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Abstract

Streptococcus thermophilus is used in many industrial dairy fermentations that require processing of milk at elevated temperatures. Its primary function is the rapid conversion of lactose to lactate while it also contributes to important sensory qualities. *S. thermophilus* strain CNRZ302 is unable to ferment galactose, neither the free sugar, nor when it is generated intracellularly by lactose hydrolysis. Nevertheless, sequence analysis demonstrated that strain CNRZ302 contained structurally intact genes for the Leloir pathway enzymes. These genes appeared to be organized in an operon with the order *galKTE*, which was preceded by a divergently transcribed regulator gene *galR*, and followed by a *galM* gene and the lactose operon *lacSZ*. This *S. thermophilus gal-lac* gene cluster is very conserved in sequence, organization and flanking regions among strains isolated from various fermented products. The structural *gal* genes were found to be transcribed weakly by strain CNRZ302, and only in medium containing lactose, reflecting the Gal$^-$ phenotype. Indeed, the upregulation of the *galKTE* promoter seems to suffice for a galactose-fermenting phenotype of *S. thermophilus*.

A catabolite responsive element (*cre*) was identified in the promoter of the *lacSZ* operon indicating a possible role for CcpA in regulation of transcription of this operon. CcpA has been studied in many low G+C Gram-positive bacteria where it mediates catabolite repression. *S. thermophilus*, unlike many other Gram-positive bacteria, prefers lactose to glucose as the primary carbon and energy source. To assess the role of CcpA mediated global regulation in *S. thermophilus* the *ccpA* gene was cloned and sequenced. Transcription analysis of the *lacSZ* operon showed relief of repression in the absence of a functional CcpA when cells were grown on lactose. In strains carrying a disrupted *ccpA* gene lactose transport was increased significantly while lactate production was reduced relative to wild-type cells. Global control of carbon metabolism in bacteria is primarily modulated by intracellular concentrations of glycolytic intermediates. The efficiency of glycolytic steps were determined by glycolytic intermediate dynamics analysis in resting cells isolated from various stages of growth. This showed a change in flux through glycolysis coinciding with this transition in growth by the lactose availability level indicated that the efficiency of several glycolytic steps were growth phase dependently regulated. This regulation was lost in *ccpA* mutant cells. Moreover, Northern analysis showed that CcpA acts as an activator of the *pfk-pyk* operon as well as of the *ldh* gene in *S. thermophilus*. CcpA not only fine-tunes lactose uptake and conversion when cells are grown in excess lactose, but is also involved in derepression of transport activity and reduction of glycolytic capacity to allow cellular adaptation to conditions where lactose becomes limiting. By over-expressing wild-type *S. thermophilus ptsH* and specifically mutated *ptsH* alleles in a *ptsH* deletion strain of *L. lactis* the functional role of *S. thermophilus* HPr was confirmed and specific amino acid residues were identified that are essential for its function in utilization of PTS and non-PTS carbohydrates. However, we hypothesize that the role of HPr in *S. thermophilus* may have been adapted to the preferential lactose metabolism similar to what was found for CcpA in this organism.

The work described in this thesis can provide the basis for metabolic engineering of fermentation properties of this lactic acid bacterium, which is used in many dairy fermentations and is of great commercial importance.

Chapter 1

General introduction

Bacteria constitute the simplest life form on this planet where they occupy an enormous variety of ecological niches and display a corresponding diversity in their underlying biochemical composition. Bacteria can utilize virtually any organic molecule as food, including sugars, amino acids, fatty acids, hydrocarbons, and their polymeric forms. Some are even able to obtain their carbon atoms from CO_2 and their nitrogen atoms from N_2. Due to their relative simplicity, bacteria have existed longer than any other organisms and have continued to constitute the most abundant type of cells on earth (Whitman *et al*., 1998).

Bacteria are also widely employed in food production and fermentation is one of the traditional methods to preserve food-related raw-materials. Lactic acid bacteria (LAB) represent a group of Gram-positive bacteria with a low G+C content in their genomic DNA including species from the genera *Lactococcus, Lactobacillus, Leuconostoc* and *Streptococcus* that are widely used in industrial dairy and other food fermentations. Their main function in these fermentations is the conversion of the milk sugar lactose into lactic acid, thereby conserving the food product by the prevention of growth of pathogenic and spoilage bacteria. Moreover, LAB contribute to the flavour, aroma and texture of the fermented product. In addition, LAB themselves may be used in probiotic products that confer health-promoting effects. These health-effects are related to changes in the human intestinal microflora that accompany stimulation of host immune cells, competitive exclusion of pathogenic bacteria or production of metabolic and nutritional compunds (neutraceuticals) (Vaughan *et al*., 2000). Furthermore, many laboratories investigate the possibilities to use engineered strains of LAB as oral delivery vehicles for mucosal vaccines or other therapeutic molecules (Pouwels *et al*., 1998).

Competition for carbon and energy sources has led to the development of sophisticated mechanisms that enable bacteria to sense the nutritional situation and adjust their catabolic capacities. Carbon catabolite repression is the phenomenon that rapidly metabolizable carbon sources repress the expression of catabolic genes involved in the metabolism of less-favorable carbohydrates. Two principal mechanisms for this global control have been described for species belonging to the enteric Gram-negative bacteria and the low G+C Gram-positive bacteria and in both mechanisms components of the phosphotransferase system (PTS) play a central role. Among the low G+C Gram-positive bacteria *B. subtilis* served as the model organism for the study of catabolite control. However, due to their industrial relevance and their relatively simple metabolism, catabolite control has also been studied in LAB. A considerable amount of research has been and continues to be invested in gaining insight in control of sugar metabolism in LAB in order to optimize dairy fermentations and provide tools for metabolic engineering strategies aiming at control of metabolic fluxes.

This thesis focusses on catabolite control of sugar metabolism in *Streptococcus thermophilus*, which is the key organism in many industrial dairy fermentations that require processing of milk at elevated temperatures, such as yogurt and Mozzarella, Gruyère-type or Emmental cheeses. *S. thermophilus* has solely been isolated from milk, fermented milk, cheese and starter cultures and their presence in other sources may be due to contamination (Starr *et al*., 1986). Throughout this thesis, it will become clear that lactose is the main carbon and energy source for this organism, which has led to the adaptation of the global control mechanism towards the fine-tuning of lactose uptake and subsequent catabolism by glycolysis. These regulatory adaptations enable *S. thermophilus* to achieve and maintain maximal growth on this sugar during all growth-phases and at all concentrations of lactose.

Carbohydrate transport

The first step in metabolism of carbon sources is their transport across the cytoplasmic membrane. Bacterial sugar transporters are generally substrate-specific and can be divided into three categories based on the energy utilized for transport (for a review see Poolman, 1993): i) carbohydrate transport ATPases that contain the ATP-Binding Cassette (ABC) and couple ATP hydrolysis to sugar translocation; ii) ion-linked sugar transport and sugar exchange mechanisms and iii) group translocation by a PTS that couples translocation of the sugar to its phosphorylation, at the expense of the glycolytic intermediate phosphoenolpyruvate (PEP) (Fig. 1).

PTS

The PEP:dependent sugar PTS catalyses the transport and concommitant phosphorylation of carbohydrates and is the main sugar uptake system in bacteria (for a review see Postma *et al*., 1993). The PTS transport potential of these bacteria can fairly easily be predicted from their genome sequence, but it is usually not possible to predict their substrate specificity. Table 1 shows the number of predicted complete PTSs in the genome of some lactic acid bacteria. The PTS comprises two general phosphocarrier proteins, Enzyme I and HPr, and the sugar-specific Enzyme IIABC (Fig. 1). The IIC is an integral membrane protein or domain and is responsible for the actual transport process whereas the IIA and IIB are cytosolic proteins or domains responsible for phosphoryl transfer.

Strain	Number of complete PTS in genome	Reference
Streptococcus thermophilus LMG18311	3	(Hols *et al*., 2002)
Lactococcus lactis IL1403	6	(Bolotin *et al*., 2001)
Lactobacillus plantarum WCFS1	25	(Vaughan *et al*., 2002)

Table 1. Predicted number of functional PTS in the genomes of three specius of industrial important LAB.

Carbohydrate translocation via the PTS requires the transfer in several steps of the phosphoryl group from PEP to the transported sugar. Enzyme I is autophosphorylated at the expense of PEP and donates the phosphoryl group to histidine 15 of HPr. The phosphoryl group is subsequently transferred from P-His-HPr to the IIA domain, which in its turn phosphorylates the IIB domain. Consequently, the IIC domain is activated and translocates the carbohydrate that becomes phosphorylated at the expense of P~IIB. The function of PTS is not restricted to group translocation of carbohydrates alone. In enteric Gram-negative bacteria and the low G+C Gram-positive bacteria the PTS also regulates transport and metabolism of PTS and non-PTS carbohydrates (see below).

Carbohydrate ABC transport ATPases

Uptake of sugars via primary transport systems depends on the hydrolysis of ATP that provides the energy for translocation (Fig. 1) (Fath and Kolter, 1993). The recognition of the ATP-binding cassette is often used to designate the transport systems as members of the ABC transport super family (Higgins and Linton, 2001). The number of ATP molecules hydrolyzed per carbohydrate taken up by the transport ATPases is two to three which makes transport via these systems energetically expensive relative to secondary transport systems

and PTS (Poolman, 1993). An example of ABC-transport are the MsmEFGK proteins in *Streptococcus mutans* that are involved in the uptake of multiple sugars, i.e. sucrose and the galactosides raffinose, melobiose and isomaltotriose (Tao *et al*., 1993). Maltose metabolism in *Lactococcus lactis* is dependent on the *malK* gene of which the deduced protein sequence shows strong homology to the *S. mutans* MsmK protein. Several putative genes encoding proteins that show homology to ABC transporter ATP binding proteins have been identified in the *S. thermophilus* LMG 18311 genomic DNA sequence (Hols *et al*., 2002). However, the proteins encoded by these genes showed no identity with known ABC sugar transporters. Moreover, *S. thermophilus* utilizes a verily limited number of

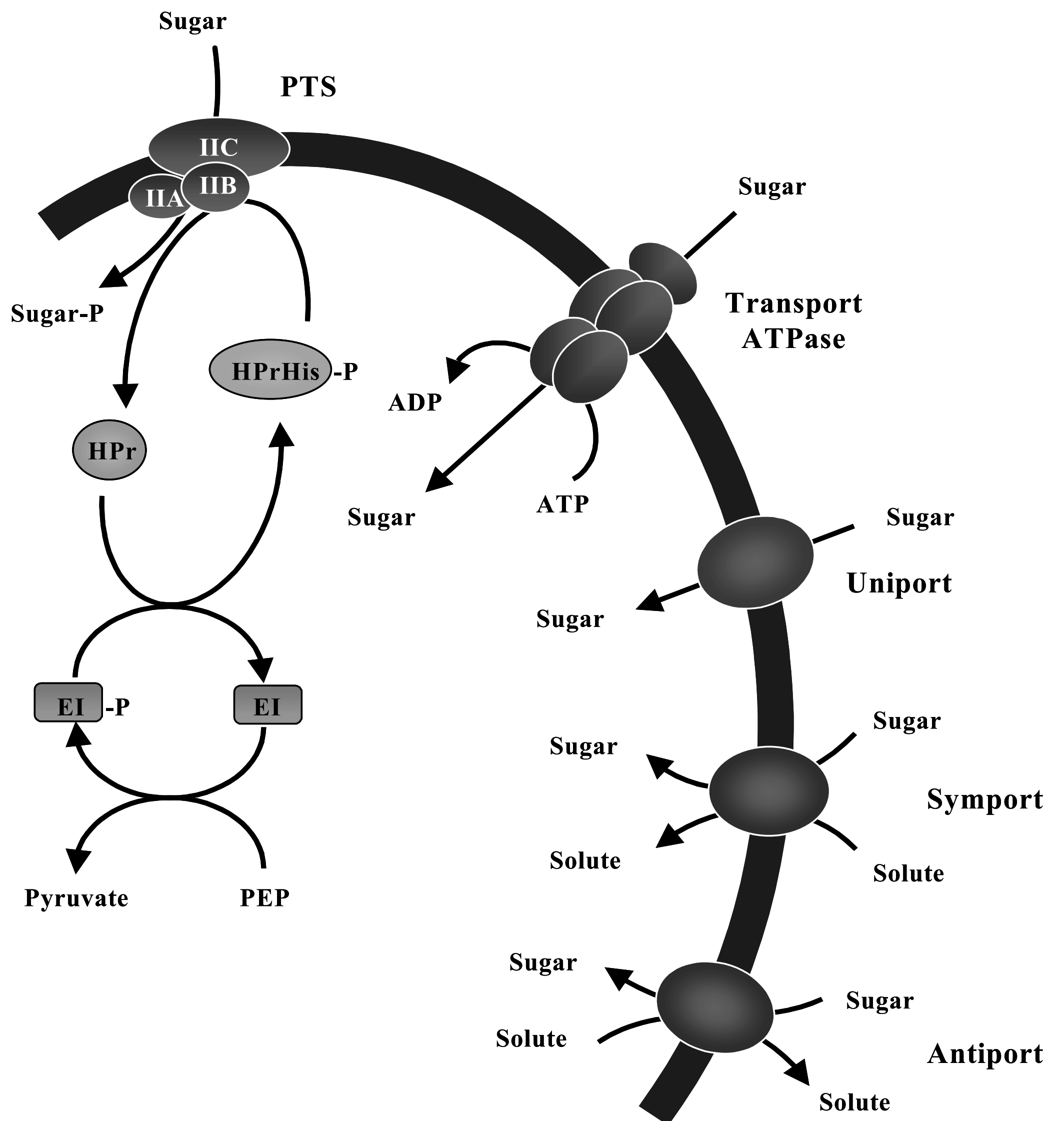

Fig. 1. Schematic representation of the different sugar uptake systems in bacteria. PTS transport is driven by the energy-rich phosphoryl group from phosphoenolpyruvate (PEP) that is transferred via Enzyme I (EI) and HPr to the substrate specific components EIIABC. Uptake of sugars via ABC transport ATPases depends on the hydrolysis of ATP that provides the energy for translocation. Secondary transport systems utilize the electrochemical energy of one solute to drive the transport of another solute. These systems can be subdivided into three classes: i) symporters that facilitate cotransport of two or more solutes; ii) uniporters which catalyze translocation of a single solute and iii) antiporters involved in countertransport of two or more solutes.

carbohydrates that are transported either by PTS or secondary transport systems (Poolman, 1993; van den Bogaard *et al*., 2002a), which indicates that ABC transport ATPases in this organism probably transport other solutes than sugars.

Ion-linked sugar transport and sugar exchange mechanisms

Secondary transport systems utilize the electrochemical energy of one solute to drive the transport of another solute (Poolman, 1993). These systems can be subdivided into three classes (Fig 1): i) symporters that facilitate cotransport of two or more solutes; ii) uniporters which catalyze translocation of a single solute and iii) antiporters involved in countertransport of two or more solutes. Carbohydrate symporters usually couple concentration gradient uphill movement of the carbohydrate to downhill movement of a proton (Lolkema *et al*., 1998). The best-studied secondary transporters in LAB is the family of homologous membrane proteins that transport galactosides-pentoses-hexuronides (GPH) (Poolman *et al*., 1996). Lactose transporters driven by the electrochemical proton gradient have been described for *S. thermophilus*, *Lactobacillus bulgaricus* and *Leuconostoc lactis* belong to the GPH family (Poolman *et al*., 1989; Vaughan *et al*., 1996). These lactose transporters are not uniquely selective for lactose but also for galactose and other galactosides e.g. melobiose and raffinose (Poolman *et al*., 1992). The lactose transport protein (LacS) of *S. thermophilus* has been demonstrated to catalyze the uptake, efflux and exchange of galactosides in this organism (Knol *et al*., 1996). In most situations the *S. thermophilus* LacS protein functions as a lactose/galactose exchanger, a reaction that has distinct advantages over sugar-H^+ symport e.g. a tight coupling of uptake to metabolism and rate of uptake (Poolman, 1993) (see below). In *L. lactis* the *galA* gene has been identified, the deduced protein sequence of which shows strong homology to the GPH family of galactose transporters indicating that galactose is taken up by secondary transport in this organism (Grossiord *et al*., 1998).

Carbohydrate utilization in LAB

Most LAB preferably grow under anaerobic or micro-aerobic conditions but some are aerotolerant. The anaerobic growth and the absence of cytochromes prevent the production of energy by the reduction of external electron acceptors and account for the limited metabolic capacities of LAB. As a consequence, metabolic energy can be generated from sugars by substrate level phosphorylation, catalyzed by phosphoglycerate kinase and pyruvate kinase from the glycolytic pathway or by acetate kinase during the conversion of acetyl phosphate to acetate. However, not all LAB species possess the metabolic capacity to perform all the reactions involved in these pathways. Additionally, energy can be generated by the formation of a proton motive force (Δp) from the efflux of solutes over the cytoplasmic membrane. The Δp is used to synthesize ATP by a membrane-located ATPase (Lolkema *et al*., 1998; Poolman, 1993). Examplatory for the formation of a Δp is the efflux of lactate, the result of sugar fermentation, by a lactate-H^+ symporter in *L. lactis*.

The mode of transport for a carbohydrate determines the subsequent catabolic pathway. Phosphorylation of the internalized carbohydrate is a prerequisite for catabolism by glycolysis and carbohydrates taken up by primary or secondary transport need to be phosphorylated by a cytoplasmatic sugar kinase. PTS is the main form of transport in LAB that results in the entry of a phosphorylated sugar. For example, *L. lactis* takes up lactose via PTS^{Lac} that generates lactose-6P in the cytoplasm (de Vos *et al*., 1990). Consequently,

lactose-6P is hydrolyzed by a phospho-β-galactosidase producing galactose-6P and glucose. The glucose moiety is phosphorylated by a glucokinase and is further catabolized via glycolysis to lactate whereas galactose-6P is metabolized via the tagatose-6P pathway to the glycolytic intermediate glyceraldehyde-3-phosphate (van Rooijen *et al.*, 1991). *S. thermophilus* does not posses a PTS^{Lac} and lactose is transported by the dedicated LacS permease. Consequently, the cytosolic lactose is hydrolyzed by a β-galactosidase resulting in a glucose and a unphosphorylated galactose moiety. Glucose is phosphorylated by a glucokinase and is further catabolized via glycolysis to lactate and the galactose moiety can be fermented via the Leloir pathway of which the first step is phosphorylation by the specific galactokinase (see below). Substrate for the galactokinase is the α-enantiomer of galactose. The hydrolysis of lactose yields β-galactose which is converted in the α-enantiomer of galactose by the activity of the mutarotase (GalM). Both systems for lactose and galactose metabolism require specific sets of enzymes which are encoded in *lac*- and *gal*-operons that are induced upon growth on lactose or galactose. Glucose is usually taken up by LAB by a PTS allowing direct glycolytic utilization of the cytoplasmic glucose-6P. Hence, glucose is generally the most efficient C-cource for growth and is the paradigm for the carbohydrate that is highest in sugar-hierarchy and is generally favored over all other carbon sources. Other fast-metabolizable carbon sources such as sucrose are usually also taken up by a specific PTS. However, these sugars require additional modification to to generate glycolytic intermediates. The genes encoding transporters and modification enzymes are generally organized in sugar-specific and inducible operons.

The main system for energy generation in LAB is the glycolytic or Embden-Meyerhoff pathway (Fig. 2). Carbohydrates are converted to pyruvate via this pathway and under anaerobic conditions pyruvate is converted to lactate by the lactate dehydrogenase LDH to regenerate the NAD^+ that is consumed in glycolysis (homolactic fermentation). However, several factors e.g. carbohydrate limitation, alkaline pH or aerobic growth conditions may partially change the distribution of end products from lactate to formate, acetate and ethanol (mixed acid fermentation) (Garrigues *et al.*, 1997). In some LAB, carbon flux through glycolysis was found to be subject to allosteric intermediate control at least at two reactions, catalyzed by phosphofructokinase (PFK) and pyruvate kinase (PYK) (Fothergill-Gilmore and Michels, 1993). In *L. lactis* and *Lactobacillus bulgaricus* PFK is allosterically activated by phosphoenolpyruvate (Fordyce *et al.*, 1982), while allosteric control of PYK activity includes activation by glucose-6P (G6P), fructose-6P (F6P) and/or fructose-1,6-bisP (FBP) and inhibition by high levels of free phosphate (Pi) (Fordyce *et al.*, 1984; Le Bras and Garel, 1993). In some LAB, such as *L. lactis*, LDH is also activated by FBP and under normal growth conditions the FBP concentration is sufficiently high to ensure full activation (Garrigues *et al.*, 1997). Moreover, the high $NADH/NAD^+$ ratio in *L. lactis* limits the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity and strongly activates LDH activity. In contrast, FBP is not an activator of the *S. thermophilus* LDH (Garvie, 1978) and other *S. thermophilus* enzymes (such as PYK) may have different kinetic parameters relative to those found in other LAB. The reactions catalyzed by PFK, PYK and LDH are highly unidirectional steps in glycolysis and obvious points for catabolite control by CR (see below). Some LAB, e.g. lactobacilli, can ferment carbohydrates e.g. gluconate, ribose, xylose or arabinose via another route, the phosphoketolase pathway (PKP) (Kandler, 1983). This heterolactic fermentation which is mainly used for the utilization of pentoses results in lactate and ethanol/acetate. However, also glucose and gluconate can be fermented by the PKP generating carbon dioxide as an additional product. Under anaerobic conditions ethanol is formed to regenerate the NAD^+ that is consumed in the first reactions of the PKP,

whereas mainly acetate is formed under aerobic conditions where NADH oxidase is used to regenerate the NAD^+, which yields more ATP and is thereby energetically more favorable (Condon, 1987).

