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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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 in which the cellular phosphorylation state of HPr greatly differed. 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. Because the Km for lactose is higher than that for galactose, the lactose transport capacity decreases as lactose concentration decreases and galactose accumulates in the medium. Our data indicate that S. thermophilus compensates for the diminished transport capacity by synthesizing more LacS and phosphorylating the protein, which results in increased transport activity. The link between transport capacity and lacS expression levels and LacS phosphorylation are discussed.

The phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS)1 catalyzes transport and concomitant phosphorylation of a number of carbohydrates in both Gram-negative and Gram-positive bacteria (1). 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 P-enolpyruvate-dependent Enzyme I-mediated phosphorylation of HPr results in the modification of a histidine residue at position 15 (HPr(His—P), Ref. 2). 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 (3, 4). The reverse reaction, the hydrolysis of HPr(Ser-P) is catalyzed by a cytosolic HPr phosphatase, which is stimulated by high concentrations of phosphate (5). HPr(Ser) kinases of several low-GC Gram-positive bacteria including Bacillus subtilis, Streptococcus pyogenes and Lactobacillus brevis (4, 6, 7) are stimulated by early glycolytic intermediates, in particular fructose 1,6-bisphosphate (FBP), of which the concentrations vary in response to the carbohydrate availability (8, 9). By contrast, the HPr kinases of Streptococcus salivarius and Streptococcus mutans Ingbritt are not stimulated by glycolytic intermediates (10, 11). In Gram-positive bacteria, HPr(Ser-P) plays an important role in the regulation of carbon metabolism 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 (cre-sites), sequences present in the promoter region of catabolite-controlled genes and operons in Gram-positive bacteria (12).

In S. thermophilus lactose transport is mediated by the LacS protein, which catalyzes heterologous exchange of lactose for intracellularly formed galactose as well as proton motive force (Δp)-driven lactose uptake (13). LacS is a hybrid protein composed of a polypitic membrane domain and a C-terminal hydrophilic domain of about 180 amino acids (14). This hydrophilic domain is homologous to IIA protein(s) domains of various PTS systems and can be phosphorylated by HPr(His—P) (15). Phosphorylation of the IIA domain affects the activity of the lactose transport protein (16, 17). 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.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Escherichia coli NM522/pAG3 (18) was grown in Luria Broth supplemented with carbenicillin (50 µg/ml) under vigorous aeration at 37 °C (19). Plasmid pAG3 carries the ptsI gene of B. subtilis under control of the Taq 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).

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1 The abbreviations used are: PTS, phosphoenolpyruvate:carbohydrate phosphotransferase system; LacS, lactose transport protein; PEP, P-enolpyruvate; FBP, fructose 1,6-bisphosphate; cre, catabolite responsive element; PAGE, polyacrylamide gel electrophoresis.

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Phosphorylation of LacS—Phosphorylation of LacS was determined quantitatively by coupling the P-enolpyruvate to pyruvate conversion, resulting from the phosphoryl transfer to Enzyme I, HPr, and LacS, to the reduction of pyruvate by lactate dehydrogenase (23). 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 dithiothreitol, 100 μM NADH plus 0.05% Triton X-100 in a total volume of 150 μl. The phosphorylation reaction was started by addition of P-enolpyruvate to a final concentration of 1 mM. The oxidation of NADH (ε₅₃₂/₅₄₇ = 6.2 × 10³ liter mol⁻¹ cm⁻¹) was measured at 540 nm using a Cary 5E spectrophotometer at 20 °C. Control experiments showed that the P-enolpyruvate concentration was sufficient to keep Enzyme I and HPr in their phosphorylated state, and neither lactate dehydrogenase 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 P-enolpyruvate, 50 mM P₃, or without any additions (see legend for 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 (3DHGD) and 2,6-dichloroindophenol (DCPIP) as electron acceptor (24, 25). 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 enabled a distinction between the residual lactose and formed galactose.

