brochu et al., 1999). In Gram-positive bacteria, HPr(Ser-P) plays an important role in the regulation of carbon metabolism that is through allosteric control of transport systems and as effector of the transcription factor CcpA. HPr(Ser-P) is able to form a complex with CcpA, allowing the transcription factor to bind to catabolite responsive elements (so-called cre-sites), sequences present in the promoter region of catabolite-controlled genes and operons in Gram-positive bacteria (Hueck and Hillen, 1995).

In S. thermophilus lactose transport is mediated by the LacS protein, which catalyses heterologous exchange of lactose for intracellularly formed galactose as well as proton motive force (Ap)-driven lactose uptake (Foucaud and Poolman, 1992). LacS is a hybrid protein composed of a polytopic membrane domain and a carboxyl-terminal hydrophilic domain of about 180 amino acids (Poolman et al., 1989). This hydrophilic domain is homologous to IIA protein(s) (domains) of various PTS systems and can be phosphorylated by HPr(His~P) (Chapter 2). Phosphorylation of the IIA domain affects the activity of the lactose transport protein (Poolman et al., 1995a; Chapter 4). The phosphorylation state of HPr, however, may also be involved in the regulation of lacS expression as the lac promoter region contains a cre element, that could be the site of control of lacS and lacZ expression by the CcpA/HPr(Ser-P) complex. To address whether or not CcpA-HPr(Ser-P)-mediated catabolite control plays a role, and to obtain further insight into the regulation of lactose transport, we determined the phosphorylation state of HPr at different stages of growth, and correlated these with the expression level and phosphorylation state of LacS.

Materials and methods

**Bacterial Strains and Growth Conditions.** E. coli NM522/pAG3 (Galinier et al., 1997) was grown in Luria Broth supplemented with carbenicillin (50 μg/ml) under vigorous aeration at 37 °C (Sambrook et al., 1989). Plasmid pAG3 carries the ptsI gene of B. subtilis under control of the t3q promoter and is fused in frame with a sequence specifying a N-terminal his-tag. For induction of gene expression, isopropyl-β-D-thiogalactopyranoside was added to the medium (1 mM) after the culture had reached an OD600 of ~ 0.7. The cells were harvested after another 4 h of incubation. For large-scale protein purification, the cells were grown in a 15-liter fermentor (Bio Bench ADI 1065; Applicon, Inc.) with pH control at 7.0 and oxygen supply at 50 % saturation.

The following S. thermophilus strains were used: ST11, wild type strain with the lacS gene on the chromosome; ST11(lacS)/pGKHs and ST11(lacS)/pGKGS8(H552R), strains in which the chromosomal lacS is deleted and a copy of the gene is present on the plasmid and under control of the lacS promoter (Poolman et al., 1995a; Knol et al., 1996). S. thermophilus cells were grown semi-anaerobically at 42 °C in (B)elliker broth supplemented with 0.5% beef extract, 0.5% lactose plus 5 μg/ml erythromycin, when necessary. Growth experiments were performed in batch in a 15-litre fermentor with pH control at 6.8.

**Isolation of Membranes.** Right-side-out membrane vesicles of S. thermophilus were isolated as described (Otto et al., 1982) with the following modifications: cells were harvested by centrifugation, washed twice with 50 mM K-Hepes, pH 7.0, and resuspended to an OD600 of ~ 150 in 50 mM K-Hepes, pH 7.0, plus 10 mM MgSO4. The cell wall was digested with 10 mg/ml lysozyme for 30 min at 37 °C under mild shaking. Cells were lysed following the addition of K2SO4 to 155 mM. After 2 min of incubation at 37 °C, the lysate was diluted 2.5 times with 50 mM K-Hepes, pH 7.0, 10 mM MgSO4 plus DNase and RNase (100 μg/ml each) and incubated for 20 min at 37 °C. After 30 min of centrifugation at 48,200 x g (4 °C), the pellet was resuspended in 50 mM KPi, pH 7.0, and further steps were performed as described previously. Membrane preparations were stored in liquid nitrogen.