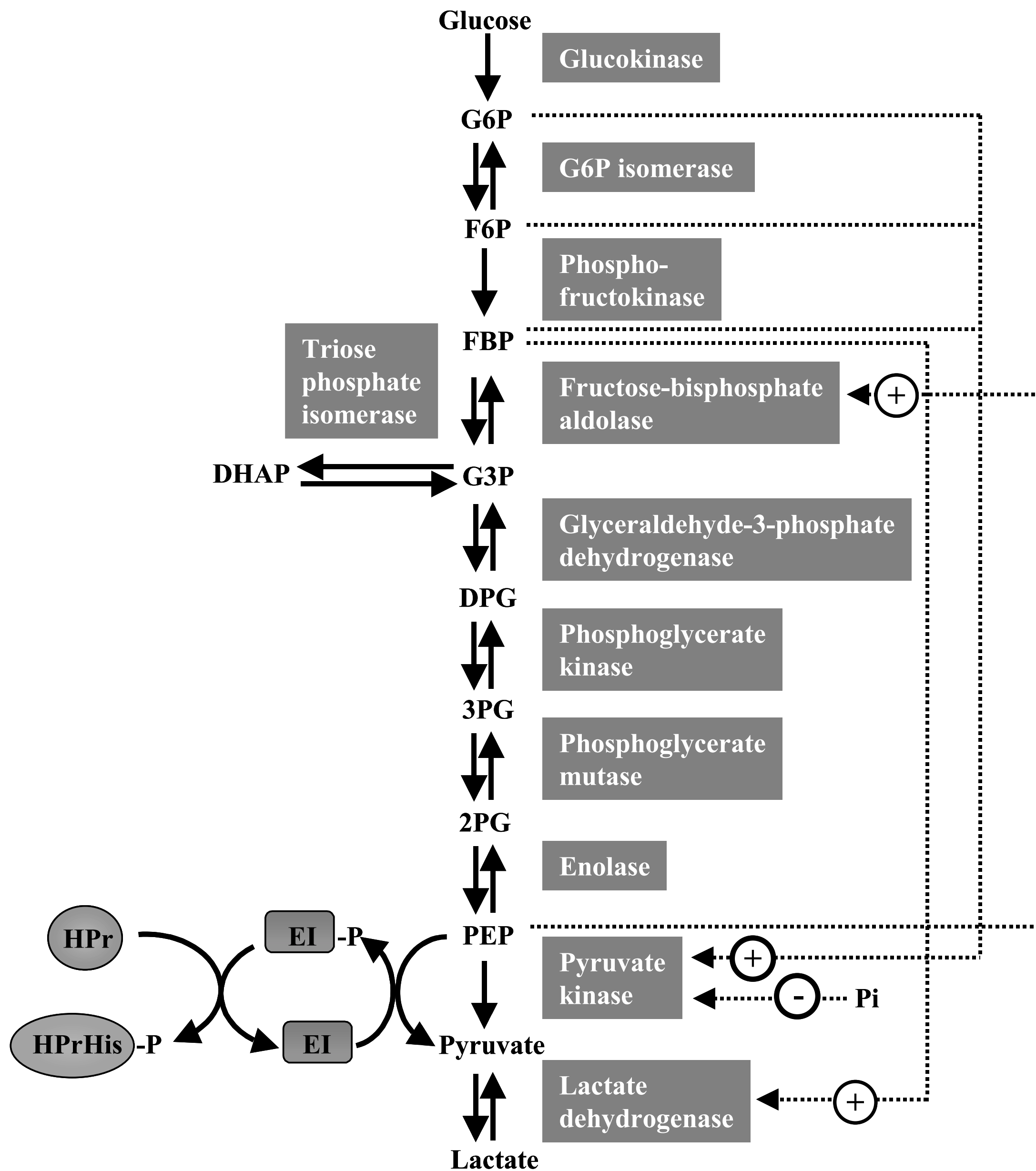

Fig. 2. Schematic overview of the glycolytic or Embden-Meyerhoff pathway, which is the main system for energy generation in lactic acid bacteria (LAB). In some LAB, glycolysis was found to be subject to allosteric intermediate control. In *L. lactis* and *Lactobacillus bulgaricus* phosphofructokinase (PFK) is allosterically activated by phosphoenolpyruvate (PEP), while allosteric control of and pyruvate kinase (PYK) activity includes activation by glucose-6P (G6P), fructose-6P (F6P) and/or fructose-1,6-bisP (FBP) and inhibition by high levels of free phosphate (Pi). In *L. lactis*, lactate dehydrogenase is also activated by FBP, while this is not an activator of the *S. thermophilus* lactate dehydrogenase.

Global control of sugar metabolism

Bacteria actively regulate their metabolism in response to the availability of carbon sources in the growth medium. Less favorable sugars are mainly transported by non-PTS permeases and generally yield lower energy levels and slower growth rates relative to PTS sugars (Postma *et al*., 1993). Carbon catabolite repression enables the cell to preferentially utilize the most favorable carbon source present in the growth medium and to repress operons encoding genes for uptake and metabolism for less favorable sugars. The most important control mechanisms in catabolite repression are global transcriptional control mechanisms and protein control mechanisms of inducer exclusion, which both have been described for species belonging to the enteric Gram-negative bacteria and the low G+C Gram-positive bacteria. Although the mechanisms involved in catabolite control may differ among bacteria, in all cases the intracellular levels of glycolytic intermediates serve as primary indicators of metabolic and energy status, and closely link the rate of carbon influx to the central metabolism.

A common regulatory system comprises the phosphorylation of transcriptional regulators. These include anti-terminators and transcriptional activators with a PTS regulation domain (PRD) that contains multiple phosphorylation sites that are recognized by P-His-HPr (Stulke *et al*., 1998). In enteric Gram-negative bacteria the enzyme IIA^{Glc} of the glucose specific (PTS) is dephosphorylated primarily by PTS-mediated transport, thereby modulating adenylate cyclase activity (Busby and Ebright, 1999; Postma *et al*., 1993). In response to the level of cyclic AMP (cAMP), catabolite activator protein (CAP) is the central transcription regulator modulating expression of catabolite-controlled genes. Recently, two other pleiotropically acting factors Cra and Mlc, have been described in *E. coli* (Plumbridge, 2001; Saier and Ramseier, 1996). Cra controls key genes for gluconeogenesis, the Krebs cycle, and the glyoxylate shunt, whereas Mlc regulates expression of *ptsHIcrr* operon (encoding HPr, EI and IIA^{Glc}). Moreover, enzyme IIA^{Glc} also participates in inducer exclusion of a number of enzymes involved in the metabolism of non-PTS carbon sources (Kolb *et al*., 1993; Postma *et al*., 1993). In *Escherichia coli*, a clear relation was revealed between the [PEP]-[pyruvate] ratio and the phosphorylation state of enzyme IIA^{Glc} (Hogema *et al*., 1998). Other components, besides IIA^{Glc} and CAP, are also involved in carbon regulation. A totally different mechanism for global carbon control is present in low G+C Gram-positive bacteria, in which HPr, HPr kinase/phosphatase (HPrK/P), and the catabolite control protein CcpA are involved in sensing the energy status of the cell and regulating carbon utilization. The mechanisms of catabolite control sugar of sugar metabolism in low G+C Gram-positive bacteria will be described below, with the emphasis on LAB.

Catabolite control in LAB

In Gram-positive bacteria an additional residue of HPr, Ser-46, can be phosphorylated besides the His-15 that is the phosphoryl acceptor and donor involved in PTS transport or phosphorylation of PRD containing regulators (Deutscher and Saier, 1983). The bifunctional HPrK/P modulates the ATP-dependent phosphorylation of the Ser-46 residue of HPr depending on the energy status of the cell (Kravanja *et al*., 1999; Reizer *et al*., 1998) (Fig. 3). The formation of P-Ser-HPr is stimulated by high intracellular FBP levels whereas acummulation of intracellular inorganic phosphate causes its dephosphorylation (Galinier *et al*., 1998; Stulke and Hillen, 2000). Intracellular levels of glycolytic intermediates serve as primary indicators of metabolic and energy status of the cell and modulate the balance

between P-His-HPr and P-Ser-HPr. When PTS substrates are metabolized, the level of glycolytic intermediates, such as FBP, rises and stimulates the kinase activity of HPrK/P to generate P-Ser-HPr. P-Ser-HPr is the main player in global transcriptional control and inducer exclusion (see below). Concommitantly, the rate of sugar consumption via PTS is reduced, because P-Ser-HPr is not a substrate for EI and does not participate in uptake of carbohydrates (Deutscher *et al*., 1994). As soon as the level of glycolytic intermediates drops and the level of inorganic phosphate rises, phosphatase activity of HPrK/P is stimulated, yielding HPr and consequently leading to increased PTS transport (Kravanja *et al*., 1999). It is clear that also non-PTS carbon sources influence the glycolytic intermediate levels in the cell, which is reflected in the P-His-HPr/P-Ser-HPr ratio and can therefore exert catabolite control.

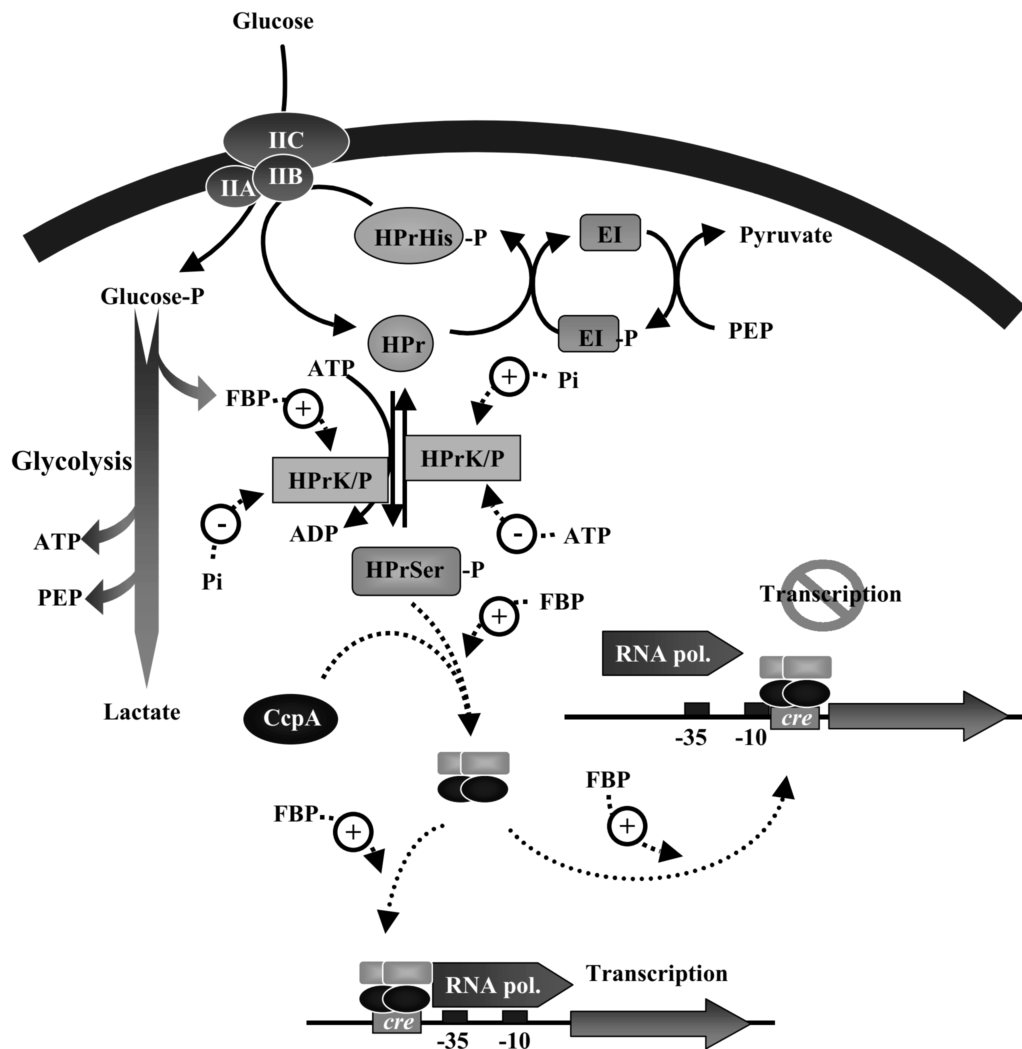

Fig. 3. Schematic representation of P-Ser-HPr/CcpA-mediated catabolite control in Gram-positive bacteria. Fast metabolizable sugars (e.g. glucose) are generally taken up by the PTS and and lead to the accumulation of fructose-1,6-bisP (FBP), which in turn results in the formation of P-Ser-HPr by the induction of HPrK activity. The P-Ser-HPr/CcpA complex subsequently regulates the expression of catabolic genes by binding to the *cre* sequence in promoter-regions. Depending on the position of the *cre* relative to the -10/-35 boxes transcription is repressed (top) or activated (bottom).

Transcriptional control by CcpA

P-Ser-HPr acts as a corepressor for the catabolite control protein A (CcpA), which exerts global transcriptional control (Deutscher *et al*., 1995) (Fig. 3). CcpA is a member of the LacI-GalR family of bacterial regulator proteins and is the central transcriptional regulator of catabolite control, as was shown first for *Bacillus subtilis,* where it mediates glucose repression of the α-amylase gene *amyE* (Henkin *et al*., 1991). Following this study, CcpA has been identified in a large number of low G+C Gram-positive bacteria, including a variety of LAB (Kuster *et al*., 1996). In all cases, CcpA has been found to be a 35 kD protein carrying a helix-turn-helix DNA binding motive and is functional as a homo-dimer. The P-Ser-HPr/CcpA complex regulates transcription of specific genes at a global level by binding to a *cis*-acting sequence termed catabolite response element (*cre*) (Hueck and Hillen, 1995; Miwa *et al*., 2000). Both the formation of the P-Ser-HPr/CcpA complex and the binding to of the complex to the *cre* sequences is stimulated by FBP (Deutscher *et al*., 1995; Kim *et al*., 1998). Mutation analysis of the *ccpA* gene in *B. megaterium* and *B. subtilis* has shown that CcpA-mediated catabolite repression can be completely uncoupled from growth on glucose (Kraus and Hillen, 1997; Kuster-Schock *et al*., 1999). Another study showed that only specific operons were rendered insensitive to catabolite control in *B. subtilis* strains producing certain CcpA mutants (Turinsky *et al*., 2000). The role of P-Ser-HPr in CcpA-mediated catabolite control was demonstrated by replacement of the *ptsH* gene (encoding HPr) with variant alleles encoding HPr of which residue serine 46 was replaced by aspartic acid that mimics a phosphorylated serine residue, and by alanine that can no be phosphorylated (Deutscher *et al*., 1994). Expression of HPr-S46A rendered CcpA-controlled genes insensitive to CR, thereby strongly resembling the effects of the inactivation of the *ccpA* gene. Production of HPr-S46D led to CcpA-mediated catabolite repression, even in the absence of glucose, thereby mimicking the activity of P-Ser-HPr (Deutscher *et al*., 1994; Luesink *et al*., 1999).

Different modes of transcriptional control by CcpA have been described, depending on the position of the *cre* relative to the promoter region (Henkin, 1996). The *cre*'s of *amyE* in *B. subtilis* and of the *gal* operon in *L..lactis*, are located within the promoter region, suggesting that CcpA binding prevents initiation of transcription P-Ser-HPr/CcpA complex (Henkin *et al*., 1991; Luesink *et al*., 1998). Some genes contain multiple *cre* sites, such as the gluconate operon (*gnt*) in *B. subtilis*, which presumably contribute synergistically to CR in response to specific environmental conditions (Miwa *et al*., 1997). The *cre* boxes of the *hut* operon involved in histidine utilization are located within the coding region, where they cause a "roadblock of transcription" (Wray and Fisher, 1994). Catabolite control by CcpA involves not only repression of genes and operons but also activation. In *B. subtilis*, transcription of the *alsS* and *ackA* genes (encoding α-acetolactate synthase and acetate kinase, respectively) is activated by CcpA when glucose is present in the medium (Grundy *et al*., 1994; Renna *et al*., 1993). In *L. lactis* the *pfk, pyk* and *ldh* genes comprise the *las* operon (Llanos *et al*., 1993) that is activated by CcpA upon glucose growth (Luesink *et al*., 1998). The reactions catalyzed by PFK, PYK and LDH are highly unidirectional steps in glycolysis and obvious points for CcpA-mediated catabolite control. This, combined with allosteric control of these enzymes by glycolytic intermediates allows strickt control of glycolytic flux in response to carbon source availability. The *L. lactis* glycolytic genes *fba*, *tpi* and *glcK* also appeared to be sugar regulated, although CcpA involvement is still inconclusive (Even *et al*., 2001). In *B. subtilis*, many of the glycolytic genes are organized in operons. Classical approaches demonstrated that the*pgk/tpi/pgm/eno* operon and the *yvbQ/gap* operon in this bacterium are induced by glucose in a CcpA-dependent manner. However, expression was found to be constitutive for

the *pfk/pyk* operon as well as the other glycolytic genes (Tobisch *et al*., 1999). These results were confirmed in *B. subtilis* by recent holistic approaches making use of DNA array technology (Moreno *et al*., 2001). The mechanism of CcpA gene activation is related to the position of *cre* sites upstream of the –35 region of the promoter. It appears that P-Ser-HPr/CcpA complex binding to these *cre* sites leads to stabilization of the RNA polymerase-promoter complex thereby enhancing transcription (Henkin, 1996).

In addition to HPr and CcpA, other *trans*-acting factors have been discovered that participate in global catabolite control. The HPr paralogue Crh (catabolite repression HPr-like protein) has been identified in *B. subtilis* (Galinier *et al*., 1997). Crh lacks a histidine 15 residue and can only be phosphorylated at serine 46. It contributes, besides HPr, to the phospho-serine dependent catabolite control pathway. However, the effect of P-Ser-Chr is less pronounced than that of P-Ser-HPr (Galinier *et al*., 1999). So far, Crh has only been found in Bacilli, suggesting that the role of this protein is restricted to this group of bacteria. In addition to CcpA, the transcriptional regulators CcpB, CcpC, AbrK and GerE are involved in carbon control in *B. subtilis*. CcpB has been shown to mediate catabolite repression of the *gnt* and *xyl* operons in concert with CcpA (Chauvaux *et al*., 1998). However, the contribution of these regulators to global catabolite control appears to be restricted to specific growth conditions and their mode of action remains to be elucidated.

Global transport control

Catabolite control of PTS and non-PTS transport is not restricted to direct transcriptional control. Carbon sources influence the glycolytic intermediate levels in the cell and dictate the P-His-HPr/P-Ser-HPr ratio, which reflects the availability of HPr for phoshoryl transfer in PTS transport (Kravanja *et al*., 1999). Furthermore, P-Ser-HPr is also suggested to be involved in inducer exclusion of PTS and non-PTS sugars. Inducer exclusion is the inhibition of the activity of enzymes that mediate uptake or the production of a sugar specific transcriptional inducer (Dills *et al*., 1980). Various studies reported inducer exclusion of PTS-mediated transport by P-Ser-HPr (Reizer *et al*., 1989; Ye *et al*., 1994; Ye *et al*., 1994; Ye and Saier, 1996). However, the hierarchial uptake of PTS carbohydrates is not a direct reflection of the inhibitory action of P-Ser-HPr. The work of Reizer *et al*., showed that a negative charge on residue 46 of HPr strongly inhibited PTS-mediated sugar uptake but that competition of two PTS permeases for P-His-HPr is quantitatively more important to the regulation of PTS function than serine 46 phosphorylation (Reizer *et al*., 1989). Apparently, the affinity of P-His-HPr varies for the sugar-specific IIA domains and competition for P-His-HPr leads to hierachial uptake of PTS carbohydrates.

Mechanisms of P-Ser-HPr mediated inducer exclusion of non-PTS sugars have so far only been reported in LAB (Fig. 4) (Reizer *et al*., 1996; Viana *et al*., 2000). In *Lactobacillus brevis*, interaction of P-Ser-HPr with non-PTS permeases inhibits the galactose-H^+ symporter by uncoupling sugar translocation and proton symport (Djordjevic *et al*., 2001) (Fig. 4). The *in vivo* investigation of HPr mutants provided evidence for P-Ser-HPr mediated inducer exclusion in *Lactobacillus casei* (Viana *et al*., 2000). In this study, maltose uptake was strongly decreased by the presence of glucose in a CcpA-independent manner, which appeared to be abolished in a mutant strain that expressed HPr variants, mutated at serine 46 or isoleucine 47. Similar results were obtained in a strain harboring a mutation in the HPrK/P gene, disabling the phosphorylation of HPr at serine 46 (Dossonnet *et al*., 2000). In a *L. lactis* mutant producing HPrS46A, a similar relieve of glucose-mediated inhibition of maltose and ribose uptake was observed (Monedero *et al*., 2001). However, the overall contribution of P-

Ser-HPr in inducer exclusion still needs to be established. Inducer expulsion refers to the mechanism by which previously internalized carbohydrates or their phosphorylated derivatives are expelled, thereby lowering the intracellular concentration of a sugar-specific transcriptional inducers (Reizer *et al*., 1983; Saier *et al*., 1996). This mechanism is described as a two step-process in which first accumulated sugar-phosphates are dephosphorylated and subsequently expelled against the concentration gradient in a carrier-mediated manner (Ye and Saier, 1995).