Immunological Methods

Immunodetection of LacS was performed with antibodies raised against the IIA domain of LacS (15), whereas antibodies raised against HPr of *S. salivarius* were used to detect the corresponding protein of *S. thermophilus*. The proteins were separated by SDS-PAGE (10% polyacrylamide) as described by Laemmli (26), 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. (19). The concentration of purified HPr and LacS was determined spectrophotometrically at 280 nm, using molar extinction coefficients of 1.4 × 10⁴ M⁻¹ cm⁻¹ for HPr and of 7.6 × 10⁴ M⁻¹ cm⁻¹ for LacS. For LacS, corrections were made for free and bound Triton X-100 by determining the absorbance at 280 and 290 and using an experimentally determined A₄₈₀/A₄₉₀ ratio. Protein concentrations of membrane vesicles or cell-free extracts were determined by the D₅ Protein Assay (Bio-Rad) using bovine serum albumin as standard.

Materials

Ni-nitroliotriacetic acid (Ni²⁺-NTA) resin was from Qiagen, Inc.; Bio-Beads SM-2 and Bio-Spin columns were from Bio-Rad Laboratories, Inc.; S- and DEAE-Sepharose fast flow resins, PD-10 and MonoQ columns (HR 16/10), and Triton X-100 were from Amersham Pharmacia Biotech. Ultrafiltration cells were from Amicon, Inc.; lactate dehydrogenase from hog muscle was obtained from Boehringer-Mannheim; sGDH and PQQ were generously provided by Prof. 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 (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 prevent changes in the relative levels of HPr species caused by the 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. To bring the cytosol to pH 4.5, the ionophore gramicidin D was added together with the acid quench. To check 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, P-enolpyruvate or P₃, 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...
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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, 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 Na Acetate, pH 4.5, at 4 °C as described under “Experimental Procedures.” 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 species after boiling, that is when all the singly phosphorylated species correspond to HPr(Ser-P). For the analysis of the LacS expression levels, the samples were washed with 50 mM K-Hepes, pH 7.0, and right-side-out membrane vesicles were prepared. The proteins were separated by SDS-PAGE (10% polyacrylamide, 6 µg of protein/lane) and LacS was visualized by immunodetection. The relative levels of HPr(Ser-P) are also plotted in Fig. 1D and a very similar growth dependence as a function of growth was observed in six 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 the 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-exponential phase of growth are approximately 40-fold higher than in the wild-type ST11 strain. In S. thermophi-
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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, and 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 the early-exponential phase of growth (Fig. 1D). Consistent with a higher basal level of LacS in S. thermophilus ST11 lacS/pGKhis, the increase in expression was less than 2-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 five 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 ST11 lacS/pGKhis was approximately 7-fold (700% in Fig. 3A) higher than in ST11 (100% in Fig. 1D).

Phosphorylation of LacS—In a previous publication (15), we demonstrated that the 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 differs 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 P-enolpyruvate 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 P-enolpyruvate, Enzyme I, and HPr (data not shown). The phosphoryl transfer from P-enolpyruvate 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 P-enolpyruvate resulted in an additional kinetic component that corresponds to the phosphoryl transfer from HPr(His−P) to LacS (Fig. 4, lower curve). 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 (15).

The phosphorylation status of LacS in S. thermophilus ST11 lacS/pGKhis was determined for cells that were harvested at different stages of growth. As presented in Table 1, 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.

Lactose Consumption and Galactose Production by S. thermophilus ST11—To determine whether or not substrate limi-
tation of lactose-grown *S. thermophilus* 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 high performance liquid chromatography analysis of lactose-metabolizing *S. thermophilus* cells, besides lactose only galactose is present in the medium (28). The presence of galactose is a direct result of the lactose/galactose exchange mode of transport and the inability of *S. thermophilus* 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 *S. thermophilus* ST11 cells, whereas the total sugar concentration remained constant at a concentration of 12.8 ± 0.1 mM. Because the *K_m* for lactose is rather high and higher than that for galactose (5 mM and 1 mM, respectively; Ref. 29), the lactose uptake rate will decrease as lactose decreases and galactose accumulates in the medium. Because lactose and galactose interact competitively with LacS protein, one can estimate the transport capacity \( \frac{V_t}{V_0} \) as a function of the external lactose and galactose concentrations:

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

(Eq. 1)

in which \( V_0 \) is the rate of transport when only lactose is present (maximal activity), whereas \( V_t \) represents the rate at a given time during growth; \( S_1 \) and \( S_2 \) correspond to the external lactose and galactose concentration, respectively, and \( K_m^1 \) and \( K_m^2 \) correspond to the affinity constant 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 its activity remain constant. The growth rate, however, is

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**Table I**

Phosphorylation state of LacS

| Sample | OD<sub>660</sub> | Growth Phase   | LacS–P |
|--------|----------------|----------------|--------|
| 1      | 0.5            | mid-exponential| 28     |
| 2      | 0.7            | mid-exponential| 30     |
| 6      | 1.6            | early-stationary| 68     |
| 7      | 1.6            | late-stationary| 67     |

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**Fig. 4.** Phosphorylation of LacS. P-enolpyruvate-dependent Enzyme I/HPr-mediated phosphoryl transfer to LacS was determined from the formation of pyruvate. The assay mixture (total volume of 150 μl) consisted of 50 mM KPi, pH 7.0, 1 mM MgSO<sub>4</sub>, 1 mM dithiothreitol, 50 μM NADH plus 0.05% Triton X-100, Enzyme I (1 μM) from *B. subtilis*, HPr (7 μM) from *S. thermophilus*, P-enolpyruvate (PEP) (1 mM) with or without LacS (14 μM). The arrow corresponds to the addition of P-enolpyruvate.

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**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 \( \frac{V_t}{V_0} \) at a given time was calculated as described under “Results,” using the determined lactose and galactose concentrations (B); the *K_m* values were taken from Ref. 29. The lactose transport capacities with constant (●) and experimentally determined (○) LacS expression levels are plotted.
constant up to (120-min time point in Fig. 5B) where 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 *S. thermophilus* ST11 is compensating for the reduced transport capacity. The calculated transport capacity corrected for the increase in LacS expression levels during growth is also shown in Fig. 5B (dotted curve). Notice that phosphorylation of LacS stimulates the transport activity (17) 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.

**DISCUSSION**

In this paper, 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. Because 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 lacS transcription. The presence of an IIA-like domain at the C terminus of LacS suggested a role for HPr(His-P) in regulating the LacS expression state and phosphorylation state of the protein for which the evidence is presented in Ref. 17.

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 expression 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* (22).

Why do the levels of HPr species vary when the growth of the organisms still proceeds at $\mu_{max}$? In the 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 (4, 5, 7, 30). A decrease in HPr(Ser-P) concentration will relieve 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 Ref. 17, we show that this phosphorylation increases the specific exchange activity of the LacS protein. The picture that thus emerges 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 explanation. First, 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. Second, both HPr(His-P) and HPr(Ser-P) are affected by the concentrations of different glycolytic intermediates. The end product of glycolysis, P-enolpyruvate, is a substrate of Enzyme I and in the absence of PTS substrates the HPr(His-P)/HPr ratio is determined by the P-enolpyruvate/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 inhibited by ATP (3, 4, 5, 7). Thirdly, the FBP, ATP, Pi, and P-enolpyruvate levels have not been measured in *S. thermophilus*, but it has been firmly established for lactic acid bacteria that FBP and ATP levels are relatively high in rapidly metabolizing cells, whereas Pi and P-enolpyruvate 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 (8, 9, 31). These physiological parameters form the basis for the proposed changes in enzyme activity when the glycolytic activity decreases. Fourth, although FBP is an allosteric activator of the HPr(Ser) kinase of most low GC Gram-positive bacteria (32), no such activation was observed for the enzyme of *S. thermophilus* and *S. salivarius* (11). 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 P_, 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 P_ 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). Fifth, lacS expression is no longer repressed in a CcpA mutant of S. thermophilus. Sixth, the regulation of lactose uptake involves a fast response affected by phosphorylation of the LacS protein and a slow response, which follows from the relieve of inhibition of transcription by the CcpA/HPr(Ser-P) complex. We conclude that this dual regulation in which the phosphorylation state of HPr has a central role and controls the uptake involves a fast response affected by phosphorylation of the LacS protein and a slow response, which follows from the relieve of inhibition of transcription by the 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 in which to adjust the lactose uptake rate to its metabolic needs.

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