**Protein Purification.** Purification of HPr and LacS from S. thermophilus and Enzyme I from B. subtilis were performed as described in the accompanying paper. For the purification of LacS, right-side-out membrane vesicles of S. thermophilus ST11(lacS)/pGKHs were used that were prepared in K-Hepes buffer, pH 7.0, as described above.

**Preparation of Cell Free Extracts and Determination of HPr levels.** Samples of 250 ml were withdrawn from a culture of S. thermophilus and growth was stopped by addition of chloramphenicol (50 μg/ml). The cellular HPr levels were stabilized by adjusting the pH to 4.5 with 10 M HCl and addition of Gramicidin D (40 μM) as described by Vadeboncoeur et al. (1991). After centrifugation (6 min, 10,000 x g), the cells were resuspended in 20 mM Na-acetate, pH 4.5, to a final protein concentration of 20 mg/ml. Cells were ruptured by sonication with an amplitude of 10 μm (5x 15 sec with intervals of 45 sec) on ice, and cell debris and membranes were removed by centrifugation for 10 min at 280,000 x g. The different species of HPr were quantified after the proteins were separated by centrifugation for 10 min at 280,000 x g.
gel electrophoresis (native-PAGE; 15 % polyacrylamide). The HPr species were visualized by immunodetection and the relative levels of the different HPr species were determined by densitometry. As HPr(Ser-P) and HPr(His~P) have identical electrophoretic mobilities on native-PAGE, the two species were discriminated by boiling a fraction of the samples for 3 minutes prior to the electrophoresis. Unlike HPr(Ser-P), HPr(His~P) is not stable under these conditions and converted into HPr.

**Phosphorylation Assays.** Phosphorylation of LacS was determined quantitatively by coupling the PEP to pyruvate conversion, resulting from the phosphoryl transfer to Enzyme I, HPr and LacS, to the reduction of pyruvate by lactate-dehydrogenase (Waygood et al., 1979). Mixtures containing lactate dehydrogenase (0.1 mg/ml), purified Enzyme I from B. subtilis (1 µM) and HPr from S. thermophilus (5 µM) were incubated for 2 min with purified LacS (10 µM) in 50 mM KPi, pH 7.0, 1 mM MgSO4, 1 mM DTT, 100 µM NADH plus 0.05% Triton X-100 in a total volume of 150 µl. The phosphorylation reaction was started by addition of PEP to a final concentration of 1 mM. The oxidation of NADH (ε340 NADH = 6.3*10^3 L*mol^-1*cm^-1) was measured at 340 nm and a temperature of 20 °C. Control experiments showed that the PEP concentration was sufficient to keep Enzyme I and HPr in their phosphorylated state, and neither LDH nor NADH was limiting for the reaction. The presence of 0.05 % Triton X-100 in the assay buffer did not affect the activity of either Enzyme I or HPr significantly.

**HPr (de)phosphorylation.** The activities of Enzyme I, HPr kinase and HPr phosphatase activities in cell free extracts were measured by following the fate of the different species in time. Cell free extracts were incubated for 10 min at room temperature with 10 mM FBP and/or 5 mM ATP, 5 mM PEP, 50 mM Pi or without any additions (see legends to Fig. 2 for details). The phosphorylation state of HPr was analyzed by native-PAGE (15 % polyacrylamide) as described above, and the proteins were detected by immunodetection.

**Determination of Sugar Concentrations.** Lactose and galactose concentrations in the medium of growing S. thermophilus cells were measured spectrophotometrically, using PQQ-dependent glucose dehydrogenase (sGDH) and 2,6-dichloroindophenol (DCPIP) as electron acceptor (Duine et al., 1982; Heuberger et al., 2000). Culture supernatant was obtained by rapid filtration of cells on a 0.2 µm filter (Schleicher & Schuell GmbH, Dassel, Germany). Analysis of the samples before and after treatment with β-galactosidase made distinction between the residual lactose and formed galactose.

**Immunological Methods.** Immunodetection of LacS was performed with antibodies raised against the IIA domain of LacS (Chapter 2), whereas antibodies raised against HPr of S. salivarius were used to detect the corresponding protein of S. thermophilus. The proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 10 % polyacrylamide) as described by Laemmli (1970), and, subsequently, they were transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. The Western-light™ chemiluminescence detection kit was used to visualize the proteins (Tropix Inc.).