The transcriptional activation is prevented of genes encoding enzymes required for the utilization of less-preferred carbohydrates (Fig. 4). Hexose-6-phosphate hydrolases have been isolated from *L. lactis* that can perform this function (Thompson and Chassy, 1983). These appeared to be induced by HPrS46D and are therefore presumably controlled by P-Ser-HPr (Ye and Saier, 1995). However, this postulated role of P-Ser-HPr has not been substantiated

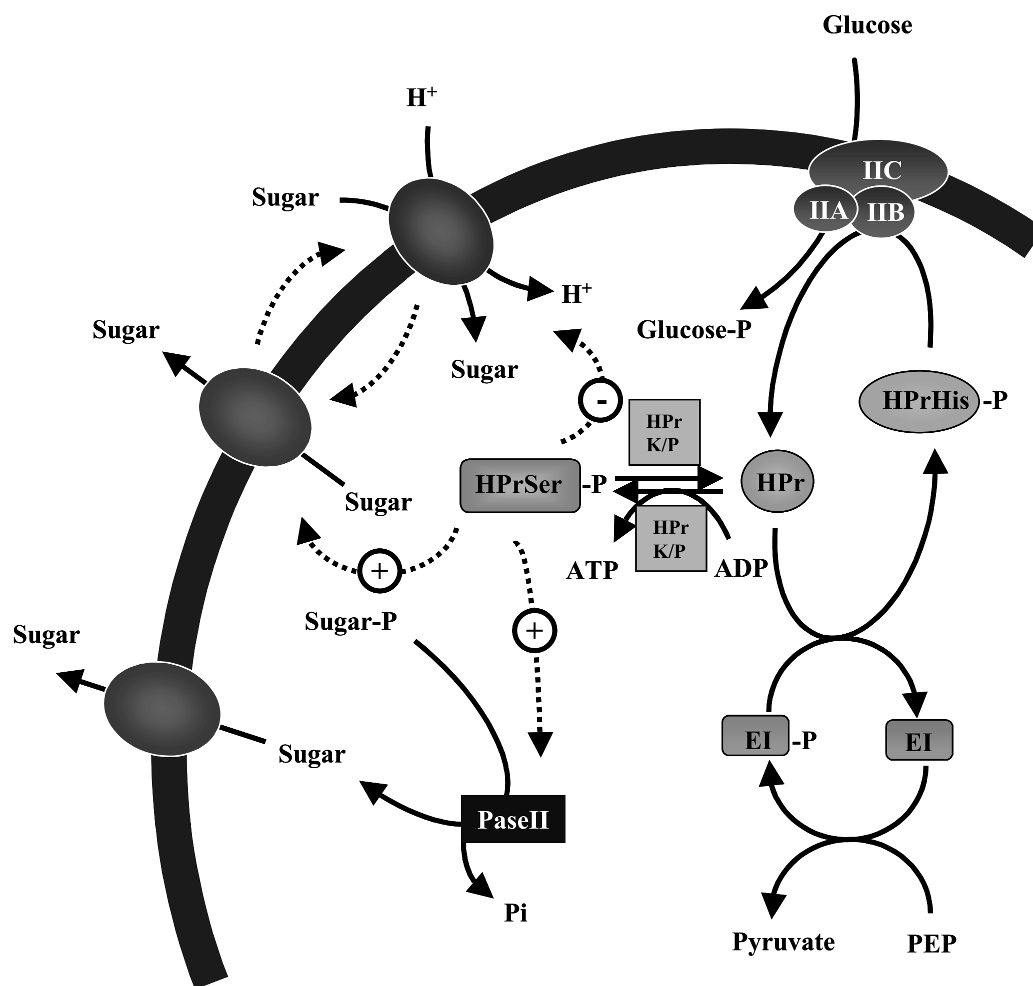

Fig. 4. Proposed mechanisms of inducer exclusion and inducer expulsion mediated by P-Ser-HPr. Carbon sources influence the glycolytic intermediate levels in the cell and dictate the P-His-HPr/P-Ser-HPr ratio, which reflects the availability of HPr for phoshoryl transfer in PTS transport. In *Lactobacillus brevis*, inducer exclusion inhibits galactose uptake through the interaction of P-Ser-HPr with the galactose-H^+ symporter. Inducer expulsion is described as a two step-process in which as a first step accumulated sugar-phosphates are dephosphorylated, thereby preventing inducers of transcription of genes encoding enzymes required for utilization of less-preferred carbohydrates.

with *in vivo* experiments and studies in *Lb. casei*, *L.lactis* and *Lb. brevis* revealed no significant differences in inducer expulsion between wild-type strain and its HPrS46A isogenic variants (Djordjevic *et al*., 2001; Dossonnet *et al*., 2000; Monedero *et al*., 2001). Hence, the direct contribution of P-Ser-HPr to inducer expulsion remains to be demonstrated.

Direct modulation of lactose transport activity by P-His-HPr has been demonstrated in *S. thermophilus* (for a review see Gunnewijk *et al*., 2001). The dedicated lactose transporter LacS, like some other members of the GPH family, has an additional carboxyl-terminal hydrophilic domain that is homologous to EIIA proteins of the PTS. P-His-HPr mediated phosphorylation of this domain modulates the lactose/galactose exchange activity of LacS in response to the carbon availability in the growth medium (see below).

Lactose and galactose metabolism in *S. thermophilus*

he primary function of *S. thermophilus* in dairy fermentations is the rapid conversion of lactose to lactate, while it also contributes to important sensory qualities. While numerous other LAB can utilize many sugars, one of the characteristics of *S. thermophilus* is the inability to ferment a large number of different sugars (Mercenier, 1990). Moreover, in contrast to what is found in most bacteria, glucose is a non-PTS carbon source for *S. thermophilus* and is a poor substrate for growth (Poolman, 1993). For *S. thermophilus*, sucrose is one of the few sugars that is taken up by a PTS and allows rapid growth, albeit that maximal growth rates for sucrose are lower than for lactose. Lactose is the primary carbon and energy source in milk and *S. thermophilus* is highly adapted to growth on this carbon source, which is illustrated by the high efficiency of lactose transport by the dedicated permease LacS (Foucaud and Poolman, 1992). Although lactose is efficiently transported and hydrolyzed intracellularly, most strains of *S. thermophilus* do not grow on galactose and only ferment the glucose moiety of lactose, while the galactose is excreted into the medium in amounts stoichiometric with the uptake of lactose (Fig. 5) (Hutkins and Morris, 1987). Transport studies indicated that LacS mediates both galactoside exchange (e.g. lactose/galactose) and galactosides-H^+ symport. However, kinetic transport studies indicate that the exchange reaction is highly favored when galactosides are in excess on either side of the membrane (Foucaud and Poolman, 1992). This may account for the galactose-negative (Gal^-) phenotype of *S. thermophilus* in milk which contains an excess of lactose (Poolman, 1993). Another explanation for the Gal^- phenotype may be the absence of functional Leloir pathway enzymes, including galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT), and UDPglucose 4-epimerase (GalE), products of the *galK, galT*, and *galE* genes, respectively. Remarkably, under appropriate selective conditions, such as limiting lactose and excess galactose, Gal^+ derivatives of *S. thermophilus* were obtained which fermented galactose and contained Leloir enzyme activities (Benataya *et al*., 1991). However, no molecular explanation was given, and the genetics of the Leloir pathway has only been poorly investigated in *S. thermophilus*. The *S. thermophilus lac* operon contains the genes encoding the lactose permease (*lacS*) and a β-galactosidase (*lacZ*) for the transport and hydrolysis of lactose. Transcription of *lacSZ* is induced during growth on lactose (Poolman *et al*., 1989; Schroeder *et al*., 1991). Next to this transcriptional regulation, control of lactose metabolism involves allosteric modulation of lactose transport activity by HPr. P-His-HPr can transfer its phosphoryl group to the enzyme IIA domain of the LacS permease which results in increased lactose/galactose exchange activity (Gunnewijk and Poolman, 2000; Poolman *et al*., 1995). P-Ser-HPr is the dominant phosphorylated HPr species in *S. thermophilus* when grown in excess lactose, whereas P-His-HPr becomes dominant when lactose becomes limiting (Gunnewijk and Poolman, 2000).

Outline of this thesis

The main focus of this thesis is the catabolite control of sugar metabolism in *S. thermophilus* for which the main carbon- and energy-source in milk-fermentations is lactose. It describes the diversity of sugar utilization, the mechanisms of transcriptional control of lactose mtabolism and glycolysis, and the role of the glycolytic intermediates herein. While

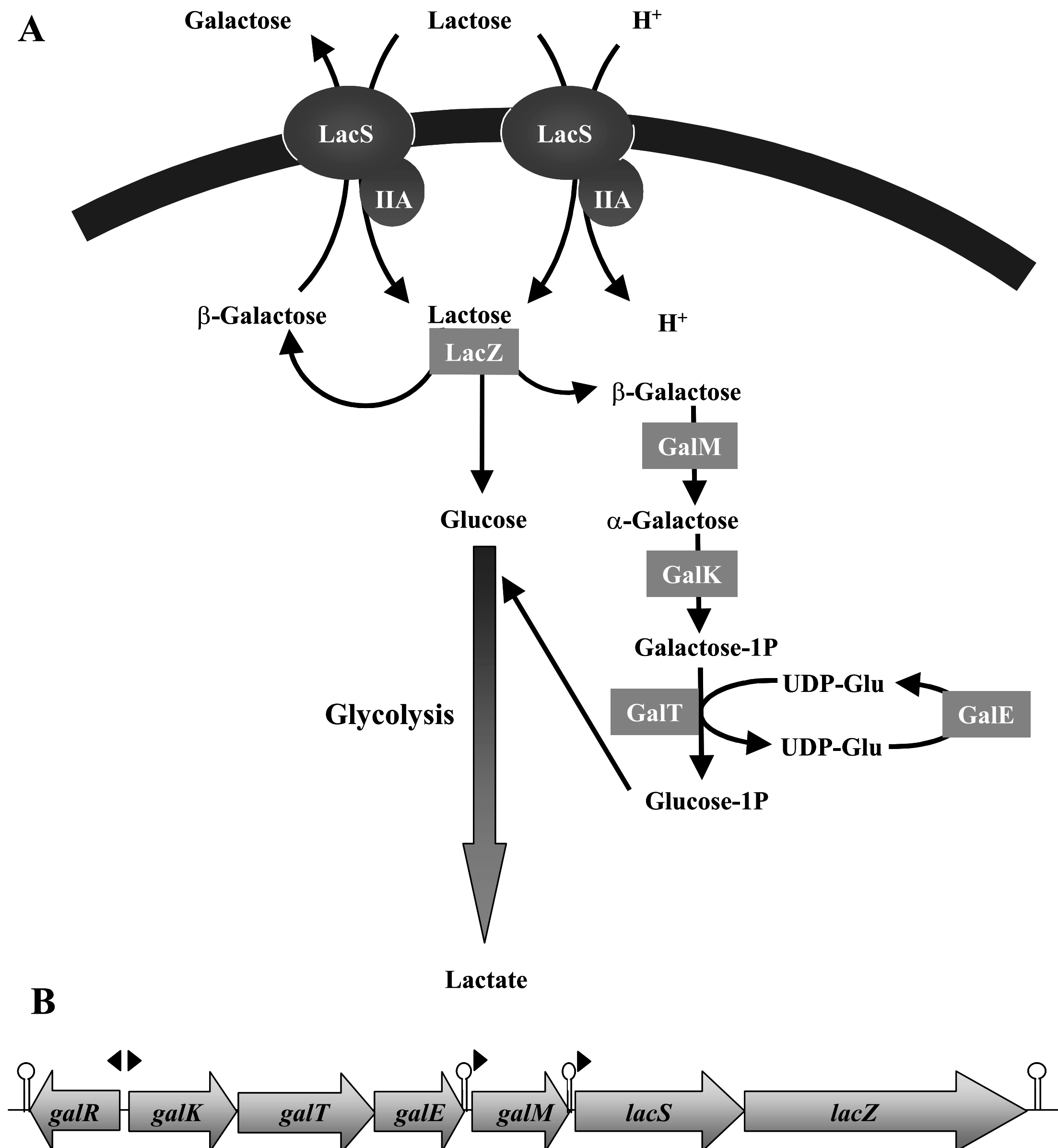

Fig. 5. Schematic overview of the lactose meatabolisms and thegenes involved. A: the two modes of transport by the dedicated lactose permease LacS and subsequent hydrolysis by the β-galactosidase LacZ. Functional Leloir pathway enzymes, including galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT), and UDPglucose 4-epimerase (GalE), are encoded by the *galKTE* operon (B). The *galM* gene could encode a mutarotase that forms the galactokinase substrate β -D-galactose from α-D-galactose pyranose generated from lactose by LacZ. The *S. thermophilus lac* operon contains the genes encoding the lactose permease (*lacS*) and a β-galactosidase (*lacZ*).

most *S. thermophilus* strains do not ferment the galactose moiety of lactose, the genes for galactose utilization and its control were found to be organized in the *galR*-*galKTEM* gene cluster that was identified upstream of the *S. thermophilus lac* operon in strain CNRZ302 (see Chapter 2). This study also revealed that the promoters of the *galKTE* as well as the *lacSZ* operons were induced by the operon-specific regulator GalR upon growth on lactose. However, the induced activity of the *galKTE* promoter was very low which was also reflected in the activities of the enzymes encoded by these genes. Galactose-fermenting mutants were isolated from *S. thermophilus* CNRZ302 that all showed strong *galKTE* expression upon induction due to insertions or substitutions in the promoter region.

The genetic organization of the *gal-lac* gene cluster and its surrounding regions were found to be conserved among a collection of 18 *S. thermophilus* strains isolated from various sources (see Chapter 3). Molecular characterization of these naturally occurring Gal^+ strains revealed up-mutations in the *galKTE* promoter that were similar to those found in the Gal+ variants of CNRZ302. The data presented in this chapter support the hypothesis that the loss of the ability to ferment galactose can be attributed to the low activity of the *galKTE* promoter, probably as a consequence of the adaptation to milk in which the lactose levels are in excess.

Chapter 4 reveals that regulation of lactose metabolism is also subjected to global catabolite control. A catabolite-responsive element (*cre*) was identified in the promoter of the *lacSZ* operon, suggesting CcpA-mediated regulation of this operon. The *S. thermophilus ccpA* gene was cloned, characterized and a *ccpA* disruption strain was made. CcpA was found to play a crucial role in the fine-tuning of lactose transport, β-galactosidase (LacZ) activity, and lactate dehydrogenase activity to yield optimal glycolytic flux and growth rate.

Intracellular levels of glycolytic intermediates serve as primary indicators of metabolic and energy status in the cell. A method for the quantitative detection of glycolytic intermediates in *S. thermophilus* is described in Chapter 5 and was applied to determine these levels in relation to the carbon source used for growth. The *ldh* gene and the *pfk*/*pyk* operon were shown to be subjected to CcpA-mediated activation and the intracellular levels of glycolytic intermediates indicated that clear changes in glycolytic flux coincide with relief of CcpA-mediated catabolite control in response to different carbon sources and their availability (see Chapter 6). The transition in HPr phosphorylation state appeared to coincide with a reduction of glycolytic potential, next to the increase of lactose transport capacity. As a consequence, catabolite repression of the *lacSZ* promoter was relieved, thereby increasing the total lactose uptake capacity when lactose is limiting.

Chapter 7 describes the characterization of the *S. thermophilus ptsHI* operon comprising the genes encoding the two general phosphocarrier proteins, HPr and Enzyme I. Attempts to make a functional deletion in the *ptsH* gene were not successful. By over-expressing wild-type *ptsH* and specific mutated forms in a *ptsH* deletion strain of *L. lactis* the functional role of *S. thermophilus* HPr was confirmed. This led to the identification of specific amino acid residues in HPr that are essential for its function in utilization of PTS and non-PTS carbohydrates.

The results described in this thesis describe the modulations of lactose metabolism that allow *S. thermophilus* to adapt effectively to the availability of lactose in the growth medium. The impact of these results is discussed in Chapter 8, followed by a summary (see Chapter 9).

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Chapter 2

Activation of Silent *gal* Genes in the *lac-gal* Regulon of *Streptococcus thermophilus*

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SUMMARY

Streptococcus thermophilus strain CNRZ 302 is unable to ferment galactose, neither that generated intracellularly by lactose hydrolysis nor the free sugar. Nevertheless, sequence analysis and complementation studies with *Escherichia coli* demonstrated that strain CNRZ 302 contained structurally intact genes for the Leloir pathway enzymes. These were organized into an operon in the order *galKTE*, which was preceded by a divergently transcribed regulator gene, *galR*, and followed by a *galM* gene and the lactose operon *lacSZ*. Results of Northern blot analysis showed that the structural *gal* genes were transcribed weakly, and only in medium containing lactose, by strain CNRZ 302. However, in a spontaneous galactose-fermenting mutant, designated NZ302G, the *galKTE* genes were well expressed in cells grown on lactose or galactose. In both CNRZ 302 and the Gal^+ mutant NZ302G, the transcription of the *galR* gene was induced by growth on lactose. Disruption of *galR* indicated that it functioned as a transcriptional activator of both the *gal* and *lac* operons while negatively regulating its own expression. Sequence analysis of the *gal* promoter regions of NZ302G and nine other independently isolated Gal^+ mutants of CNRZ 302 revealed mutations at three positions in the *galK* promoter region, which included substitutions at positions 9 and 15 as well as a single-base-pair insertion at position 37 with respect to the main transcription initiation point. Galactokinase activity measurements and analysis of *gusA* reporter gene fusions in strains containing the mutated promoters suggested that they were *gal* promoter-up mutations. We propose that poor expression of the *gal* genes in the galactose-negative *S. thermophilus* CNRZ 302 is caused by naturally occurring mutations in the *galK* promoter.

INTRODUCTION

After its discovery almost 40 years ago, the *Escherichia coli* lactose operon, encoding enzymes of lactose metabolism, became the first model for gene regulation (reviewed in reference (Beckwith, 1987). The key component of this system is the *lac* repressor (LacI), the product of the *lacI* gene. The *lac* operon contains a primary operator (O_1), which is the major element of repression by LacI, and two pseudo-operators, which enhance repressor binding to O_1 by cooperativity. Control of the *lac* operon also involves activation by the cyclic AMP receptor protein. Many other paradigm systems of negative control have since been described, including GalR, one of the two repressors of the *gal* regulon encoding enzymes of galactose transport and metabolism in *E. coli*. Regulation of the *gal* regulon is mediated through GalR, GalS (Gal isorepressor), and the cyclic AMP receptor protein. GalR and GalS negatively regulate transcription of the two promoters of the *gal* operon, although GalS is not as efficient as GalR (Weickert and Adhya, 1993).

The bioconversion of lactose, which is the primary carbon and energy source in milk, into lactic acid is an essential process in industrial dairy fermentations carried out by lactic acid bacteria. Genetic studies of the metabolic pathways for lactose utilization in these gram-positive bacteria have revealed a variety of *lac* operons that differ from the paradigm known in *E. coli* (de Vos and Vaughan, 1994). The thermophilic yogurt bacteria *Streptococcus thermophilus* and *Lactobacillus bulgaricus* contain a highly homologous *lacSZ* operon in which the β-galactosidase (*lacZ*) gene is located downstream from the *lacS* gene encoding a lactose permease (LacS), which belongs to the galactoside-pentose-hexuronide translocators (Leong-Morgenthaler *et al*., 1991; Poolman *et al*., 1989; Schmidt *et al*., 1989; Schroeder *et al*., 1991).

Although lactose is efficiently transported and hydrolyzed intracellularly, many strains of *S. thermophilus* and *L. bulgaricus* do not grow on galactose and ferment only the glucose portion of lactose, while the galactose is excreted into the medium in amounts stoichiometric with the uptake of lactose (Hickey *et al*., 1986; Hutkins and Morris, 1987). Kinetic studies indicated that LacS mediates both galactoside exchange (e.g., lactose-galactose) and movement of galactosides and protons (Foucaud and Poolman, 1992). The exchange reaction is highly favored with excess galactosides on either side of the membrane and may account for the galactose-negative (Gal) phenotype of *S. thermophilus* in milk which contains an excess of lactose (Poolman, 1993). Another explanation for the Gal phenotype may be the absence of functional Leloir pathway enzymes, including galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT), and UDPglucose 4-epimerase (GalE), products of the *galK, galT*, and *galE* genes, respectively. Remarkably, under appropriate selective conditions, such as limiting lactose and excess galactose, Gal^+ derivatives of *S. thermophilus* were obtained which fermented galactose and contained Leloir enzyme activities (Hutkins *et al*., 1985; Thomas and Crow, 1984). However, no molecular explanation was given, and the genetics of the Leloir pathway has only been poorly investigated in *S. thermophilus*. The *lacSZ* operon of strain A147 was found to be preceded by *galE* and *galM* (Poolman *et al*., 1990). The *galM* gene appeared to be constitutively expressed and could encode a mutarotase that, similar to the homologous enzyme of *E. coli*, forms the galactokinase substrate α-D-galactose from β-D-galactose pyranose generated from lactose by β-galactosidase (LacZ) (Bouffard *et al*., 1994). However, *S. thermophilus* A147 is not a Gal^+ strain.