**Miscellaneous.** Coomassie Brilliant Blue staining was performed as described by Sambrook et al., (1989). The concentration of purified HPr and LacS was determined spectrophotometrically at 280 nm, using molar extinction coefficients of 1.4*10^4 M^-1*cm^-1 for HPr and of 7.6*10^4 M^-1*cm^-1 for LacS. In case of LacS, corrections were made for free and bound Triton X-100 by determining the absorbance at 280 and 290, and using an experimentally determined A280/A290 ratio. Protein concentrations of membrane vesicles or cell free extracts were determined by the Dc Protein Assay (Bio-Rad) using bovine serum albumine as standard.

**Materials.** Ni-nitrilotriacetic acid (Ni-NTA) resin was from Qiagen, Inc.; Bio-Beads SM-2 and Bio-Spin columns were from Bio-Rad Laboratories, Inc.; S-Sepharose and DEAE-Sepharose fast flow resins, PD-10 and MonoQ columns (HR 16/10) and Triton X-100 were from Pharmacia Biotech, Inc.; Ultrafiltration cells were from Amincon, Inc.; Lactate dehydrogenase from hog muscle was obtained from Boeringer Mannheim; sGDH and PQQ were generously provided by professor J.A. Duine from the Technical University of Delft, the Netherlands; β-galactosidase of E. coli was from Sigma. All other materials were reagent grade and obtained from commercial sources.

**RESULTS**

**HPr Species in S. thermophilus**

The relative levels of all HPr species, that is HPr(Ser-P), HPr(His~P), HPr(Ser-P/His~P) and HPr, in S. thermophilus ST11 were determined at different stages of growth. A typical growth experiment of S. thermophilus ST11 in medium with 0.5 % lactose is shown in Fig. 1A. The sample identification (1 to 6) denotes the times at which the samples were withdrawn from the culture.

To develop changes in the relative levels of HPr species, due to activity of Enzyme I, HPr(Ser) kinase and/or HPr phosphatases, the medium and the cytosol of the cells were acidified instantaneously to pH 4.5 after withdrawal of the samples from the culture.
Fig. 1. HPr species and LacS expression in *S. thermophilus* ST11 at different stages of growth. *S. thermophilus* ST11 was grown with 0.5% lactose as energy source. The sample identification (1 to 6 panel A) denotes the times at which the samples were withdrawn from the growth culture for analysis of the different HPr species (B) and the LacS expression levels (C). For the analysis of the different HPr species, the medium and the cytosol of the cells were acidified to pH 4.5, directly after sample withdrawal, and cell free extracts were prepared in 20 mM NaAcetate, pH 4.5, at 4 °C as described in Materials and Methods. Part of the cell free extracts was boiled for 3 min (indicated by +), and, untreated and boiled fractions were loaded onto a native PAGE-gel (15 % polyacrylamide; 30 µl of cell free extract/lane) for electrophoresis. The different HPr species were visualized by immunodetection. The arrows point to the different species of HPr and the boxes highlight the situation after boiling, that is when all the singly phosphorylated species corresponds to HPr(Ser-P). For the analysis of the LacS expression levels, the samples were washed with 50 mM K-Hepes, pH7.0, and right-side-out membrane vesicles were prepared. The proteins were separated by SDS-PAGE electrophoresis (10 % polyacrylamide; 6 µg of protein/lane) and LacS was visualized by immunodetection. The relative levels of HPr(His~P) [■], HPr(Ser-P) [□] and LacS [bars] are shown in panel D. The ratio of the HPr species in each sample was determined by comparing the HPr species in the untreated fraction with those in the boiled fraction. Corrections were made for the presence of HPr(Ser-P/His~P), which converts to HPr(Ser-P) upon boiling. The total HPr level was set to 100 % for each fraction. For the determination of the relative level of LacS expression, the intensity of sample 6 was set to 100%. 