The present study was undertaken to gain insight into the presence and regulation of the *gal* genes of *S. thermophilus* and the mechanism by which the genes, in particular the *galK* gene, are prevented from being expressed. Here we describe the characterization of the *gal* operon, consisting of the *galK, galT* and *galE* genes, and its promoter from *S. thermophilus* CNRZ 302, for which galactose-fermenting (Gal^+) revertants have been reported (Benataya *et al*., 1991). A regulatory gene, *galR*, was identified that is divergently transcribed from the *gal* operon. Analysis of mRNA for the *gal* metabolic genes from a Gal^+ fermenting derivative of CNRZ 302 indicated that regulation occurred at the transcriptional level. In contrast, the *gal* metabolic genes of the original Gal^- strain were not sufficiently transcribed to allow galactose metabolism. Furthermore, we demonstrate that GalR acts as a transcriptional activator of both the *gal* and *lac* operons and negatively regulates its own expression. To the best of our knowledge, this is the first report describing the mechanisms regulating galactose utilization in *S. thermophilus* at the molecular level.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *S. thermophilus* strains were subcultured in M17 broth (Oxoid, Basingstoke, England), containing either 1% lactose, glucose, or galactose as necessary, at 42°C unless stated otherwise. The taxonomic position of strain CNRZ 302 was confirmed by 16S rRNA sequence analysis and corresponded to *Streptococcus thermophilus* (GenBank accession number X68418). *E. coli* strains HB101 or LE392 and TG1 were used for the isolation of pACYC184- and pUC19-derived plasmids and for the propagation of bacteriophage M13 chimeras, respectively. *E. coli* was routinely grown in TY medium (Sambrook *et al*., 1989) or brain heart infusion (Difco) broth with aeration at 37°C. MacConkey agar base (Difco Laboratories) supplemented with 1% galactose was used to

detect galactose-positive (Gal^{+}) *E. coli* strains. Agar media were prepared by adding 1.5% agar to broth. The antibiotics used for selection in media were chloramphenicol at 4 µg/ml and erythromycin at 2.5 µg/ml for *S. thermophilus* and chloramphenicol at 15 µg/ml, tetracycline at 12 µg/ml, and ampicillin at 100 µg/ml for *E. coli*. Em^{r} *E. coli* strains were selected on brain heart infusion agar containing 150 µg of erythromycin per ml.

DNA isolation and manipulations. Transformation and isolation of plasmid DNA from *E. coli* were performed essentially by established protocols (Sambrook *et al*., 1989). Chromosomal DNA was extracted from exponentially growing cells of *S. thermophilus* by the procedure of Hayes *et al*. (Hayes *et al*., 1990).

Strain	Relevant features	Reference or source
E. coli		
HB101	F^{-} *hsdS20* (r_B^-, m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^{r}) *xyl-5 mtl-1 supE44* λ^{-}	(Sambrook *et al*., 1989)
LE392	F^{-} *hsdR514* (r_k^-, m_k^+) *supE44 SupF58 lacY1 galK2 galT22 metB1 trpR55* λ^{-}	(Sambrook *et al*., 1989)
TG1	*Δ(lac-pro) supE thi hsdD5* F'[*traD36 proA^{+}B^{+} lacI^{q} lacZΔM15*]	(Sambrook *et al*., 1989)
S. thermophilus		
CNRZ 302	Wild-type Gal^{-} strain	(Benataya *et al*., 1991)
ST11	Gal^{-} strain	(Mollet *et al*., 1993)
NZ302G	Gal^{+} Class I mutant	This study
SS1	Gal^{+} Class I mutant	This study
SS2	Gal^{+} Class II mutant	This study
SS3	Gal^{+} Class I mutant	This study
SS4	Gal^{+} Class I mutant	This study
SS5	Gal^{+} Class II mutant	This study
SS6	Gal^{+} Class I mutant	This study
SS7	Gal^{+} Class III mutant	This study
SS8	Gal^{+} Class I mutant	This study
SS9	Gal^{+} Class II mutant	This study
NZ302GΔR	NZ302G carrying a disruption in *galR*	This study
ST11ΔR	ST11 carrying a disruption in *galR*	This study
Plasmids		
pACYC184	Tc^{R}	(Chang-Cohen, 1978)
pUC19	Ap^{R}	Gibco-BRL
pNZ680	4.9-kb *gal* insert in pACYC184, Tc^{R}	This study
pNZ273	Contains *gusA* reporter gene, Cm^{R}	(Platteeuw *et al*.., 1994
pNZ6871	Contains *galR* gene, and *galK* promoter of strain CNRZ 302 fused to *gusA*, Cm^{R}	This study
pNZ6872	Contains *galR* gene, and *galK* promoter of strain SS2 fused to *gusA*, Cm^{R}	This study
pG^{+}host9	Temperature sensitive shuttle vector, Em^{R}	(Maguin *et al*., 1996)
pNZ684	pG^{+}host9 with internal fragment of *galR* gene, Em^{R}	This study
pNZ6811	Derived from pNZ6871, carries *galR* gene of strain CNRZ 302, Cm^{R}	This study
Phage		
M13mp18/19		(Yanisch-Perron, 1985)

TABLE 1. Bacterial strains and plasmids used in this study

The Anderson and McKay (Anderson and McKay, 1983) lysis procedure was used to detect plasmid DNA in *S. thermophilus*. Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were used as recommended by the supplier (Gibco-BRL, Life Technologies, Gaithersburg, Md.). DNA fragments were recovered from agarose gels with the GlassMatrix DNA isolation system (Gibco-BRL). Electroporation of *S. thermophilus* was performed by the procedure of Mollet *et al*. (Mollet *et al*., 1993) with the modification that the harvested cells were incubated in the electroporation buffer at 4°C for at least 4 h prior to electroporation. PCR was performed under the conditions described previously (Kuipers *et al*., 1991) using *Taq* polymerse (Life Technologies) or *Pwo* polymerase (Boehringer Mannheim). Oligonucleotides were synthesized by Eurogentech (Gent, Belgium).

Cloning of *gal* genes. *S. thermophilus* CNRZ 302 total genomic DNA was digested with *Eco*RI, and the DNA fragments were separated by agarose gel electrophoresis (0.7% agarose). The DNA was transferred to a GeneScreen Plus (Dupont, Boston, Mass.) membrane by established methods (Sambrook *et al*., 1989). The membrane was hybridized with a 700-bp *Acc*I fragment, containing part of the *S. thermophilus* F140 *galK* gene kindly provided by B. Hutkins (Mustapha *et al*., 1995). The labeling of this fragment with horseradish peroxidase and the hybridization and detection methods were as described in the manufacturer's manual for the ECL system (Amersham, Little Chalfont, United Kingdom). Fragments of approximately 5 kb were recovered, ligated with *Eco*RI-linearized calf intestinal alkaline phosphatase-treated pACYC184, and used to transform *E. coli* HB101 and LE392. Gal^+ clones were selected as red Tc^r colonies on McConkey galactose agar at a frequency of approximately 1%. Analysis of the plasmid content of all 10 Gal^+ colonies indicated that they contained a recombinant plasmid with a 4.9-kb insert that showed an identical restriction pattern. One of the clones, designated HB101(pNZ680), was used in further experiments.

Nucleotide sequence analysis. DNA fragments were subcloned in the phage vectors M13mp18 and M13mp19 with TG1 as a host by using standard techniques (Sambrook *et al*., 1989). Nucleotide sequences of both strands were determined by the dideoxy-chain termination method (Sanger *et al*., 1980), adapted for Sequenase version 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) with either the M13 universal primer or specifically synthesized primers. The *gal* promoter regions of *S. thermophilus* CNRZ 302 and its 10 Gal^+ derivatives were isolated as 350-bp PCR fragments from agarose gels. The purified fragments and primers were annealed by boiling for 5 min and rapidly freezing in liquid nitrogen, and sequencing proceeded as described above. The sequence data were assembled and analyzed with PC/GENE version 6.6 (Genofit, Geneva, Switzerland). Amino acid sequence comparisons were performed with the EMBL (release 31.0), SwissProt (release 28.0), and NBRF/PIR (release 40.0) databases using the FASTA program (Pearson and Lipman, 1988), through the facilities of the CAOS/CAMM Center (Nijmegen, The Netherlands). The curvature of DNA was predicted as described by Munteau *et al*. (Munteanu *et al*., 1998).

Isolation of Gal^+ *S. thermophilus* strains. *S. thermophilus* CNRZ 302 cultures grown in M17 broth supplemented with 1% lactose were diluted 100-fold into M17 broth containing 1% galactose and 0.01% glucose and incubated for 24 h. Cultures that exhibited growth were transferred to M17 broth containing 1% galactose (Gal-M17) and incubated for 16 to 20 h. Ten Gal^+ single-colony isolates were obtained by plating 10 cultures treated as described above on M17 agar with 1% galactose, and these were designated NZ302G and SS1 to SS9.

RNA isolation, Northern blotting, and primer extension analysis. *S. thermophilus* strains were grown in M17 broth (50 ml) containing 1% lactose, glucose, or galactose to an optical density (600 nm) of 0.6 to 1.0. Total RNA was isolated from the harvested cells as described by Kuipers *et al*. (Kuipers *et al*., 1993) with the following modification: before being subjected to bead beating, the cells were treated with 2 mg of lysozyme per ml for 2 min on ice, which increased the RNA yield. The RNA was either fractionated on a 1.0% formaldehyde gel (Sambrook *et al*., 1989) or glyoxylated and fractionated on a 1.2% agarose gel as described previously (van Rooijen and de Vos, 1990). RNA size markers were obtained from Bethesda Research Laboratories. RNA was transferred to GeneScreen Plus membranes by following the protocols outlined by the manufacturers. Hybridizations were performed at 65°C in a 0.5 M sodium phosphate buffer (pH 7.2) containing 1.0% bovine serum albumin (fraction V), 1.0 mM EDTA, and 7.0% sodium dodecyl sulfate, and the blots were washed at 55 to 65°C in 1.0 to 0.1× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% sodium dodecyl sulfate (Sambrook *et al*., 1989).

Gel-purified restriction fragments and PCR products that had been labeled by nick translation with α-^{32}P (Amersham) were used as hybridization probes (Sambrook *et al*., 1989). These included a *galK*-specific probe isolated as a 0.6-kb *Hpa*I-*Hind*III fragment from pNZ680, a *galTE* probe consisting of a 1.2-kb *Pst*I-*Eco*RI fragment, a *galR*-specific probe amplified from pNZ680 with primers 5'-GCC CAA TGA GTA GGC C-3' and 5'-CGG ATA TTA ACT ATC GCT G-3', and a 1.6-kb *lacS*-specific probe generated with primers 5'-TAA CAC AGG TGA TCC AAA GCA-3' and 5'-GGT GAC CAG AAC TCA AGA AG-3'. The primer GALRAS (5'-GTT GAA ATA GAT ACA CCT GC-3'), which is complementary to the 5' end of the sense strand of the *galR* gene, was end labeled with α-^{32}P using polynucleotide kinase (Bethesda Research Laboratories).

Primer extension was performed by annealing 5 ng of oligonucleotide 3'-ACT AAC CAC TCG TAT GCC TGA T-5' and 5 ng of oligonucleotide 5'-GTA TCC TCT GTT ACG G-3' complementary to the mRNA for *galK* and *galR*, respectively, to 20 µg of *S. thermophilus* RNA and performing complementary DNA synthesis as previously described (van Rooijen and de Vos, 1990). The reaction products were separated by electrophoresis on a 5% sequencing gel, together with a sequencing reaction product obtained using the same primers.

Construction of plasmids for analysis of *galK* promoters. The promoters from the CNRZ 302 and a class II Gal$^+$ mutant, strain SS2, were amplified by PCR using primers 5'-CGG GAT CCT GCT AAT TTT GCG ATA TCT G-3' and 5'-CGG AAT TCC TTT AAA CTT TTC TCT TAA C-3', with built-in *Bam*HI and *Eco*RI sites (underlined), respectively. The 210-bp products were cloned into *Bam*HI-*Eco*RI-digested pUC19, generating pNZ680.1 and pNZ680.2. A 1.5-kb *Nsi*I-*Eco*RV fragment from pNZ680 containing the CNRZ 302 *galR* gene and a potential transcription terminator (Fig. 1A) was attached in frame to the *galR* promoters (this step was necessary for the stability of further constructs), using the *Pst*I and *Eco*RV sites in the pUC19 derivatives, generating plasmids pNZ6861 and pNZ6862. The "*gal* promoter and *galR* gene cassettes" were removed as 1.7-kb *Eco*RI-*Hin*dIII fragments (the 3' recessed terminus of the *Hin*dIII sites were first filled) and subsequently ligated into the *Eco*RI-*Sca*I-digested pNZ273. Plasmid pNZ273 contains the *gusA* reporter gene that encodes the -glucuronidase enzyme. The plasmids, designated pNZ6871 and pNZ6872 for the CNRZ 302 and SS2 mutants, respectively, contain the *galK* promoter fused to the *gusA* gene and the *galR* gene under its own promoter. The integrity of the amplified promoter regions was confirmed by sequence analysis. The constructs were initially made in *E. coli* and were subsequently used to transform *S. thermophilus* ST11 and selected on M17 agar containing

1% sucrose and chloramphenicol. Histochemical screening for -glucuronidase activity by selecting for blue colonies with 5-bromo-4-chloro-3-indolyl--D-glucuronide (X-Gluc) (Research Organics Inc., Cleveland, Ohio) was performed as previously described (de Ruyter *et al*., 1996).

Construction and use of integrating and complementing plasmids. A 750-bp *Pml*I-*Nla*IV fragment of the *galR* gene from pNZ680 was ligated into the calf intestinal alkaline phosphatase-treated *Eco*RV site of the thermosensitive pG$^+$host9 vector, generating pNZ684 (Fig. 1C). Electrotransformation of pNZ684 into *S. thermophilus* NZ302G resulted in four Em^r transformants, all of which contained the expected plasmid at 30°C. To obtain integration of pNZ684, cultures grown overnight in M17 sucrose broth with erythromycin at 28°C were diluted 100-fold into fresh medium and reincubated at 28°C to allow the exponential phase of growth to resume. The cultures were shifted to 42°C and grown until they reached stationary phase. Dilutions of the cultures were plated at 42°C, and integrants appeared as Em^r colonies after 24 to 48 h of incubation. Correct integration within the *galR* gene in the chromosome (Fig. 1D) was confirmed by both Southern hybridization and PCR for integrant NZ302GΔR (data not shown). The *galR* gene of strain ST11 was disrupted in the same manner.

To construct a plasmid with the *galR* gene without *gusA*, the *gusA* gene in pNZ6871 was removed by digesting with *Eco*RI-*Hin*dIII and the recessed 3' termini of the remaining 4.9-kb fragment were filled in and ligated to generate pNZ6811.

Enzyme and protein assays and chromatography. The *S. thermophilus* strains were grown in M17 broth containing either 1% lactose, galactose, glucose, or sucrose with the appropriate antibiotics to an optical density at 600 nm (OD_{600}) of 1.0. For the preparation of extracts, cells were disrupted with zirconium glass beads in a Bead Beater (Biospec Products, Bartlesville, Okla.) for 3-min treatments with intervals of 1 min on ice between treatments and cellular debris was removed by centrifugation. The extracts were kept on ice, and enzyme assays were performed within 4 h. Galactose 1-phosphate uridylyltransferase activity was assayed in the resultant extracts with 30 to 350 µg of protein per assay by the spectrophotometric method of Isselbacher (Isselbacher, 1974). β-Galactosidase was assayed at 37°C by the method of Miller (Miller, 1972) using 1 to 6 µg of protein per assay and galactokinase assays by the method of Ajdic *et al*. (Ajdic *et al*., 1996). All enzyme activity measurements presented were the mean of at least two independent experiments. Proteins concentrations were estimated by a dye binding assay (Bradford, 1976).

Lactose and galactose were detected by high-performance liquid chromatography with a refractive index detector (M410; Waters) using a Polyspher CHPb18 column (Merck). The separations were carried out on a M6000 isocratic pumping system (Perkin-Elmer) in combination with an automatic sample injector (717+; Waters), and water was used as the eluent.

Nucleotide sequence accession number. The GenBank accession number assigned to the nucleotide sequence encoding *S. thermophilus galR*, *galK*, *galT*, and the partial *galE* gene is U61402.

RESULTS

Isolation and localization of the *S. thermophilus galK* and *galT* genes.

Southern hybridizations of genomic DNA of *S. thermophilus* strain CNRZ 302 identified an *Eco*RI fragment of approximately 5 kb that hybridized with a probe consisting of a 0.7-kb internal fragment of the *galK* gene of *S. thermophilus* F140 (Mustapha *et al*., 1995). A minibank of fragments including the hybridizing fragment was constructed in the chloramphenicol resistance (*cat*) gene of pACYC184, and the putative *galK* gene of *S. thermophilus* CNRZ 302 was isolated by functional complementation of the *galK2* mutation of *E. coli* HB101. The complementing plasmid, designated pNZ680 (Fig.1A), contained a 4.9-kb insert that allowed HB101 to form red colonies on McConkey galactose agar and utilize galactose as the sole carbon source in minimal M9 medium. Introduction of a frameshift mutation in the *Kpn*I site on pNZ680 resulted in a plasmid which could not restore a Gal^{+} phenotype to HB101, indicating that the *galK* gene was overlapping this site (data not shown). Moreover, pNZ680 also complemented both the *galK2* and *galT22* mutations of *E. coli* LE392, indicating that it also contained the *galT* gene of *S. thermophilus*. Although these *gal* genes are hardly expressed in *S. thermophilus*, the promoter upstream of the *cat* gene in pACYC184 is likely to be responsible for their expression in *E. coli*. The presence of a functional *galT* gene was further confirmed by assaying for GalT enzyme activity. Cell extracts of LE392(pNZ680) contained 119 nmol of GalT activity per min per mg, whereas no activity was detected for the LE392 strain alone.

Organization and similarity studies of the *S. thermophilus gal* region.

Commencing at the *Kpn*I site on pNZ680, nucleotide sequence analysis in both directions revealed the *galK* open reading frame (ORF), 1164 bp in length (Fig. 1A). The deduced *galK* sequence had the strongest similarity to the GalK proteins of several gram-positive bacterial species including *Streptococcus mutans* (83%) and *Lactobacillus casei* (70%) (Ajdic *et al*., 1996; Bettenbrock and Alpert, 1998). The *S. thermophilus* CNRZ 302 GalK was also 79% similar to the GalK of the Gal^{+} *S. thermophilus* F410 strain (Mustapha *et al*., 1995). A potential ribosome binding site (5'-GAGA-3'), complementary to the 3' end of the 16S rRNA of lactic acid bacteria(de Vos and Simons, 1994), was located 8 bp upstream from the first translational initiation codon at nucleotide (nt) 1483.

Upstream of *galK* located in a divergent orientation, a 1014-bp ORF was designated *galR* on the basis of the similarity of its deduced amino acid sequence to proteins of the LacI-GalR family of transcriptional regulators (Weickert and Adhya, 1992) (Fig. 1A). The translational initiation site at nt 1340 is proposed on the basis of the position of the putative ribosome binding site (5'-AGGAGGA-3', nt 1351 to 1345) and the similarity between related proteins (see also below). The *S. thermophilus* GalR had the greatest similarity to the GalR repressor of *S. mutans* (75%; 57% identity) and the potential GalR repressor of *L. casei* (59%;40% identity) (Ajdic *et al*., 1996; Bettenbrock and Alpert, 1998). There was also significant similarity, 53 and 48% (35 and 27% identity), to the evolved β-galactosidase (EbgR) and galactose (GalR) repressors, respectively, of *E. coli* (Hall *et al*., 1989; von Wilcken-Bergmann and Muller-Hill, 1982). An inverted-repeat structure and a stretch of five T nucleotides (nt 95 to 56) that could function as a rho-independent transcriptional terminator (Platt, 1986). followed the *galR* gene sequence.

DNA sequence analysis downstream of *galK* revealed the *galT* gene (1482 bp), whose deduced sequence was similar (67 to 74%) to the GalT proteins from several gram-positive bacteria including *S. mutans, L. casei*, and *Lactococcus lactis* (Fig. 1A) (Ajdic *et al*., 1996; Bettenbrock and Alpert, 1998; Vaughan *et al*., 1998). The stop codon for the *S. thermophilus*

galK gene and the start codon of the *galT* gene were separated by just 19 bp. The translational initiation site for *galT* was preceded by a putative ribosome binding site (nt 2654 to 2660). Finally, a fourth ORF was present immediately downstream of the *galT* gene and reading beyond the pNZ680 clone (Fig.1A). The nucleotide and predicted amino acid sequence were identical to the aminoterminus of the previously characterized *galE* gene from *S. thermophilus* A147 (Poolman *et al*., 1990).

The organization of *lac* genes in relation to the *galE* gene, which have been characterized for *S. thermophilus* A147 (Poolman *et al*., 1990; Poolman *et al*., 1989), was demonstrated to be identical in CNRZ 302. Long-range PCR was performed using primers based on the sequences of the *galK* gene of CNRZ 302 and the *lacS* gene (Poolman *et al*., 1989) of A147, which resulted in the expected 5.7-kb product. Restriction enzyme analysis of the PCR product showed an identical pattern to that of A147, confirming the presence of the *galE, galM*, and *lacS* genes downstream of *galK*-*galT* in strain CNRZ 302 (Fig. 1B).