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**Phosphorylation State of HPr and Protein Expression Levels**

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In order to bring the cytosol to pH 4.5, the ionophore Gramicidin D was added together with the acid quench. To test whether or not the levels of the HPr species were arrested under these conditions, the different species of HPr were analyzed in the cell extract at pH 4.5 in the presence or absence of ATP plus FBP, PEP or Pi, the substrates/effectors of HPr(Ser) kinase, Enzyme I and HPr(Ser-P) phosphatase, respectively. Fig. 2 shows the different HPr species present in *S. thermophilus* harvested at late exponential stage of growth, that is HPr and the phosphorylated species HPr(His–P) and HPr(Ser–P), which have identical electrophoretic mobilities in native PAGE-gels. Distinction between the two species was made by boiling a fraction of each sample for 3 min before the electrophoresis. Since HPr(His–P) is heat labile in contrast to HPr(Ser–P), the former species will only be observed in the unboiled sample, whereas HPr(Ser–P) will still be present in the boiled sample. As shown in Fig. 2, the ratio of the two bands [HPr and HPr(His–P) plus HPr(Ser–P)] were not affected upon addition of ATP plus FBP, PEP or Pi. The same was true when the samples were boiled before electrophoresis, showing that the HPr/HPr(Ser–P) ratio remained constant under the different incubation conditions (data not shown). This indicates that the HPr modifying enzymes are inactive at pH 4.5. If, however, the cell extracts were analyzed for these enzymatic activities at pH 7.0, the HPr species were converted to the corresponding phosphorylated or unphosphorylated forms when respectively ATP plus FBP, PEP or Pi was added (data not shown). At higher pH, in conclusion, pH 8.5, HPr(Ser–P) was unstable and Enzyme I was still able to phosphorylate HPr. Although HPr(His–P) of *E. coli* is known to be acid labile (Mattoo *et al.*, 1984), HPr(His–P), but also HPr(Ser–P) and HPr(Ser–P/His–P), of *S. thermophilus* are stable for at least 1 hour when kept at pH 4.5 on ice.

**Fig. 2. Stability of HPr species.** *S. thermophilus* ST11 was grown in the presence of 0.5% lactose and cells were harvested in the late-exponential phase of growth. The medium and the cytosol were acidified to pH 4.5, as described in Materials and Methods. Cell extracts were incubated for 10 min at room temperature with 10 mM FBP, 5 mM ATP, 5 mM PEP or 50 mM Pi as indicated in the figure. The different HPr species were visualized by immunodetection.

**Fig. 1B.** shows the relative levels of HPr species in cells withdrawn from the growth culture at the indicated time points (the numbering corresponds to the arrows in panel A). Until the midexponential phase of growth, the main phosphorylated species of HPr is HPr(Ser–P) (Fig. 1B; sample 1). The level of HPr(Ser–P) decreased at later times and an accompanying increase of HPr(His–P) was observed (Fig. 1B; samples 2 to 4). The double phosphorylated form of HPr [HPr(Ser–P/His–P)] was observed at low levels (maximally 5% of total HPr present).

The relative levels of HPr(Ser–P) and HPr(His–P) are also plotted in Fig. 1D, and very similar growth dependencies as a function of growth were observed in 6 independent experiments. As the rapid quench method prohibited careful adjustments of the extract volumes to identical protein quantities, the total amount of HPr loaded in each lane was not identical. Control experiments showed that the level of HPr did not change as a function of growth phase. From the experiments with *S. thermophilus* ST11, we conclude that the transition from HPr(Ser–P) to HPr(His–P) already starts in the mid-exponential phase of growth.

The relative levels of HPr species were also determined in *S. thermophilus* ST11ΔlacS/pGKhis, a strain in which lacS gene is overexpressed by using a low copy number plasmid (Fig. 3).
The basal LacS levels in *S. thermophilus* ST11ΔlacS/pGKhis in the mid-exponentional phase of growth are approximately 40-fold higher than in the wild type ST11 strain. In *S. thermophilus* ST11 ΔlacS/pGKhis, the transition from HPr(Ser-P) to HPr(His~P) occurred in the late- rather than in the mid-exponentional phase of growth (Fig. 3B; sample 5). Similar results were obtained with *S. thermophilus* ST11ΔlacS/pGKGS8(H552R), a LacS mutant that cannot be phosphorylated by HPr(His~P) as the active-site histidine is replaced by an arginine (data not shown). Thus, major catabolic changes associated with the transition from HPr(Ser-P) to HPr(His~P) are taking place at much later stages in growth when the basal level of LacS expression is (artificially) increased.

**LacS Expression Levels**

Next, we investigated whether the LacS expression levels correlated with the presence of HPr(Ser-P) in the cells. As shown in Fig. 1C, but observed in several independent experiments, the level of LacS increased after the culture reached the mid-exponential phase of growth. At stationary phase, the expression level was 11 times higher than the basal LacS level at early-exponential
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Phase of Growth (Fig. 1D). Consistent with a higher basal level of LacS in *S. thermophilus* ST11 ΔlacS/pGKhis, the increase in expression was less than two fold when early-exponential and late-exponential or stationary phase were compared (Fig. 3). Importantly, the increase in expression paralleled a decrease in HPr(Ser-P) and occurred at later steps of growth than in the wild-type ST11 strain. Overall, the data from 5 experiments indicate that the time point of transition from HPr(Ser-P) to HPr(His~P) is dependent on the basal level of LacS expression. The final level of LacS in ST11ΔlacS/pGKhis was approximately seven-fold (700 % in fig. 3A) higher than in ST11 (100 % in Fig. 1D).

**Phosphorylation of LacS**

In the previous Chapter, we demonstrated that purified IIA domain of LacS could be phosphorylated by HPr(His~P) from *S. thermophilus*. As the HPr(His~P) levels vary with growth, one would expect that the phosphorylation state of LacS differ as well. The phosphorylation state of LacS was measured spectrophotometrically from the formation of pyruvate using lactate dehydrogenase as coupling enzyme. As shown in Fig. 4, the addition of PEP resulted in a rapid phosphorylation of Enzyme I and HPr, observed as an absorbance decrease due to NADH oxidation, which was followed by a slow auto-oxidation of NADH that was independent of PEP, Enzyme I and HPr (data not shown). The phosphoryl transfer from PEP to Enzyme I and HPr occurred within the mixing time of the experiment. When, purified LacS was added together with Enzyme I and HPr, the addition of PEP resulted in an additional kinetic component that corresponds to the phosphoryl transfer from HPr(His~P) to LacS (Fig. 4, lower line).

![Fig. 4. Phosphorylation of LacS.](image)

These data show that phosphorylation of LacS is orders of magnitude slower than that of Enzyme I and HPr, which is consistent with phosphorylation studies on the isolated IIA domain of LacS (Chapter 2).

The phosphorylation status of LacS in *S. thermophilus* ST11ΔlacS/pGKhis was determined for cells that were harvested at different stages of growth. As presented in Table I, the phosphorylated
state of LacS is dominant in the late-exponential phase of growth, whereas at earlier times the largest fraction of the protein was unphosphorylated.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>OD&lt;sub&gt;660&lt;/sub&gt;</th>
<th>Growth Phase</th>
<th>LacS-P (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>Mid-Exponential</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>0.7</td>
<td>Mid-Exponential</td>
<td>30</td>
</tr>
<tr>
<td>6</td>
<td>1.6</td>
<td>Early-Stationary</td>
<td>68</td>
</tr>
<tr>
<td>7</td>
<td>1.6</td>
<td>Late-Stationary</td>
<td>67</td>
</tr>
</tbody>
</table>

Cells of <i>S. thermophilus</i> ST11<sub>lacS/pGKhis</sub> were grown on 0.5% lactose and samples were taken as described in materials and methods. Experimental conditions are the same as described in the legends to Fig. 3. The phosphorylation status of LacS was determined as described in the legends to Fig. 4.