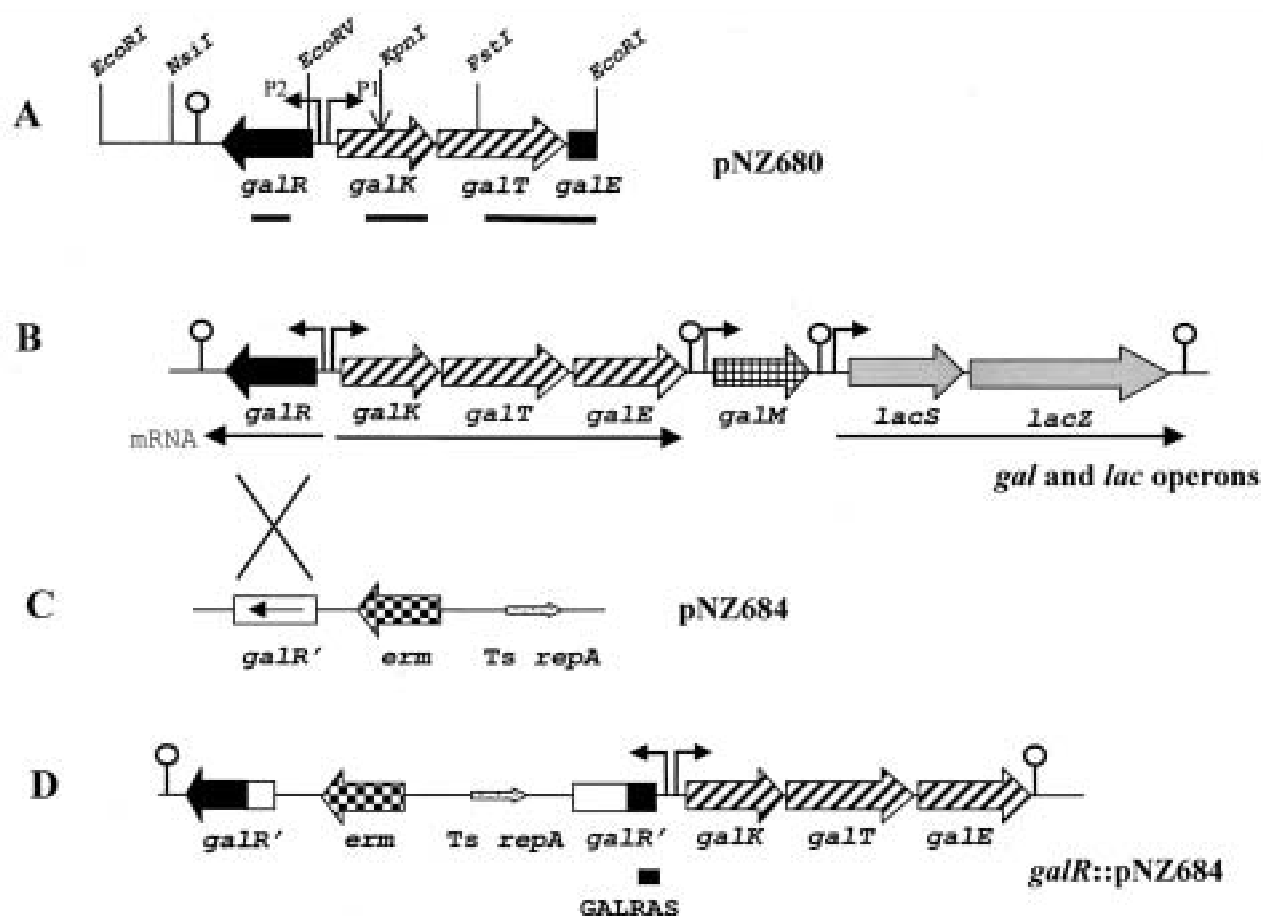


FIG. 1. Organization of the *gal* and *lac* genes in their operons, and construction of a *galR* gene disruption. (A) Illustration of the *gal* genes on the 4.9-kb *Eco*RI fragment of *S. thermophilus* CNRZ 302 cloned in pNZ680. The positions of relevant restriction enzyme cleavage sites are indicated, and the frameshift mutation in the *Kpn*I site is marked with a vertical arrow. The *galK* and *galR* promoters are indicated by arrows P1 and P2, respectively. The black lines below the genes represent the DNA probes used in the Northern blot experiments. (B) Organization of the *gal* and *lac* genes in the chromosome of *S. thermophilus* CNRZ 302. The mRNA transcripts for the *gal* and *lac* genes are indicated by arrows. Positions of promoters and potential terminators are indicated. (C) Plasmid pNZ684 carrying the thermosensitive replicon (Ts *repA*) of pG^{+}host9 (small arrow) is shown in a linear form for convenience; the directions of the sense strand in the 750-bp *Pml*I-*Nla*IV *galR* fragment in pNZ684 and the Em^{r} gene (*erm*) are indicated. (D) Orientation of integration of pNZ684 in the *galR* gene. The two partially deleted copies of *galR* are designated *galR'*, and the location of the primer GALRAS is indicated by the bar.

Transcriptional analysis of the *gal* genes.

The transcription of the *gal* genes was analyzed in the wild-type *S. thermophilus* strain CNRZ 302, and in strain NZ302G, an isogenic spontaneous Gal^+ mutant strain. Strain NZ302G has a doubling time of 58 min in M17 medium containing 1% galactose at 42°C, in contrast to the wild-type CNRZ 302 parental strain which does not grow at all on galactose. The Gal^+ phenotype of NZ302G was stably maintained even after several subcultures in M17 containing lactose.

Northern analysis failed to detect hybridization signals for *galK* or *galTE* from the glucose-grown wild-type and Gal^+ strains (Fig.2). Only after prolonged exposure, were weak signals obtained for lactose-grown wild-type cells (data not shown). However, mRNA was detected for the lactose- and galactose-grown NZ302G cells (Fig. 2), in accordance with the Gal^+ phenotype. The weaker signal obtained for the galactose-grown cells may be due to the poorer-quality RNA obtained as a result of their slower growth. The transcripts were stronger when hybridized with the larger *galTE* probe than when hybridized with the *galK* probe. The size of the predominant mRNA hybridizing to the *galK* and *galTE* probes was approximately 3.7 kb, indicating that the *galK, galT*, and *galE* genes, which are 1.2, 1.4, and 1.0 kb, respectively, are transcribed together as a single mRNA. In conclusion, sufficient induction of *galKTE* mRNA for galactose metabolism occurred only in the Gal^+ NZ302G strain when it was cultured in lactose- or galactose-containing M17 medium. A major transcript of approximately 1.2 kb was identified for *galR* from both the wild-type strain grown in lactose medium and the mutant strain grown in lactose or galactose medium (Fig. 2). However, only a weak signal for this *galR* mRNA was detected in glucose-grown cells for both strains. The size of the transcript for the 1.0-kb *galR* gene suggests that it is transcribed

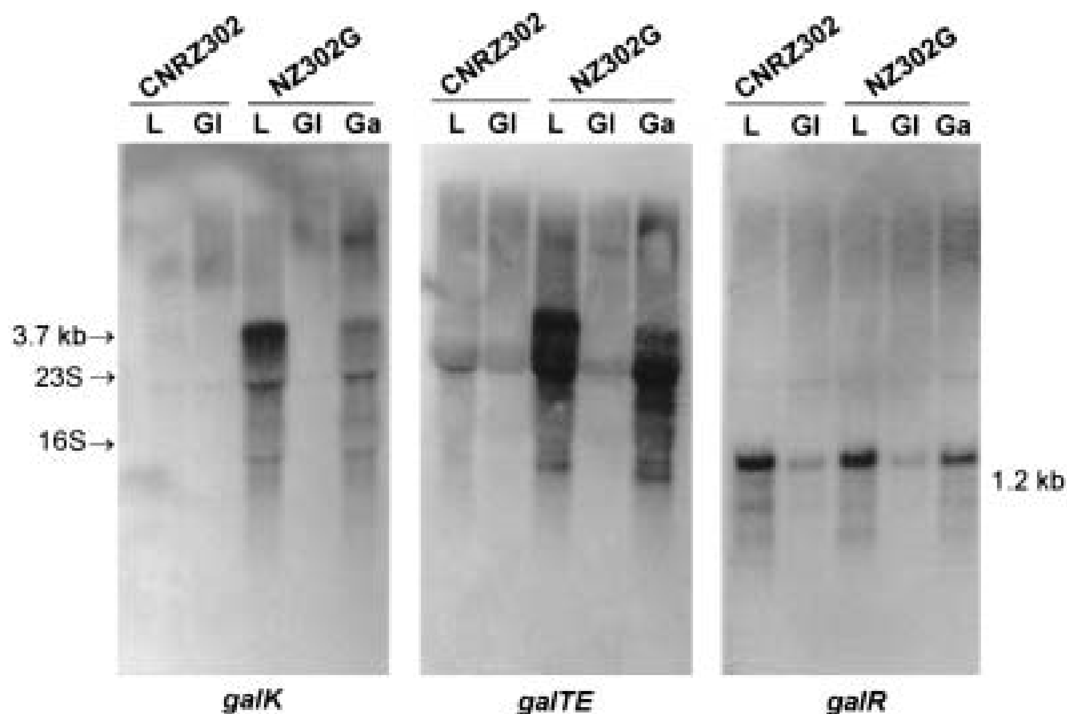


FIG. 2. Northern blot analysis of RNA isolated from *S. thermophilus* strains CNRZ 302 (Gal) and NZ302G (Gal^+) grown on glucose (Gl), lactose (L), or galactose (Ga). The probes used for the hybridization are indicated below the blot. The 23S and 16S rRNAs and the mRNA transcript sizes are indicated by arrows.

alone and supports the functional role of the terminator following *galR*. These data indicate that the expression of the *galR* gene is also regulated at the level of transcription.

Mapping and characterization of the *galR* and *galK* promoters.

The transcriptional start points of the *galK* and *galR* genes were determined by primer extension analysis. Total RNA was isolated from lactose- and glucose-grown *S. thermophilus* CNRZ 302 cells and from lactose-, glucose-, and galactose-grown NZ302G cells. A major transcriptional start site was observed for the *galK* gene of strain NZ302G that mapped at an A residue (nt 1452), 6 bp downstream of the inferred -10 sequence (TACGAT) (Fig.3A). The latter was separated by 17 bp from a -35 sequence (TTGATT) that conforms well to the *E. coli* and *S. thermophilus* promoter consensus sequences (Fig.4A). Essentially no clear signal for the *galK* gene of CNRZ 302 was detected, in accordance with its Gal phenotype (Fig.3A).

For *galR*, a strongly labeled extension product was detected that initiated at a G residue (nt 1352) 7 bp upstream of a putative -10 sequence (TATACT) (Fig.3B) for both CNRZ 302 and NZ302G. A putative -35 sequence (TAGGTA) could be found 18 bp upstream of the -10 box (Fig.4A). The presence of the primer extended products for *galK* and *galR* matched exactly the results obtained in the Northern experiments and confirmed control at the transcriptional level.

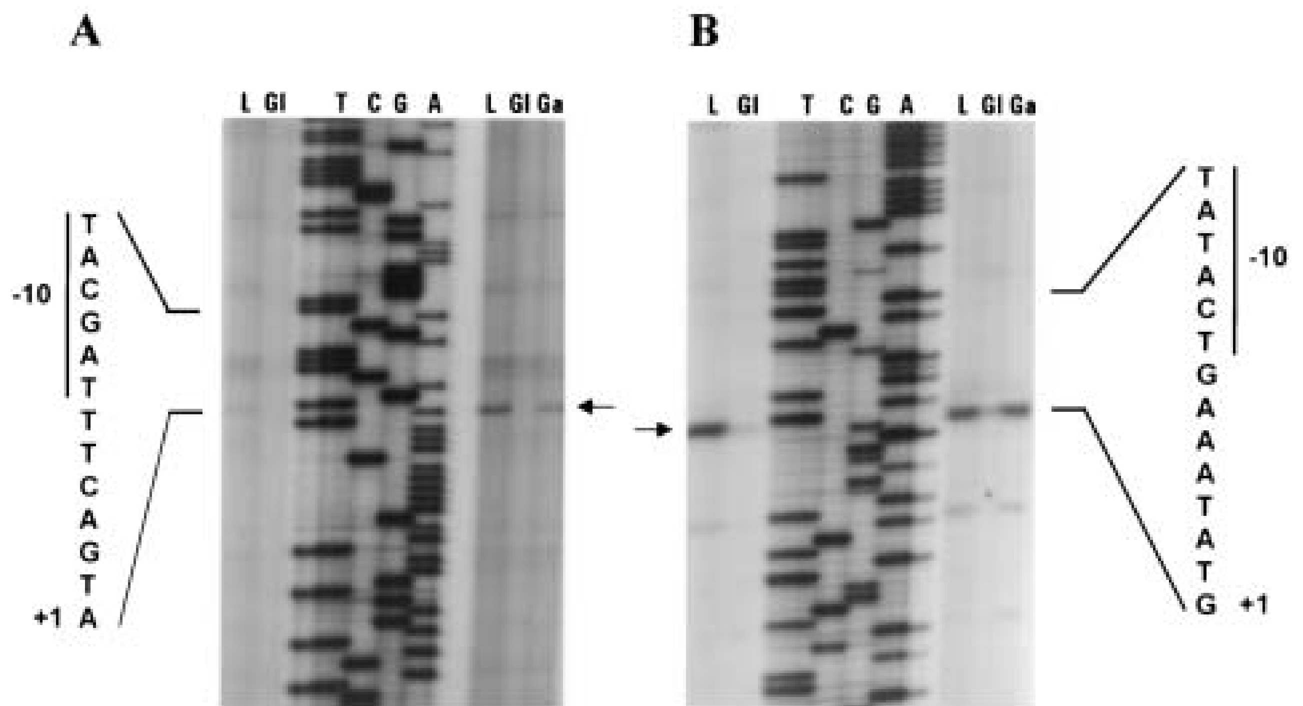


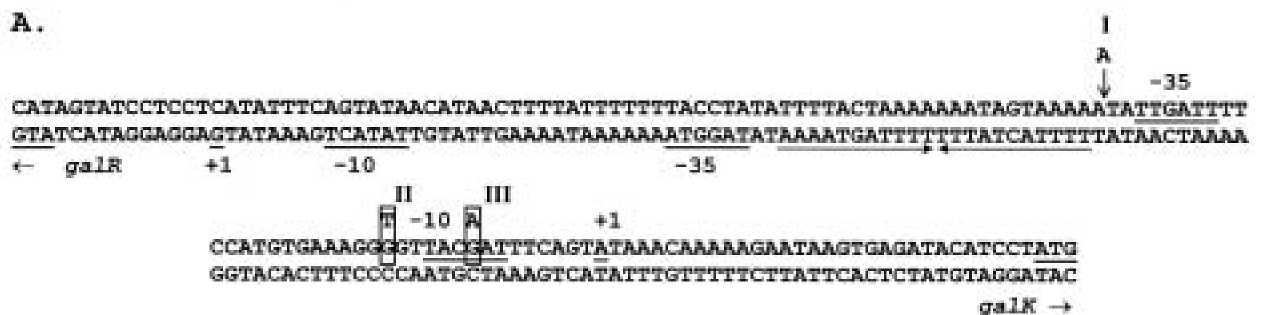
FIG. 3. Primer extension analysis of the 5' ends of the RNA transcripts for the *galK* (A) and *galR* (B) genes of *S. thermophilus* CNRZ 302 (two left-hand lanes) and NZ302G (three right-hand lanes) grown in lactose (L), glucose (Gl) or galactose (Ga). The ladder obtained from pNZ680 sequenced with the relevant primer is in the middle of each panel. Relevant nucleotide sequences of the promoter regions are presented on either side of the figure. The -10 region is denoted by a vertical bar, and the transcription initiation sites are indicated by +1. The arrows point to the major primer-extended products.

Effect of the *galR* gene disruption on galactose utilization.

To determine the function of the *galR* gene in the Gal$^+$ *S. thermophilus* NZ302G, the gene was disrupted using pNZ684, consisting of the temperature-sensitive pG$^+$ host9 vector that carried an internal fragment of the *galR* gene (Fig.1C). The disruption caused by pNZ684 resulted in two partially deleted copies of the *galR* gene, one of which lacks the DNA for the 17 N-terminal amino acids including most of the conserved DNA binding motif while the other suffers from a 200-bp deletion that can encode 67 amino acids of the C-terminal region (Fig.1D). In contrast to the NZ302G strain, the isogenic NZ302GΔR integrant could no longer grow in M17 broth containing 1% galactose. To determine whether NZ302GR could utilize the galactose moiety of lactose, its growth was compared with that of the parental strain NZ302G in medium containing 0.4% lactose. Although NZ302G is Gal$^+$, when the strain is grown in medium containing excess lactose (1%), the glucose moiety is preferentially metabolized while galactose is excreted, and presumably acid inhibits growth and prevents subsequent metabolism of the galactose portion of lactose. Reduction of the concentration of lactose to 0.4% eliminates this imbalance while supporting normal growth of strain NZ302G (Table 2).

High-performance liquid chromatography analysis of the spent medium indicated that the galactose moiety of lactose was completely metabolized by strain NZ302G while, in contrast, a substantial amount of galactose (72% of the amount that could be hydrolyzed from lactose) was not utilized by the NZ302GΔR integrant. Moreover, the doubling time of

A.



B. Microorganism	Gene	Promoter regions
S. thermophilus	*galK*	TTTTACTAAAAAAATAGTAAAAATATTGATT
S. thermophilus	*lacS*	TATTAGTAAAATTTTAGTAAAAAACACTGAAATTATTGACT
S. thermophilus	*galR*	TAAAAGTTATGTTATACTGAAA
S. mutans	*galK*	ATTTAGTAAAATTTTAGTAAAAATATTGACC
S. mutans	*galR*	TTTTACTAAAATTTTACTAAAT

FIG. 4. (A) Intergenic region containing the promoter sequences for the *galK* and *galR* genes. The -10 and -35 regions are underlined, and the transcriptional start sites are indicated as +1. The point mutations for the three classes (I, II, and III) of *S. thermophilus* Gal$^+$ mutants are as described in the text. Inverted repeats are indicated by arrows. (B) Alignment of similar regions in the promoters of *lac* and/or *gal* genes of *S. thermophilus* and *S. mutans*. Inverted repeats in the *S. thermophilus galK* promoter are indicated by arrows. Nucleotides that are complementary in each arm of the inverted repeats are in boldface type.

NZ302GΔR on lactose increased to approximately 1 h in comparison to that of NZ302G, which is 25 min, and the final OD_{600} was less than half that of NZ302G (Table 2).To exclude any possible polar effects of the integration of pNZ684 in the NZ302GΔR integrant, complementation of its *galR* mutation was studied. Plasmid pNZ6811 contains the *galR* gene under its own promoter on a high-copy-number vector based on pNZ123 (de Vos and Simons, 1994). Three transformants of NZ302GΔR harboring pNZ6811 grew to an OD_{600} of approximately 1.4 in M17 medium containing 0.4% lactose broth, and no residual galactose was detected in the cell-free supernatant (Table 2). Furthermore, the transformants could again utilize galactose as the sole carbon source (data not shown). Thus, GalR is necessary for the ability to utilize galactose.

Effect of *galR* disruption on transcription of the *gal* and *lac* genes.

Northern hybridizations were performed to determine the effect of the *galR* disruption on the transcription of the *galR* gene and of the *gal* and *lac* operons. The primer GALRAS, which is complementary to the 5' end of the sense strand of *galR*, was chosen since it can hybridize to the single copy of the 3'-deleted *galR* (*galR*') in the chromosome that is under control of the *galR* promoter (Fig.1D). The 1.2-kb *galR* transcript, which was only weakly visible for NZ302G growing on glucose, gave a signal of much greater intensity for strain NZ302GΔR (Fig.5A). The 5'-deleted copy of *galR* did not appear to be transcribed since the same result was obtained by probing with a 600-bp fragment internal to the *galR* gene (Fig.5B). This constitutive overexpression of the 3'-truncated *galR*' was also observed for lactose-grown cells (data not shown) and suggests that the product of the *galR* gene is a negative regulator of its own expression. It should be noted that termination of transcription of *galR*' in NZ302GΔR is located at an unknown point within the integration plasmid pNZ684, and therefore it is coincidental that the *galR* and *galR*' transcripts appear to have similar sizes.

A *galK* probe was used to monitor the transcription of the *galKTE* operon in NZ302G and the mutant NZ302GR (Fig.5C). While the 3.7-kb transcript of the *galKTE* operon was observed when NZ302G was grown on lactose, no transcript was detected for NZ302GΔR. This indicates that GalR is an activator of transcription for the *galKTE* operon and explains why the galactose moiety of lactose is not effectively metabolized by strain NZ302GΔR.

Strain	Final OD_{600}	% Galactose[a]
NZ302G	1.84	NDb
NZ302GΔR	0.76	0.143
NZ302GΔR1 (pNZ6811)	1.37	ND
NZ302GΔR2 (pNZ6811)	1.30	ND
NZ302GΔR3 (pNZ6811)	1.43	ND

TABLE 2. Growth and galactose utilization of *S. thermophilus* NZ302G and derivatives in medium containing 0.4% lactose

a Percentage of galactose remaining in medium following growth.
b ND, not detectable (<0.01%)

LacS is the sole galactoside transporting activity in *S. thermophilus* and therefore is essential for both lactose and galactose transport (Foucaud and Poolman, 1992). We speculated that GalR may also play a role in the regulation of the *lac* operon (*lacS-lacZ*). Northern hybridization of NZ302G probed with an internal fragment of *lacS* showed that a 5.2-kb transcript, corresponding to the sizes of the *lacS* and *lacZ* genes, was expressed weakly when the strain was grown on glucose (barely visible on the blot) and induced when the strain

was grown on lactose or galactose (Fig.5D). The *lac* operon was still transcribed in NZ302G R when the strain was grown on glucose, but there was no longer induction of transcription when it was grown on lactose. This indicates that in addition to being the positive regulator of the *gal* operon, GalR functions as an inducer of transcription of the *lac* operon.

Effect of *galR* disruption on GalT and LacZ enzyme activities.
To further substantiate the effect of the *galR* gene disruption on the expression of the *gal* and the *lac* operons, enzyme assays were performed. Since the *galT* gene is the central one of the three genes in the *gal* operon, GalT activity was used as a measure of the activity of the Leloir pathway enzymes (Table 3). In strain NZ302G, GalT activity is induced three- to fourfold in lactose- and galactose-grown cultures in comparison to growth in glucose-containing medium. In contrast, very low activity was detected in the *galR* disruption strain NZ302GΔR when grown on glucose and no significant increase was observed following growth on lactose, indicating a lack of induction of the enzyme. However, the levels of activity of the NZ302GR mutant complemented by pNZ6811 were similar to those of NZ302G on all the sugars tested and thus were also induced on lactose and galactose.

LacZ activity was measured to determine the expression of the *lac* operon genes. In NZ302G, LacZ activity was induced about two- and fourfold during growth on lactose and galactose, respectively, in comparison to growth on glucose (Table 3). In contrast, LacZ levels in NZ302GΔR were not induced during growth on lactose. LacZ was again inducible in NZ302GΔR carrying the *galR*-expressing plasmid pNZ6811, although the activity was not as high as that of the original strain. Thus, the enzyme activity measurements confirmed the transcriptional analysis results, demonstrating the role of GalR as an activator of the *gal* and *lac* operons.