**Lactose Consumption and Galactose Production by <i>S. thermophilus</i> ST11**

To determine whether or not substrate limitation of lactose-grown <i>S. thermophilus</i> ST11 is related to the transition in HPr species and the altered expression levels of LacS, the lactose and galactose concentrations were determined during growth. In a previous study, we showed by HPLC analysis of lactose-metabolizing <i>S. thermophilus</i> cells, that besides lactose only galactose is present in the medium (Gunnewijk et al., 1997). The presence of galactose is a direct result of the lactose/galactose exchange mode of transport and the inability of <i>S. thermophilus</i> to utilize galactose. Both lactose and galactose concentrations were determined in the growth medium at different stages of growth. As shown in Fig. 5A, the lactose concentration decreased and the galactose concentration increased in lactose-growing <i>S. thermophilus</i> ST11 cells, whereas the total sugar concentration remained constant at a concentration of 12.8 ± 0.1 mM. Since the K<sub>m</sub><sup>out</sup> for lactose is rather high and higher than that for galactose (5 mM and 1 mM, respectively; Veenhoff and Poolman, 1999), the lactose uptake rate will decrease as lactose decreases and galactose accumulates in the medium. Since lactose and galactose interact competitively with LacS protein, one can estimate the transport capacity (V<sub>t</sub>/V<sub>0</sub>) as a function of the external lactose and galactose concentrations:

\[
\frac{V_0}{V_t} = \frac{S_1}{S_1 + K^1_M \left(1 + \frac{S_2}{K^2_M}\right)}
\]

in which V<sub>0</sub> is the rate of transport when only lactose is present (maximal activity), whereas V<sub>t</sub> represents the rate at a given time during growth; S<sub>1</sub> and S<sub>2</sub> correspond to the external lactose and galactose concentration, respectively, and K<sub>1</sub><sup>m</sup> and K<sub>2</sub><sup>m</sup> correspond to the affinity constants for lactose and galactose.

Fig. 5B shows that the relative transport capacity decreased during growth if one assumes that the expression level of LacS and the activity remain constant. The growth rate, however, is constant up to times (up to timepoint 120 min in Fig. 5) that the calculated transport capacity is less than 10%. Within this period the LacS levels and the degree of phosphorylation of the protein increased, indicating that <i>S. thermophilus</i> ST11 is compensating for the reduced transport capacity. The calcu-
lated transport capacity corrected for the increase in LacS expression levels during growth is also shown in Fig. 5B (dotted line). Notice that phosphorylation of LacS stimulates the transport activity (accompanying paper) and thereby the transport capacity of the cell, implying that the transport capacity increases even further as growth proceeds. The link between the transport capacity and the LacS expression levels and phosphorylation state of the protein are discussed below.

Fig. 5. Lactose consumption and galactose production by lactose-growing *S. thermophilus* ST11. Samples were taken to determine the lactose and galactose concentrations in the medium (A). The lactose transport capacity ($V_t/V_0$) at a given time was calculated as described under Results, using the determined lactose and galactose concentrations (B); the Km-values were taken from Veenhoff and Poolman (1999). The lactose transport capacities with constant [●] and experimentally determined [■] LacS expression levels are plotted.

**DISCUSSION**

In this Chapter, we link the level of expression and the extent of phosphorylation of the lactose transport protein of *S. thermophilus* to the phosphorylation state of HPr. Since the promoter region of the *lac* operon of *S. thermophilus* comprises a catabolite repression element, we anticipated a role for HPr(Ser-P) in CcpA-mediated regulation of *lac* transcription. The presence of an IIA-like domain at the carboxyl-terminus of LacS suggested a role for HPr(His~P)-mediated regulation of the lactose transport activity, for which the evidence is presented in the accompanying paper (Chapter 4).

Our data show that in *S. thermophilus* ST11 HPr(Ser-P) is the major phosphorylated HPr species in the exponential phase of growth, whereas HPr(His~P) dominates in the late exponential and stationary phase. The transition from HPr(Ser-P) to HPr(His~P) parallels an increase in LacS level, an increase in the extent of phosphorylation of LacS, a drop in lactose and an increase in galactose concentration in the growth medium. The observation that the phosphorylated forms of HPr [HPr(His~P) and HPr(Ser-P)] dominate at different stages of growth in *S. thermophilus* is in agreement with results obtained for *S. salivarius* and *S. mutans* (Vadeboncoeur *et al.*, 1991).

Why do the levels of HPr species vary when the growth of the organisms still proceeds at $\mu_{\text{max}}$? In case of *S. thermophilus* lactose metabolism is initiated by the uptake of lactose via LacS, which has a relatively poor affinity for the substrate at the outer surface of the membrane. Moreover, as galactose, the end-product of metabolism, accumulates in the medium, the transport capacity decreases even with millimolar concentrations of lactose available. 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 and Engelman, 1984).
A decrease in HPr(Ser-P) concentration will release the catabolite repression of the lacS promoter
and as a result more LacS is synthesized. At the same time the concentration of HPr(His~P)
increases, resulting in phosphorylation of the LacS protein. In Chapter 4 we show that this phos-
phorylation increases the specific exchange activity of the LacS protein. The picture that thus emer-
ges for lactose metabolism is the following (summarized in Fig. 6): S. thermophilus maintains
the transport capacity high at relatively adverse lactose/galactose ratios by synthesizing more LacS and
stimulating the transport activity. The tuning of lactose transport to the needs of catabolism is
mediated by the phosphorylation state of HPr.

Some aspects of the scheme (Fig. 6) and the proposed regulation mechanism deserve further expla-
nation. Firstly, the correlation between LacS levels and the concentration of different HPr species is
observed in cells with low and high basal levels of LacS. The transition from HPr(Ser-P) to
HPr(His~P) is shifted to later stages of growth when more LacS is present, strongly suggesting that
the two parameters are directly linked. Secondly, both HPr(His~P) and HPr(Ser-P) are affected by
the concentrations of different glycolytic intermediates. The end-product of glycolysis, PEP, is a
substrate of Enzyme I and, in the absence of PTS substrates, the HPr(His~P)/HPr ratio is deter-
mined by the PEP/pyruvate ratio. ATP and early glycolytic intermediates are effectors of HPr(Ser)
kinase, whereas Pi is an inhibitor. Moreover, HPr(Ser-P) phosphatase is stimulated by Pi and inhi-
bited by ATP (Deutscher and Saier, 1983; Reizer et al., 1984; 1989b; Deutscher et al., 1985).
Thirdly, the FBP, ATP, Pi and PEP levels have not been measured in S. thermophilus, but it has

--- Phosphorylation State of HPr and Protein Expression Levels ---
been firmly established for lactic acid bacteria that FBP and ATP levels are relatively high in rapidly metabolizing cells, whereas Pi and PEP are low under these conditions. These latter compounds become high at the end of the exponential phase of growth and remain high in the stationary phase (Mason et al., 1981; Thompson et al., 1984; Konings et al., 1989). These physiological parameters form the basis for the proposed changes in enzyme activity when the glycolytic activity decreases. Fourthly, although FBP is an allosteric activator of the HPr(Ser) kinase of most low GC Gram-positive bacteria (Saier et al., 1996), no such activation was observed for the enzyme of *S. thermophilus* and *S. salivarius* (Brochu et al., 1999). We observed that serine phosphorylation of HPr in cell extracts of *S. thermophilus* was stimulated upon addition of ATP, and HPr(Ser-P) dephosphorylation was stimulated by Pi, whereas the presence of FBP had no effect on the activity HPr(Ser-P) kinase (data not shown). We thus propose that the level of HPr(Ser-P) in *S. thermophilus* is governed by the relative cellular concentrations of ATP and Pi, which are indicators of the energy status of the cells. Therefore, when the lactose transport capacity diminishes, the accompanying decrease in glycolytic activity will result in a drop in ATP and an increase in Pi concentrations, which in turn will decrease the concentration of HPr(Ser-P) as a result of the diminished HPr(Ser-P) kinase activity and the stimulated HPr(Ser-P) phosphatase activity (Fig. 6). Fifthly, lacS expression is no longer repressed in a *ccpa*− mutant of *S. thermophilus* (van den Bogaard et al., 2000). Sixthly, the regulation of lactose uptake involves a fast response, affected by phosphorylation of the LacS protein, and a slow response, which follows from the release of inhibition of transcription by CcpA/HPr(Ser-P) complex. We conclude that this dual regulation, in which the phosphorylated state of HPr has a central role and controls the lactose transport capacity, is an important mechanism for *S. thermophilus* to adjust the lactose uptake rate to its metabolic needs.

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