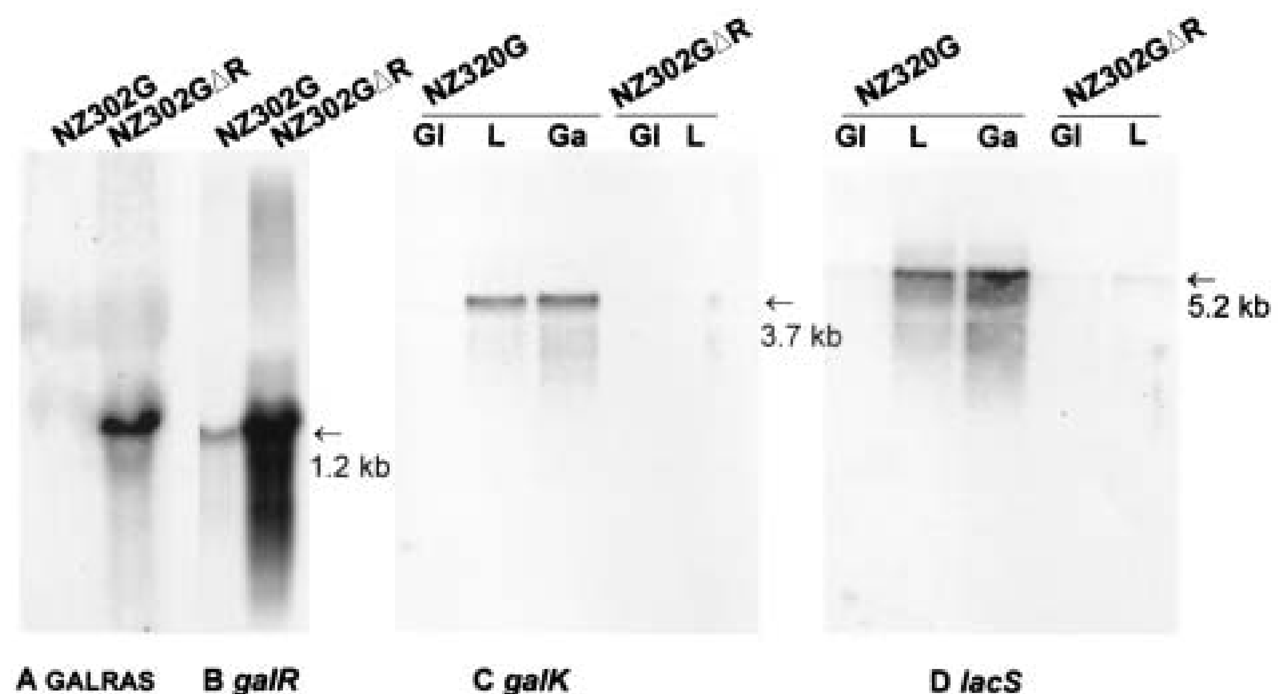


FIG. 5. (A and B) Northern blot analysis of RNA from *S. thermophilus* NZ302G and NZ302GR grown on glucose hybridized with the GALRAS primer (A) and *galR* probe (B). (C and D) Northern blot analysis of RNA from *S. thermophilus* NZ302G and NZ302R grown on glucose (Gl), lactose (L), or galactose (Ga) hybridized with the *galK* probe (C) and the *lacS* probe (D). The mRNA transcript sizes are indicated by arrows.

To analyze whether the activating role of GalR was specific for the galactose-utilizing strain NZ302G, the *galR* gene of *S. thermophilus* ST11, a well-characterized Lac^{+} Gal strain (Mollet *et al*., 1993), was also disrupted using the pNZ684 construct. PCR analysis indicated an identical organization of the *gal* genes in this strain and in CNRZ 302 (data not shown). Since strain ST11 grows very poorly on glucose, LacZ activities were compared using medium containing sucrose (Table 3). Both ST11 and ST11ΔR showed an induction of LacZ of approximately 2.5- to 3-fold after growth on lactose in comparison to growth on sucrose, but the levels of the LacZ activity of ST11ΔR were at least half those produced by ST11 on both carbon sources. The failure to fully induce LacZ in ST11ΔR strongly suggests that GalR also plays a role in activating the *lac* operon of this strain.

Carbohydrate	Strain	Gal-1-phosphate uridylyltransferase activity (nmol/min/mg of protein)[a]	β-Galactosidase activity (µmol/min/mg of protein)[a]
Glucose	NZ302G	59 ± 6	4.2 ± 0.9
	NZ302GΔR	19 ± 3	3.1 ± 0.5
	NZ302GΔR(pNZ6811)	70 ± 12	2.5 ± 0.4
Lactose	NZ302G	178 ± 73	7.7 ± 0.8
	NZ302GΔR	23 ± 9	3.1 ± 0.4
	NZ302GΔR(pNZ6811)	149 ± 35	6.7 ± 1.8
Galactose	NZ302G	230 ± 70	15.6 ± 2.8
	NZ302GΔR(pNZ6811)	393 ± 21	9.9 ± 0.8
Sucrose	ST11	ND[b]	4.8 ± 1.0
	ST11ΔR	ND	2.2 ± 0.3
Lactose	ST11	ND	13.3 ± 0.9
	ST11ΔR	ND	5.5 ± 0.6

TABLE 3. Enzyme activity measurements of *S. thermophilus* strains NZ302G, ST11, and derivatives.

a Results are expressed as mean ± standard deviation. The enzyme assays were performed on the same extracts within 4 h.
b ND, not determined.

Characterization of the *galK* promoters for the Gal^{+} mutants.

To gain an understanding of the ability of NZ302G to transcribe the *gal* metabolic genes, in contrast to the parent strain CNRZ 302, a series of hybridization experiments using specific *galR* and *galK* probes on the *gal* region and PCR amplification of the *galKTE* gene cluster were performed on the Gal^{+} mutant. However, no DNA structural rearrangements were detected within *galR* or the metabolic *gal* gene cluster compared to the parent CNRZ 302 strain (data not shown). The *galR* and *galK* promoter regions of CNRZ 302 and NZ302G were amplified by PCR in duplicate with the same primers as those used in the primer extension experiments, and both strands of each product were sequenced. The analysis revealed that an extra A residue was inserted in a stretch of adenines preceding the -35 region (nt 1410 to 1414 [Fig.4A]) of the *galK* promoter in the NZ302G DNA sequence, resulting in 6 A residues in the mutant in comparison to 5 in the Gal^{-} parent. To determine whether other

Gal$^+$ mutants of CNRZ 302 contained similar mutations in the promoter region, nine more independently isolated Gal$^+$ mutants of CNRZ 302 were investigated. DNA sequence analysis of these nine promoters showed that the *galK* promoter of each Gal$^+$ mutant also contained a point mutation. The mutants could be divided into three classes based on their mutations: class I consisted of five mutants with an A insertion, as described above for NZ302G; in class II, the three mutants contained a G-to-T substitution 3 bp preceding the -10 box; and the one mutant in the third class had a G-to-A substitution in the -10 box (Fig.4A).

galK **promoter activity.**

The GalK activity of the mutant strains was used as a reporter for comparing the expression of the mutated promoters with that of the wild-type. A low level of GalK activity, 10 nmol/min/mg, was detected for the Gal *S. thermophilus* CNRZ 302 grown on glucose-containing medium, and it increased to 37 nmol/min/mg on lactose. For the Gal$^+$ NZ302G and the other five class I mutants, the GalK activity increased from an average 46 nmol/min/mg on glucose to 264 and 305 nmol/min/mg on lactose and galactose, respectively. The activity increased from 53 nmol/min/mg on glucose to 193 and 323 nmol/min/mg on lactose and galactose, respectively, for the class III mutant. The highest activity was observed with the three class II mutants, an average of 68 nmol/min/mg on glucose to 400 and 458 nmol/min/mg on lactose and galactose, respectively. Thus, it is likely that the basal GalK activity has increased in the mutant strains due to promoter-up mutations, which allows sufficient expression of the *galK* gene on induction for galactose utilization.

To support our hypothesis that point mutations in the promoters were largely responsible for the Gal$^+$ phenotype of the mutants, the *galK* promoters of CNRZ 302 and SS2 (class II mutant) were cloned in front of the *gusA* reporter gene in plasmids pNZ6871 and pNZ6872, respectively. Both plasmids were stable in *E. coli*, but the frequency of transformation for pNZ6872 in *S. thermophilus* ST11 was consistently 10-fold lower than for pNZ6871. Since ST11 grows very poorly on glucose, the strength of the promoters was examined in medium containing sucrose or lactose. Expression of the *gusA* gene results in -glucuronidase activity, which is indicated by the development of a blue color in colonies on plates containing the substrate X-Gluc. Surprisingly, growth of *S. thermophilus* ST11 transformants harboring pNZ6871 resulted in blue colonies on X-Gluc plates containing sucrose, while on lactose plates very small and bluer colonies developed, suggesting increased activity from the *galK* promoter. Colonies of ST11 harboring pNZ6872 developed as very small blue colonies on both sucrose and lactose plates, indicating strong activity of the class II mutant promoter on both carbon sources. However, ST11 transformed with pNZ6872 resulted in colonies that were smaller than those obtained with pNZ6871, and some white colonies also appeared. Analysis of the plasmid content of these colonies demonstrated that the pNZ6871 construct remained intact while deletion derivatives of the pNZ6872 construct were present in ST11 (data not shown). Thus, pNZ6872 is unstable, probably due to toxic effects of high -glucuronidase activity.

DISCUSSION

This study demonstrates conclusively that *S. thermophilus* CNRZ 302 possesses the full complement of genes necessary for galactose metabolism despite its Gal phenotype. In many organisms, the *galK, galT*, and *galE* genes, which constitute the Leloir pathway of galactose metabolism, may be clustered or organized in a single operon, and the order of these genes within the operon may be highly variable. While the similarity between the deduced

primary sequences of the enzymes is very high within the lactic acid bacteria, the genomic organization of the *gal* clusters and gene order is species specific (Grossiord *et al*., 1998). The gene order for the *S. thermophilus galKTE* operon and the divergent *galR* gene is identical to that in *S. mutans* (Ajdic *et al*., 1996), which reflects the evolutionary relationship between these bacteria. A potential transcriptional regulatory gene, *galR*, has also been identified in *L. casei* and is transcribed in the *L. casei gal* operon *galKETRM* (Bettenbrock and Alpert, 1998). In contrast, the putative homologues in *E. coli, galR* and *galS*, are not linked to the *gal* operon (Weickert and Adhya, 1993), and regulatory genes have as yet not been identified for other *gal* operons in lactic acid bacteria such as *L. lactis*, *Leuconostoc lactis*, and *Lactobacillus helveticus*.

The effect of glucose, lactose, and galactose on the *galR* mRNA levels strongly suggested that GalR was a transcriptional regulator of the *S. thermophilus gal* operon. Transcriptional analysis of the two *galR* copies generated following disruption of *galR* in the Gal^+ NZ302G indicated that the copy resulting in a C-terminally truncated GalR protein is driven by the *galR* promoter. The second copy, which lacks most of the DNA binding motif, was not transcribed. This was expected based on the orientation of the *galR* gene fragment in pNZ684. Since the substantial deletion from the C terminus includes residues contributing to inducer binding and dimerization (Weickert and Adhya, 1992), the truncated *S. thermophilus* GalR proteins are no longer functional. Northern analysis of NZ302GΔR confirmed that GalR functions as an activator of transcription for the *gal* operon and explained the inability of the disruption mutant to use galactose or the galactose moiety of lactose as a carbon source. The very low activity still detected for the GalT enzyme in NZ302GΔR might be due to a basal transcription level in the absence of GalR. Alternatively, it might reflect a low level of reversion to wild type. When the *galR* gene was provided in *trans*, the Gal^+ phenotype was restored.

The constitutive transcription of the nonfunctional *galR* gene in the absence of GalR indicates that the latter normally functions as a negative regulator of its own expression. Autoregulation is a common feature in prokaryotic gene regulation strategies. Negative autoregulation has been reported for other members of the LacI-GalR family such as GalS, PurR, and CytR (Gerlach *et al*., 1990; Meng *et al*., 1990; Weickert and Adhya, 1993). It is noteworthy that the majority of LacI-GalR members function as negative regulators while some positive regulators also belong to this family and some (like CcpA) perform both functions (Ramseier *et al*., 1995; Saier *et al*., 1996; van den Bogaard *et al*., 2000).

The expression of the *lac* operon genes of both *S. thermophilus* NZ302G and ST11 is induced by growth on lactose- or galactose-containing medium. In both strains, β-galactosidase activity could no longer be induced to the usual level when the *galR* gene was disrupted, confirming that GalR also functions as a transcriptional activator of the *lac* operon. β-Galactosidase is more strongly induced in NZ302G in galactose-containing medium than in lactose-containing medium. This differential gene expression of the *lac* genes may be due to catabolite repression by the glucose moiety of lactose on the *lac* operon promoter (van den Bogaard *et al*., 2000), which will result in repression of the *lac* operon by lactose but not by galactose. Furthermore, the excretion of galactose by LacS in lactose medium would effectively reduce the availability of inducer in the cell.

The mutation to a Gal^+ phenotype does not result in constitutive expression of the *gal* genes that are induced in the presence of lactose and galactose, which strongly suggests that *S. thermophilus* was Gal^+ but became Gal^- in the recent past. While the advantages of the exchange reaction of the lactose transport protein offer a rationale for the observed excretion of galactose (Poolman, 1993), the precise mechanism by which the enzymes of the Leloir

pathway are suppressed has not been determined. Characterization of the 10 Gal^+ mutants of CNRZ 302 revealed that a point mutation had occurred in the *galK* promoter region of every isolate, the majority of which were single-base insertions in a homopolymeric run of adenine residues. Interestingly, a study of the molecular basis for the adaptive response of *E. coli* populations to conditions of nonlethal selection such as nutrient deprivation also identified single-base variations mainly in short mononucleotide repeats (Foster and Trimarchi, 1994; Rosenberg *et al*., 1994), and slipped-strand mispairing was proposed as the responsible mechanism. In the *S. thermophilus galK* promoter, the G-to-A substitution in the class III mutant results in a -10 box (TACAAT) with greater homology to the -10 consensus (TATAAT) sequence (McClure, 1985). In class II mutants, the G-to-T substitution gives a TG doublet 1 bp upstream of the -10 sequence, which is a feature present in the promoters of gram-positive bacteria (de Vos and Simons, 1994). This may correspond to the "extended - 10" sequence that functions as a -35-independent promoter and requires the TG motif for efficient initiation at such promoters (Keilty and Rosenberg, 1987). Thus, these substitutions that resemble promoter-up mutations may increase the level of transcription of the *gal* genes and allow metabolism of galactose. The A insertion in class I mutants may also be a promoter-up mutation, although the reason for the enhanced activity in this case is not so apparent. The extra A increases the size of the inverted repeat preceding the -35 box from 11 to 15 nt (see below). In particular, the intrinsic DNA curvature that is predicted in this region is enhanced by the A insertion (data not shown), and this may result in increased promoter strength. The presence of curved DNA upstream of promoters, of which A tracts appear to be a major determinant, is associated with increased transcription (Perez-Martin *et al*., 1994).

The CNRZ 302 and SS2 *galK* promoter fusions to the *gusA* gene support the hypothesis that mutations in the *galK* promoter of *S. thermophilus* CNRZ 302 suppress the expression of the *gal* genes. Although β-glucuronidase activity was not expected from the ST11(pNZ6871) strain since galactokinase activity is barely detectable in CNRZ 302, factors such as the high copy number of this plasmid (de Vos and Simons, 1994) and the gene dosage effect of the GalR activator are likely to be responsible. The only difference between the pNZ6871 and pNZ6872 plasmids was the G-to-T mutation in the *galK* promoter of the latter. Very high -glucuronidase activity as a consequence of this promoter-up mutation would result in lethal effects on the host (P. G. G. A. de Ruyter, personal communication). This would explain the reduced frequency of transformation for pNZ6872, the small size of the blue colonies on medium containing X-Gluc, and the instability observed for this plasmid in the *S. thermophilus* host.

The intergenic region between *galR* and *galK* of *S. thermophilus* consists of 142 bp, which contains the promoter sequences of both genes in a back-to-back configuration (Fig.4A). An 11-bp inverted-repeat sequence (IR) (nt 1392 to 1413; 5'-TTTTACTA-3', 8 out of 11 matching nt) was detected in this region that could be an operator for GalR. The potentially global regulation by GalR prompted us to search for homologous sequences in the promoters of the *lac* operon and *galR* gene of CNRZ 302. Similar 11-bp IRs were found 13 bp upstream of the -35 box in the *lac* promoter and also overlapping the -10 box of the *galR* promoter (Fig.4B). A consensus sequence could be deduced, with the 11-bp half of the IR consisting of a central 3 bp highly conserved portion, A(C/G)T, flanked on either side by four predominantly adenine and thymine bases. It is usually the -40 to -35 region of a promoter that is approached by an activator site; exceptions include the MerR family regulators (Collado-Vides *et al*., 1991; O'Halloran, 1993). In contrast, sites for repressors may be located from the -35 to -10 region. When activator proteins are used for repression, which generally occurs in cases of autoregulation, the operators often appear in positions for

repression rather than activation. The potential operator sites for GalR conform to these general rules.

The *gal* genes of *S. mutans* are homologous to those of *S. thermophilus* and are organized in a similar divergent orientation (Ajdic *et al*., 1996). Alignment of the nucleotide sequences of the *S. thermophilus* and *S. mutans* promoter regions revealed homologous palindromic sequences, as described above (Fig.4B). It is noteworthy, however, that a single-crossover disruption of the *S. mutans galR* gene resulted in constitutive expression of galactokinase, indicating that GalR functions as a repressor of the *gal* operon in this species. In contrast, GalR of *S. thermophilus* activates the *gal* and *lac* operons while repressing its own expression. The presence of these potential operators gives further credence to the hypothesis that repression of the *gal* operon was caused by recent mutations in the *galK* promoter.

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Chapter 3

Sugar Utilization and Conservation of the *gal-lac* Gene Cluster in *Streptococcus thermophilus*

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SUMMARY

Characteristic for *Streptococcus thermophilus* is the adaptation to lactose as primary carbon and energy source of which only the glucose moiety is utilized while the galactose moiety is excreted into the growth medium. In this study we evaluated the diversity of sugar utilization and the conservation of the *gal-lac* gene cluster in a collection of 18 *S. thermophilus* strains isolated from a variety of sources. For this purpose diagnostic PCR, Southern hybridization and nucleotide sequence analysis was performed on DNA from these isolates and the results were compared with those obtained with a strain from which the complete genome sequence has been determined. The sequence, organization and flanking regions of the *S. thermophilus gal-lac* gene cluster was found to be highly conserved among all strains. The vast majority of the *S. thermophilus* strains were able to utilize only glucose, lactose, and sucrose as carbon sources, some strains could also utilize fructose and two of these were able to grow on galactose. Molecular characterization of these naturally occuring Gal^{+} strains revealed up-mutations in the *galKTE* promoter that were absent in all other strains. These data support the hypothesis that the loss of the ability to ferment galactose can be attributed to the low activity of the *galKTE* promoter, probably as a consequence of the adaptation to milk in which the lactose levels are in excess.

INTRODUCTION

Streptococcus thermophilus is used in a variety of industrial dairy fermentations at elevated temperatures where it forms a major component in cheese starters, and, in symbiotic association with *Lactobacillus bulgaricus*, is involved in production of yogurt (Auclair and Accolas, 1983; Giraffa *et al*., 2001; Randazzo *et al*., 2002). The primary function of *S. thermophilus* in these dairy fermentations is the rapid conversion of lactose into lactate but it also produces other components that may contribute to taste, aroma and texture of the fermented product. Characteristic for this organism is the inability to ferment a large number of sugars that can be utilized by other lactic acid bacteria (Mercenier, 1990). The phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) catalyzes the transport and concommitant phosphorylation of carbohydrates and is the main sugar uptake system in lactic acid and other bacteria (de Vos and Vaughan, 1994; Postma *et al*., 1993). For *S. thermophilus*, sucrose and fructose are the only sugars that are taken up by a PTS, albeit that the maximal growth rate on these sugars is lower than that on lactose. Remarkably, glucose is a non-PTS sugar for *S. thermophilus* and the maximal growth rate on glucose is several-fold lower than that on lactose (Poolman, 1993; van den Bogaard *et al*., 2000). *S. thermophilus* is highly adapted to growth on this lactose although this sugar is not transported by PTS but by the efficient dedicated permease LacS (Foucaud and Poolman, 1992). The *S. thermophilus lacSZ* operon codes for this lactose permease (*lacS*) as well as the β-galactosidase (*lacZ*) that is involved in the hydrolysis of the internalized lactose. The transcription of the *lacSZ* operon is induced during growth on lactose and subject to catabolite control by CcpA, resulting in fine-tuning of lactose uptake and hydrolysis to subsequent catabolism via glycolysis (Poolman *et al*., 1989; van den Bogaard *et al*., 2000; Vaughan *et al*., 2001). Most *S. thermophilus* strains used as industrial starters do not utilize galactose and only ferment the glucose moiety of lactose, while the galactose moiety is excreted into the medium in stoichiometric amounts relative to the lactose taken up (Hutkins *et al*., 1985). Recently, the genes for galactose utilization and its control were found to be organized in the *galR-galKTEM* gene cluster that was identified upstream of the *S.*

thermophilus lac operon in strain CNRZ302 (Vaughan *et al*., 2001). The same study also revealed that the promoters of the *galKTE* as well as the *lacSZ* operons were induced by the operon-specific regulator GalR upon growth on lactose. However, the induced activity of the *galKTE* promoter was very low which was also reflected in the activities of the enzymes encoded by these genes. Galactose-fermenting mutants were isolated from *S. thermophilus* CNRZ302 that all showed strong *galKTE* expression upon induction due to insertions or substitutions in the promoter region (Vaughan *et al*., 2001).

This study was undertaken to investigate the diversity of sugar utilization and the genetic conservation of the *gal-lac* gene cluster in a collection of *S. thermophilus* strains isolated from various sources. Sugar utilization was found to be restricted to glucose, lactose, sucrose and fructose in most strains, while only two strains were able to ferment galactose. The genomic sequence of *S. thermophilus* LMG18311 (Hols *et al*., 2002) allowed detailed genetic analysis of the *gal-lac* locus and its position in the chromosome. The genetic organization of the *gal-lac* gene cluster was conserved in all strains tested as were the surrounding regions, suggesting conservation of its chromosomal location. Genetic analysis of the divergent *galR-galKTE* promoter region showed that the natural galactose-fermenting *S. thermophilus* isolates contained *galKTE* promoter-up mutations that are to likely mediate the galactose-fermenting phenotype of *S. thermophilus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and culture conditions. The strains and plasmids used in this study are listed in Table 1. *S. thermophilus* was routinely grown at 42°C in M17 broth (Difco, Surrey, U.K.) containing 1% (w/v) of the chosen carbon source unless stated otherwise. *Escherichia coli* HB101 (Sambrook *et al*., 1989) was grown in TY broth with aeration at 37°C, and for plasmid selection ampicillin (Ap) 50 μg/ml was used.

S. thermophilus strain	Relevant features	Reference or source
ATCC 19258	Type-strain, pasteurized milk	ATCC (Herman and McKay, 1986)
LMG18311	Yogurt starter	LMG (Hols *et al*., 2002)
CNRZ302	Yogurt starter	CNRZ (Benataya *et al*., 1991)
NZ302G	Gal$^+$ derivative of CNRZ302	(Vaughan *et al*., 2001)
A054	Yogurt starter	(Schroeder *et al*., 1991)
ST8	Proteolytic starter	LODI, Italy
ST11	Commercial starter	(Mollet *et al*., 1993)
NIZO B110	Yogurt	NIZO
NIZO B112	Pasteurized milk	NIZO
NIZO B113	Pasteurized milk	NIZO
NIZO B126	Milk	NIZO
NIZO B132	Milk	NIZO
NIZO B1122	Danish yogurt starter	NIZO
NCIMB 701968	Bulgarian cheese starter	NCIMB
NCIMB 702533	Italian cheese	NCIMB
NCIMB 702560	Emmental starter	NCIMB
NCIMB 702561	Calf rennet	NCIMB
NCIMB 702562	Gorgonzola cheese	NCIMB
NCIMB 702637	Gruyère starter	NCIMB

Table 1. Strains used in this study.

Sequencing of the 16S rRNA variable regions. Species verification of the *S. thermophilus* strains used in this study was carried out by sequencing the 16S rRNA variable regions as described previously (te Giffel *et al*., 1997).

DNA manipulations and transformations. Transfer to and isolation of plasmid DNA from *E. coli* was performed using established protocols (Sambrook *et al*., 1989). Plasmid and chromosomal DNA from *S. thermophilus* was isolated as described previously for *L. lactis* (Vos *et al*., 1989). All DNA-modifying enzymes were used as recommended by the suppliers (Gibco/BRL, Boehringer Mannheim or Roche). DNA fragments were recovered from agarose gels using the Glass Matrix DNA Isolation System (Gibco/BRL).

Organizational analysis of the *S. thermophilus gal-lac* gene cluster and surrounding regions. A PCR-based approach was used in to determine the organization of *gal-lac* gene cluster and its surrounding regions in the different laboratory and starter strains of *S. thermophilus*. Primer combinations were selected from the oligonucleotides (Table 2) that allowed the organizational analysis of the *gal-lac* gene cluster in such a manner that the position of each gene and the length of the intergenic regions could be determined from the generated PCR products using Taq polymerase (Gibco/BRL)(Fig. 1). The oligonucleotides were designed based on the *gal-lac* operon sequence from strain LMG18311 (Hols *et al*., 2002).

Primer	Sequence (5'- 3')	strand	Position	used for PCR products
metalF	TGACGACAGTTGCAATTGACGG	C	839 – 860	A, B
transR	CATGCCATTGTCTGGACGATTCC	NC	1564 – 1542	B
transF	CGTCCAGACAATGGCATGATGG	C	1547 – 1568	C
galRR	CTCTTTTAACGATACAACTCTAGC	NC	2392 – 2369	A, C
41	GTCGTCAGGGACCTTG	C	2404 – 2419	D
galR1	GTTGAAATAGATACACCTGC	C	3154 – 3174	*galRK* promoter region
81	TAGTCCGTATGCTCACCAATCA	NC	3456 – 3435	*galRK* promoter region
42	CTTGCAAGAAGCTGGGC	C	3649 – 3665	E
12	CGACATTATCCTTGAGGTC	NC	3968 – 3950	D
88	GATACGGTCCAATTCCTC	NC	4607 – 4590	E
galT4	CTCCTAACAATTGTTGAAGC	C	5241 – 5260	G, F
galER	TACGAAGATATTCAACAGCAAGG	NC	6773 – 6751	G
galEF	GCCTTAGACTTAGGTTCATCAACAGG	C	6791 – 6816	H
galMF	CAATGGAAACTGGTGCTC	C	7245 – 7262	I
galMR	TCCATAGCAATAGCTTCACG	NC	8046 – 8027	H, F
lacS2R	GATAAAGTATGTTGACAAGG	NC	8362 – 8343	I
lacS2F	CATTTGCCGTAGCTGCCGG	C	9471 – 9489	J
lacZR	CAAAGAGATCTTGAACGTGAAC	NC	10850 – 10829	J
exoF	GCAAGAATCTGATGCTACATGG	C	13102 – 13123	K
exoR	CGTCCTACATGCCAATCTGACG	NC	14143 – 14122	K

Table 2. Oligonucleotides used in this study.

Cloning and sequence analysis of the *galK* promoter regions. Oligonucleotides galR1 and 81 (Table 2) were used in a PCR reaction using high fidelity Pwo polymerase (Roche) to amplify the *galR-galK* promoter regions with chromosomal DNA isolated from the different *S. thermophilus* strains as template. The 0.3-kb PCR fragments obtained were cloned in the pGEM-T vector (Promega) and subsequently both strands were sequenced using an ALF DNA sequencer (Pharmacia Biotech) according to protocol recommended by the supplier. These

galR-galK promoter regions were compared in an DNA sequence alignment to the *galR-galK* promoter region of CNRZ302 and its galactose-fermenting mutants (Vaughan *et al*., 2001).

Growth and sugar utilization. Overnight cultures grown in glucose M17 medium were diluted to an OD_{600} of 0.05 in fresh medium supplemented with 1% (w/v) of the appropriate sugar, and growth was followed spectrophotometrically. All strains were subjected to an API-50 test to evaluate the sugar fermentation ability according to the protocol recommended by the supplier (Biomerieux, France).

Nucleotide sequence accession numbers. The *gal/lac* sequence data have been submitted to the GenBank database under accession number AF503446.

RESULTS

***S. thermophilus gal-lac* operon sequence comparison.**

The organization and regulation of the *gal* and *lac* genes has been studied in considerable detail in the *S. thermophilus* strains CNRZ302 and A147, respectively (Poolman *et al*., 1990; Poolman *et al*., 1989; Vaughan *et al*., 2001). However, the availability of the genome sequence of *S. thermophilus* LMG18311 allowed for the complete annotation of the *gal-lac* gene cluster (Hols *et al*., 2002). The *gal-lac* gene cluster is located at approximately 1.24 Mb relative to the predicted origin of the 2.0 Mb genome of this yogurt starter and resides on the minus strand. This cluster is flanked at the upstream side by two small incomplete and partially overlapping ORF's, predicted to code for products with high degree of homology (82%) to a putative IS*861* transposase from *S. agalacticae* (Fig. 1). Further upstream, an ORF was identified that could encode a product with strong similarity (77%) with the zinc-metalloprotease encoded by the *zmpB* gene of *S. pneumoniae*. Downstream of the *gal-lac* cluster an ORF was located, predicted to code for a product with high similarity (70%) to an exonuclease, encoded by the *sbcD* gene of *Lactococcus lactis*.

The comparison of the complete or partial *gal-lac* sequence information of the strains LMG18311, CNRZ302 and A147 revealed that their overall sequence similarity was very high, while the organization of the *gal-lac* genes was found to be identical (Fig. 1). This conserved organization was also found in the recently reported sequence of *S. thermophilus* strain SMQ-301 (Vaillancourt *et al*., 2002). Moreover, the G+C content of the LMG18311 *gal-lac* gene cluster matches that of the whole genome, suggesting that these important catabolic genes were not recently aquired via horizontal gene transfer. However, detailed comparisons showed considerable difference in degree of sequence similarities between the various individual genes of the *gal-lac* gene cluster (Table 3). While the regulatory gene *galR* was highly conserved and showed no substitutions at all, the enzyme-coding genes showed larger diversity. This diversity was found to be largest in the *galE* (22/1010 nucleotides) and *lacS* genes (16/1904 nucleotides). Remarkably, the *galE* sequence of strain A147 contained 3 nucleotides less than that of the other strains, resulting in two frame-shifts. This generated a new sequence of 11 amino acids (from residue 187 to 188) in the predicted GalE protein of strain A147. It remains to be established whether this frameshift is the result of sequencing errors since the A147 GalE was found to be functional (Poolman *et al*., 1990). Apart from these unique amino acids, all other substitutions in GalE appeared to be rather uniformly distributed. This contrasts with the substitutions in the LacS protein sequence, many of which were found to be located in the hydrophobic membrane spanning regions while none were located in the regulatory IIA domain. Not only the regulatory gene *galR* but also all regulatory sequences showed little sequence variation. The *galR-galK*

promoter region was found to be identical in the strains LMG18311, CNZ302 and SMQ-301. Furthermore, the *lacS* promoter region was identical in all strains and contained an almost perfect *cre* site that has been implicated in catabolite repression (Poolman, 1993; van den Bogaard *et al.*, 2000). A single nucleotide substitution was present in the putative ribosome binding site (RBS) of A147 *LacS* relative to LMG18311 and SMQ-301, making it less homologous to postulated consensus RBS sequences in LAB (De Vos and Simons, 1988). These results indicate that not only the organization of the *gal-lac* gene cluster is very well conserved but notably all regulatory regions.

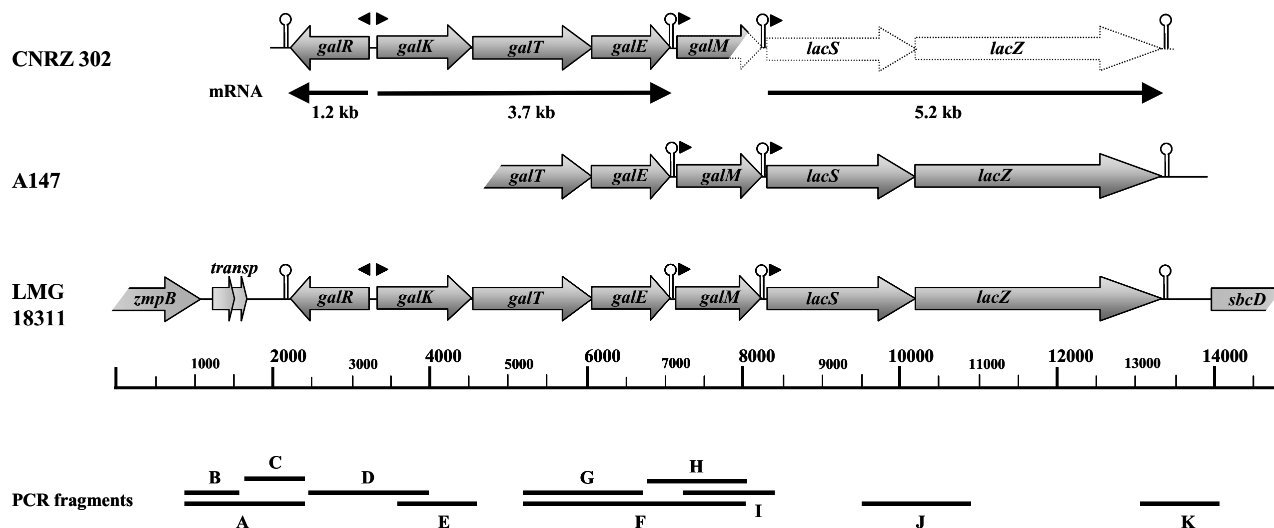

Figure 1. Organization of (partialy) sequenced *S. thermophilus gal-lac* gene clusters. A: Open arrows indicate genes, promoters are denoted by black arrowheads and the terminators are indicated by stem-loop structures. For strain CNRZ302 the presence of the *galM-lacSZ* genes downstream of the complete *galE* was confirmed by long-range PCR and partial sequence analysis. The transcripts of this *gal-lac* gene cluster are denoted underneath as arrows (Vaughan *et al.*, 2001). Underneath the LMG18311 *gal-lac* gene cluster (and surrounding regions) the nucleotide scale is presented (bp) together with the PCR fragments generated in this study with the oligonucleotides from Table 2.

Sugar utilization of different *S. thermophilus* strains.

To investigate the degree of conservation in the *gal-lac* gene cluster and correlate this with the capacity to utilize lactose, galactose and other sugars, we screened a set of 18 *S. thermophilus* strains from a variety of sources (Table 1). To verify the taxonomic identity of these strains the V1, V2 and V3 variable regions of 16S rRNA were sequenced and compared to known sequences in databases (data not shown). The obtained 16S rRNA sequence were completely identical, confirming that all strains belonged to the species *S. thermophilus*. In a growth assay all strains tested were able to utilize glucose, lactose and sucrose, and four were able to utilize fructose (Table 4). Besides the earlier described Gal^+ mutant of CNRZ302, NZ302G (Vaughan *et al.*, 2001), NIZO B112 and NIZO B113 were the only strains that showed significant growth on galactose, as was also suggested by the API-50 CH assay (data not shown). Remarkably, these natural Gal^+ strains were also capable of utilizing fructose (Table 4). For most of these strains tested the results of the API-50 CH assay could be confirmed by growth on a specific sugar. However, strains NIZO B112 and NIZO B113 showed reproducible acidification of the API-50 CH indicator medium containing maltose, mannose or cellobiose, while no significant growth was observed on these sugars. Acidification in absence of growth

could be due to the presence of transport proteins and metabolic pathways with insufficient efficiency or specificity. These results indicate that all *S. thermophilus* strains are able to utilize glucose, lactose and sucrose, except for strain NCIMB 701968 that showed no growth on sucrose.

LMG18311 template		A147		CNRZ302	
gene	gene size (bp)	nucleotide mismatches	AA subst	nucleotide mismatches	AA subst
galR	995	-	-	2	0
galK	1166	-	-	3	A290T *
galT	1481	-	-	7	V42I*, N71K, A243V*, E314G*, N476S, D480A*
galE	1010	22	I100V, T238A, L251N, D252N, C269R, L284R, R285P, F302L, D312E, N313K, N324S	-	-
galM	1046	6	T55M*, E101D, E125D, I174V	-	-
lacS	1904	16	F105L, L107F, I110L, D183V, L185F, H190N, Y203F, D208A, N210I, H230R, M251L,H547R	-	-
lacZ	3080	4	H284Q, T964S*, Q973H*	-	-

Table 3. List of nucleotide mismatches and amino acid substitutions from four (partial) sequenced *S. thermophilus gal-lac* gene clusters using the cluster of LMG18311 as template. All shown nucleotide mismatches and amino acid substitutions were also found in the reported sequence of *S. thermophilus* SMQ-301 (Vaillancourt *et al*., 2002) except for these indicated with an asterix. Conservative amino acid changes (score ≥ 0 in Blusum62 matrix) are underlined. A minus indicates the genes of which no sequence data was available.

Organization and location of the *S. thermophilus gal-lac* operon.

S. thermophilus is highly adapted to lactose fermentation. However, only few strains are able to ferment the galactose moiety of lactose. We used a diagnostic PCR-based approach to assess the conservation of the *gal-lac* gene cluster organization in *S. thermophilus* (Fig. 1). Primers were designed based on strategic regions in the cluster and flanking sequences that allowed the determination of the position of each gene and the length of the intergenic regions from the generated PCR products. The anticipated PCR products (fragments D to J in Fig. 1) were readily generated from all strains, indicating that in all the *gal-lac* gene order (*galKTEM-lacSZ*) is conserved. Furthermore, no differences in length of these PCR products could be detected on agarose gels, indicating that individual gene sizes were highly similar.

To analyze the location of the *gal-lac* cluster in the genome of the various *S. thermophilus* strains, we amplified its flanking regions. In all *S. thermophilus* strains, the *gal-lac* cluster was found to be flanked by the same genes (*zmpB*-transposase and *sbcD* homologues). Only for strain NIZO B126, the PCR fragments A and B (Fig. 1) were similarly increased in size by approximately 0.5 kb, indicating a small insertion between the *zmpB* and the transposase homologues in this strain.

These results indicate that the organization of the *S. thermophilus gal-lac* gene cluster as well as the flanking regions of this locus in the genome of *S. thermophilus* are very well conserved among strains isolated from different sources. This also holds for the galactose-fermenting strains NIZO B112 and NIZO B113.

Strain	glucose	lactose	sucrose	galactose	fructose
LMG18311	+	+	+	-	-
CNRZ302	+	+	+	-	+/-
NZ302G	+	+	+	+	+/-
ATCC 19258	+	+	+	-	+/-
A054	+/-	+	+	-	-
ST8	+	+	+	-	-
ST11	+	+	+	-	-
NIZO B110	+	+	+	-	-
NIZO B112	+	+	+	+	+
NIZO B113	+	+	+	+	+
NIZO B126	+	+	+	-	-
NIZO B132	+	+	+	-	-
NIZO B1122	+	+	+	-	-
NCIMB 701968	+/-	+	-	-	-
NCIMB 702533	+	+	+	-	-
NCIMB 702560	+	+	+	-	-
NCIMB 702561	+	+	+	-	-
NCIMB 702562	+	+	+	-	-
NCIMB 702637	+	+	+	-	-

Table 4. Growth of the strains used in this study on various sugars (1% (w/v), in M17 medium). Full growth (OD_{600nm} <1): +, No growth: -, In some cases significant growth was observed but the final OD_{600nm} was between 0.3 and 0.6; this growth was scored at +/-.

Sequence analysis of the *galR-galK* promoter region.

To study the relation between the natural ability to ferment galactose of strains NIZO B112 and NIZO B113 and the previously identified promoter-up mutations within the *galK* promoter (Vaughan *et al*., 2001) the *galR-galK* intergenic regions of these and the other *S. thermophilus* strains were amplified by PCR, cloned in pGEM-T, and characterized by sequence analysis (Fig. 2). The *galR-galK* promoter sequences of 10 of the analysed strains were completely identical to that of LMG18311 and the rest showed in between one to five nucleotide mismatches or single nucleotide deletions. The promoter sequences of NIZO B112, NIZO B113 and ATTC19258 showed several substitutions relative to LMG18311 (Fig. 2).

Common substitutions were a single base pair deletion in the proposed –35 sequence of the *galR* promoter, and a A to G substitution at the predicted transcript initiation site *galK*. In contrast, only the galactose-fermenting strains NIZO B112 and NIZO B113 contained a G to A substitution in the –10 sequence. An identical substitution in the –10 sequence of the *galKTE* promoter was identified in one class of galactose-fermenting mutants of CNRZ302 that conformed better to consensus –10 sequences known (McClure, 1985; Vaughan *et al.*, 2001). These results indicate that an optimized *galK* promoter sequence most likely conferred the ability to ferment galactose to NIZO B112 and NIZO B113.

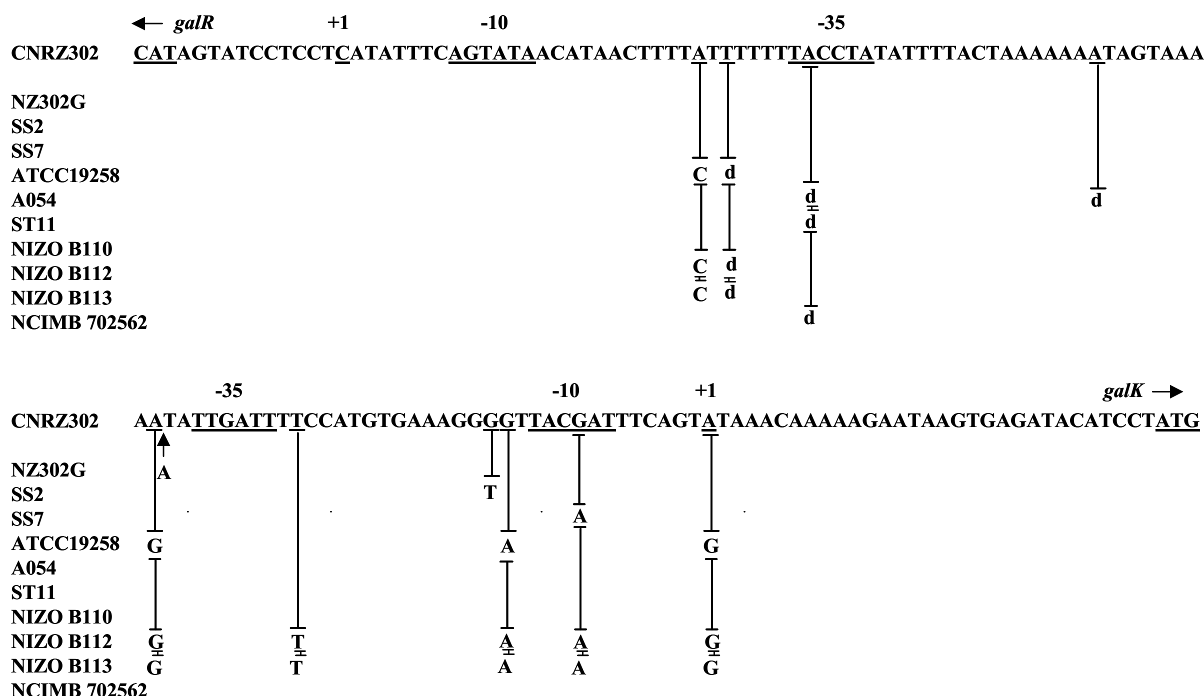

Figure 2. Sequence of the *galR-galK* intergenic region. The ntergenic region containes the promoter sequences for the *galK* and *galR* genes from the strains used in this study are shown as deviations from the CNRZ302 sequence. The -10 and -35 regions of the promoter are underlined, and the transcriptional start sites are indicated as +1. The nucleotide mismatches (relative to the CNRZ302 sequence) are depicted underneath. Nucleotide insertion is indicated with a vertival arrow and nucleotide deletions are indicated with d.

DISCUSSION

Characteristic for *S. thermophilus* is the adaptation to lactose as primary carbon and energy source and the inability to ferment a large number of sugars that can be utilized by other lactic acid bacteria (Mercenier, 1990). Moreover, although *S. thermophilus* readily ferments lactose, most strains can not utilize galactose, neither the free sugar nor the moiety derived from lactose hydrolysis by β-galactosidase activity (Hutkins and Morris, 1987). In this study we used a combination of diagnostic PCR, Southern hybridization and nucleotide sequence analysis to evaluate the diversity of sugar utilization and the conservation of *gal-lac* gene cluster in a collection of 18 *S. thermophilus* strains isolated from different sources.

The carbon sources that most *S. thermophilus* strains are able to utilize are limited to glucose, lactose, and sucrose, while some strains can also grow on fructose. If utilized, glucose and fructose are relative poor carbon sources on which the growth rates are several-fold lower than that on lactose or sucrose, which are readily metabolized (Poolman, 1993; van den Bogaard *et al*., 2000). Although fructose is taken up by a PTS, utilization of this sugar as a carbon source requires fructose-bisphosphatase (FBPase) activity to form essential biomass precursors such as nucleotide sugars. In the genome sequence of *S. thermophilus* LMG18311 no homologue for a FBPase encoding gene could be found (Hols *et al*., 2002). It could very well be that FBPase activity levels in most *S. thermophilus* strains are limiting since a lack of this activity in *L. lactis* uncoupled fructose fermentation from growth (Looijesteijn *et al*., 1999). Strains NIZO B112 and B113 were able to utilize galactose and fructose as sole carbon source and were also able to convert maltose and cellobiose into acids, although these strains did not support growth.

In many organisms the *galK*, *galT* and *galE* genes that encode the enzymes of the Leloir pathway of galactose metabolism are clustered or organized in a single operon, but their order may vary. The organization of the *gal-lac* genes appears to be identical in all *S. thermophilus* strains tested in this study. Moreover, the regions flanking the *S. thermophilus gal-lac* cluster were also found to be conserved among the tested strains reflecting conservation for that chromosomal locus. The remnant of one or more transposase-like genes upstream of the *gal-lac* cluster indicates the insertion and loss of a transposable element early in *S. thermophilus* evolution, which did not disrupt the operon itself. At least 13 of these transposase remnants with a homology of 86% or higher (at the amino acid level) can be found in the genome sequence of strain LMG18311 (Hols *et al*., 2002). The gene order for the *S. thermophilus gal-lac* operon and the divergently encoded *galR* gene is identical to that of *S. salivarius*, which reflects the evolutionary relationship between these bacteria (Vaillancourt *et al*., 2002). However, the *S. salivarius gal-lac* operon has different flanking regions compared to those of *S. thermophilus* indicating that the *gal-lac* gene cluster has evolved as one single unit in these phylogenetically closely related streptococci. Alignment of all reported *S. thermophilus gal-lac* operon sequences showed that the overall homology was highest between SMQ-301, A147 and CNRZ302 relative to LMG18311. The observed substitutions were randomly distributed over the *gal-lac* cluster and although the substitution frequency was higher in the *galE* and the *lacS* genes, no significant hotspots could be identified. The regulatory gene *galR* and all regulatory sequences showed no sequence variations.

Two naturally isolated strains NIZO B112 and NIZO B113 were able to ferment and use galactose for growth. Apparently, organizational changes are not responsible for conferring the ability for galactose utilization to *S. thermophilus*. Only limited sequence variation was found between the complete *galRK* intergenic regions of the Gal$^-$ strains used in this study. However, in the galactose-fermenting strains NIZO B112 and NIZO B113 a single nucleotide substitution in the –10 promoter sequence was found that can be considered a *galK* promoter-up mutation, since an identical substitution was identified in class III of galactose fermenting-mutants of CNRZ302 (Vaughan *et al*., 2001).

In conclusion, lactose is the preferential sugar in *S. thermophilus* and due to continued growth on lactose-containing media such as milk, this organism has probably lost the ability to utilize a number of PTS and non-PTS sugars that can be fermented by other lactic acid bacteria. The *S. thermophilus gal-lac* operon is very conserved in sequence, organization and genomic location among strains isolated from a variety of sources. Galactose-fermenting mutants have lower growth-rates when growing on lactose compared to the Gal$^-$ parental strains (Levander *et al*., 2002). Aparently, the fermentation of the galactose moiety via the relatively slow Leloir pathway is energetically less favorable and will lead to the outgrowth of the Gal$^-$ variants. The loss of the ability to ferment galactose can be attributed to the

adaptation to milk in which the lactose levels are in excess. Apparently, the galactose-fermenting strains NIZO B112 and NIZO B113 that were fortuitously found in milk have not adapted as strongly to growth on lactose and are able to utilize additional sugars. At the molecular level this the adaptation to lactose is reflected not only by the highly efficient lactose/galactose exchange activity of the galactoside transporter LacS but also by the low activity of the *galKTE* promoter, due to accumulation of specific nucleotide substitutions that have been identified here.

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Chapter 4

Control of Lactose Transport, β-Galactosidase Activity, and Glycolysis by CcpA in *Streptococcus thermophilus*: Evidence for Carbon Catabolite Repression by a Non-Phosphoenolpyruvate-Dependent Phosphotransferase System Sugar

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SUMMARY

Streptococcus thermophilus, unlike many other gram-positive bacteria, prefers lactose over glucose as the primary carbon and energy source. Moreover, lactose is not taken up by a phosphoenolpyruvate-dependent phosphotransferase system (PTS) but by the dedicated transporter LacS. In this paper we show that CcpA plays a crucial role in the fine-tuning of lactose transport, β-galactosidase (LacZ) activity, and glycolysis to yield optimal glycolytic flux and growth rate. A catabolite-responsive element (*cre*) was identified in the promoter of the *lacSZ* operon, indicating a possible role for regulation by CcpA. Transcriptional analysis showed a sevenfold relief of repression in the absence of a functional CcpA when cells were grown on lactose. This CcpA-mediated repression of *lacSZ* transcription did not occur in wild-type cells during growth on galactose, taken up by the same LacS transport system. Lactose transport during fermentation was increased significantly in strains carrying a disrupted *ccpA* gene. Moreover, a *ccpA* disruption strain was found to release substantial amounts of glucose into the medium when grown on lactose. Transcriptional analysis of the *ldh* gene showed that expression was induced twofold during growth on lactose compared to glucose or galactose, in a CcpA-dependent manner. A reduced rate of glycolysis concomitant with an increased lactose transport rate could explain the observed expulsion of glucose in a *ccpA* disruption mutant. We propose that CcpA in *S. thermophilus* acts as a catabolic regulator during growth on the preferred non-PTS sugar lactose. In contrast to other bacteria, *S. thermophilus* possesses an overcapacity for lactose uptake that is repressed by CcpA to match the rate-limiting glycolytic flux.

INTRODUCTION

Carbon catabolite repression (CR) in bacteria is the phenomenon of using a rapidly metabolizable carbon source in the growth medium by inhibiting utilization of other substrates. The mechanism underlying CR is best understood in enteric bacteria, where the glucose-specific enzyme IIA of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) modulates adenylate cyclase activity. Controlled by the level of cyclic AMP, the cyclic AMP receptor protein is a transcriptional regulator modulating expression of target genes (Postma *et al*., 1993; Saier, 1993). In low-G+C gram-positive bacteria, the mechanism of CR is distinctly different. The catabolite control protein A (CcpA) is the central regulator of CR, as was shown first for *Bacillus subtilis*, in which it mediates glucose repression of the α-amylase gene (Henkin *et al*., 1991). CcpA is a member of the LacI-GalR family of bacterial regulator proteins and appears to be widespread among low-G+C gram-positive bacteria (Egeter and Bruckner, 1996; Hueck *et al*., 1995; Lokman *et al*., 1997; Monedero *et al*., 1997). Genes affected by CR typically contain a catabolite-responsive element (*cre*) near their promoter regions (Weickert and Chambliss, 1990). CcpA has been shown to bind to these *cre* sites in vitro in a way that can be enhanced by indicators of a high energy state in the cell, e.g., glucose 6-phosphate (Gosseringer *et al*., 1997; Miwa *et al*., 1997). Another important factor in this catabolite control mechanism is the PTS phosphocarrier HPr. In *B. subtilis*, high concentrations of the glycolytic intermediate fructose-1,6-diphosphate (FBP) trigger an ATP-dependent protein kinase that phosphorylates HPr at residue Ser-46. P-Ser-HPr subsequently enhances the binding of CcpA to *cre* and hence links glycolytic activity to CR (Deutscher *et al*., 1995; Fujita *et al*., 1995; Jones *et al*., 1997). Catabolite control by CcpA involves not only repression of genes and operons but also activation. In *B. subtilis*, transcription of the *alsS* and *ackA* genes (encoding α-acetolactate synthase and acetate kinase, respectively) is

activated by CcpA when glucose is present in the medium (Grundy *et al*., 1993; Renna *et al*., 1993). More direct evidence for a link between catabolite control and glycolytic activity was reported recently for *Lactococcus lactis*. In the presence of glucose in the medium, CcpA was found to be a transcriptional activator of the *las* operon, thus modulating glycolytic flux rates by controlling the production of the three key glycolytic enzymes, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase (Luesink *et al*., 1998).

Although the mechanism of CR differs between gram-negative and low-G+C gram-positive bacteria, they have in common that a rapidly metabolizable PTS sugar reduces the expression of genes involved in the utilization of other PTS or non-PTS carbon sources. Glucose is the classical example of such a rapidly metabolizable PTS sugar in most bacteria. However, glucose is a non-PTS carbon source for *Streptococcus thermophilus* and is a poor substrate for growth (Poolman, 1993). Lactose is also a non-PTS sugar for this organism but is a very good growth substrate on which growth is even more rapid than on a PTS sugar like sucrose. This indicates that *S. thermophilus*, a homofermentative thermophilic lactic acid bacterium, is highly adapted to growth on lactose as the primary carbon and energy source. Together with other lactic acid bacteria, this organism is used as a starter culture for the production of yogurt and certain cheeses, where it mainly contributes to the rapid acidification of milk by conversion of lactose to lactic acid.

The *S. thermophilus lac* operon contains the genes encoding a lactose permease (*lacS*) and a β-galactosidase (*lacZ*) for the transport and hydrolysis of lactose, and its transcription is induced during growth on lactose (Poolman *et al*., 1989; Schroeder *et al*., 1991). Studies of the *lac* operon revealed a *cre* site located in the *lacSZ* promoter, suggesting a possible involvement of CcpA in the regulation of this operon (Poolman, 1993). The *S. thermophilus galM* and *galE* genes, encoding enzymes of the Leloir pathway for galactose fermentation, were found upstream of this *lac* operon (Poolman *et al*., 1990). The complete *galKTE* operon was recently identified in strain CNRZ302, which is unable to grow on galactose, like most *S. thermophilus* strains (Vaughan *et al*., 2001). From this strain, galactose-fermenting mutants were isolated, and their molecular characterization showed that these mutants were all *galK* promoter-up mutants. One of these mutants, used in this study, was designated NZ302G. Insertional mutagenesis studies of the *galR* gene located upstream of the *galKTE* operon, encoding a regulator protein of the LacI-GalR family of transcriptional regulators, showed that the GalR protein was an activator of the *galK* promoter (Vaughan *et al*., 2001). Transcription of this promoter was induced when cells were grown on medium containing lactose or galactose. Furthermore, GalR was also found to be a transcriptional activator of the *lac* operon, which is expressed at a basal level when cells are grown on glucose, while it is expressed at least twice as high in lactose- or galactose-grown cells.

In this study we show that in *S. thermophilus*, CcpA is acting as a transcriptional repressor of the *lac* operon and an activator of genes encoding key glycolytic enzymes, induced by the non-PTS sugar lactose. This catabolite control is probably regulated by the glycolytic intermediates that are derived from the glucose moiety of lactose rather than from a PTS sugar in the growth medium. We provide evidence that CcpA is involved in fine-tuning the rate of lactose transport with glycolytic activity, enabling rapid fermentation and high growth rate of *S. thermophilus*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. *S. thermophilus* was routinely grown at 42°C in M17 broth (Difco, Surrey, U.K.) containing 1% of the chosen carbon source unless stated otherwise. *Escherichia coli* strains were grown in TY broth (Sambrook *et al*., 1989) with aeration at 37°C. The antibiotics used for selection in growth media were chloramphenicol (Cm, 4 µg/ml) and erythromycin (Em, 2.5 µg/ml) for *S. thermophilus* and ampicillin (Ap, 50 µg/ml), Cm (10 µg/ml), and Em (150 µg/ml) for *E. coli*.

DNA manipulations and transformations. Transfer to and isolation of plasmid DNA from *E. coli* was performed using established protocols (Sambrook *et al*., 1989). Plasmid and chromosomal DNA from *S. thermophilus* was isolated as described previously for *L. lactis* (Vos *et al*., 1989). Electroporation of *S. thermophilus* was performed by the procedure described by Mollet *et al*. (Mollet *et al*., 1993) with the modification that the harvested cells were incubated in the electroporation buffer at 4°C for at least 4 h prior to electroporation. Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were used as recommended by the suppliers (Gibco-BRL or Boehringer Mannheim). DNA fragments were recovered from agarose gels using the glass matrix DNA isolation system (Gibco-BRL).

Cloning and disruption of the *S. thermophilus ccpA* gene. Total genomic DNA from *S. thermophilus* CNRZ302 was digested with *Hin*dIII and *Kpn*I, and the DNA fragments were separated by agarose gel (0.7%) electrophoresis. The DNA was transferred to a Gene Screen Plus (Dupont, Boston, Mass.) membrane by standard procedures (Sambrook *et al*., 1989). A 3.3-kb hybridizing fragment was identified using a 1-kb fragment of the *B. subtilis* 1G33 *ccpA* gene (kindly provided by E. Luesink) that was gel purified and labeled by nick translation using [α-^{32}P]dATP (Amersham International plc, London, U.K.). *S. thermophilus* chromosomal DNA was digested with *Hin*dIII and *Kpn*I, and fragments with sizes of between 3.0 and 3.5 kb were recovered, ligated with *Hin*dIII- and *Kpn*I-digested pUC19 (Yanisch-Perron *et al*., 1985), and transformed into *E. coli* MC1061. Clones carrying the *ccpA* gene were selected by colony blotting of the Ap-resistant colonies (Sambrook *et al*., 1989) onto Gene Screen Plus membranes and probing the transferred minibank with the radiolabeled *B. subtilis ccpA* gene. Sequence analysis of the positive clones confirmed that a 3.3-kb insert in pUC19 contained a *ccpA*-like gene. This construct was designated pNZ6100 and used in further experiments. A 799-bp internal PCR fragment of the *S. thermophilus ccpA* gene was generated from pNZ6100 as a template using primers CCPAKF (5'-GCTCGAAGTCATTGATCG-3') and CCPAKR (5'-AGTCAACATACGCATGCT-3') and ligated into pGEM-T (Promega), yielding pNZ6101. Plasmid pNZ6102 was generated by cloning the insert with *Apa*I and *Sal*I from pNZ6101 into the thermosensitive pGh9 vector (Maguin *et al*., 1992; Maguin *et al*., 1996) digested with the same restriction enzymes. *S. thermophilus* strains CNRZ302 and NZ302G transformed with pNZ6102 were selected at 28°C on M17 sucrose medium supplemented with Em. To facilitate integration of pNZ6102, cultures grown overnight in M17 glucose broth with Em at 28°C were diluted 100-fold into fresh medium and reincubated at 28°C to allow the exponential phase of growth to resume. The cultures were then shifted to 42°C and grown until stationary phase. Dilutions of the cultures were plated at 42°C, and integrants appeared as Em-resistant colonies after 24 to 48 h of incubation. Integration of pNZ6102 into the *ccpA* locus of CNRZ302 and NZ302G should result in two truncated and inactive copies of the *ccpA* gene. Correct integration was confirmed by PCR and Southern analysis and yielded strains NZ6150 and NZ6151, which were handled further at 42°C with Em to maintain the integrated plasmid. For

complementation studies of the *ccpA* disruption mutant, plasmid pNZ6100 was digested with *Acc*I, and the ends were filled in using Klenow polymerase followed by a second digestion with *Hin*dIII. The 1.3-kb fragment containing the *ccpA* gene was ligated in pNZ273 (Platteeuw *et al*., 1994), from which the *gusA* gene was removed by digestion with *Sca*I and *Hin*dIII. The resulting plasmid (pNZ6103) harbors the *S. thermophilus ccpA* gene under the control of its own promoter. The *S. thermophilus* CNRZ302 *ldh* promoter was obtained by PCR using *Pwo* DNA polymerase (Boehringer Mannheim) and primers LDH1 (5'-ACACTCATGGCATAATCGATA-3') and LDH2 (5'-AGTTCTTGAGCGATACCTTG-3') based on the sequence of the *ldh* locus of strain M-192 (Ito and Sasaki, 1994). The promoter fragment was adenylated using *Taq* polymerase and ligated into pGEM-T (Promega), yielding pNZ6104.

RNA isolation, Northern blot, and primer extension analysis. *S. thermophilus* strains were grown in M17 broth (30 ml) containing 1% glucose or lactose to an optical density at 600 nm (OD_{600}) of 1.0. Total RNA was isolated from the harvested cells using the Macaloid method as described by Kuipers *et al*. (Kuipers *et al*., 1993) with the following adaptation. Prior to bead beating, the resuspended cells were incubated with lysozyme for 5 min on ice to increase RNA yield. Per sample, 4.5 µg of RNA was size separated on a 1.0% formaldehyde gel (Sambrook *et al*., 1989) and transferred to Gene Screen Plus membranes (Dupont) according to the protocols provided by the manufacturers. RNA size markers were obtained from Bethesda Research Laboratories. Hybridizations were performed at 65°C in a 0.5 M sodium phosphate buffer (pH 7.2) containing 1.0% bovine serum albumin (fractionV), 1.0 mM EDTA, and 7.0% sodium dodecyl sulfate (SDS), and subsequently, blots were washed at 55 to 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Internal PCR fragments were generated using *S. thermophilus* CNRZ302 chromosomal DNA as the template and primers based on published sequence data: *lacS*, LACSF (5'-TAACACAGGTGATCCAAAGCA-3') and LACSR (5'-GGTGACCAGAACTCAAGAAG-3') (Morel *et al*., 1999); and *ldh*, STLDH-F (5'-GTCATCCTTGTTGGTGACGG-3') and STLDH-R (5'-TGCTTCATCAATGATAGCTTGC-3') (Hueck *et al*., 1995). These fragments were glass matrix purified, labeled by nick translation with [α-^{32}P]dATP (Amersham International plc), and subsequently used as hybridization probes (Sambrook *et al*., 1989). The images of the Northern blots were exposed to a Storage Phosphor Screen (Molecular Dynamics) and scanned using a STORM 840 PhosphorImager (Molecular Dynamics). The Northern signals were quantified using the ImageQuant 1.2 program (Molecular Dynamics). Per Northern blot, a final 16S rDNA probe, created by PCR with 16S-specific primers (NR7 [5'-GAAGCAGCGTGG-3'] and NR19 [5'-GTCGTTATGCGGTA-3']) with *S. thermophilus* CNRZ302 chromosomal DNA as the template, was used to correct the gene-specific signals for the total amount of RNA loaded per sample, which never differed by more than 20%. Primer extension was performed as previously described (Sambrook *et al*., 1989) by annealing 20 ng of oligonucleotide PECCPA (5'-CGATACTCCAGCTTCACGCGC-3'; *ccpA*) or PELDH (5'-CGGCACCGTCACCAACAAGG-3'; *ldh*) to 15 µg of total *S. thermophilus* CNRZ302 mRNA. The primer extension reaction was loaded on a 5% polyacrylamide gel together with a sequencing reaction obtained using the same oligonucleotide primer and an appropriate template.

β-Galactosidase and protein assays. The *S. thermophilus* strains were grown to an OD_{600} of 1.0 in M17 broth containing 1% lactose, galactose, or glucose. For the preparation of cell extracts, cells were disrupted with zirconium glass beads in a Bead Beater (Biospec Products, Bartlesville, Okla.) by 3-min treatments, with intervals of 1 min in between on ice; cellular

debris was removed by centrifugation. The extracts were kept on ice, and enzyme assays were performed within 2 h using 1 to 6 μg of protein per reaction. β-Galactosidase was assayed at 42°C by the method of Miller (Miller, 1972). Lactate dehydrogenase was assayed at 25°C by the method of Hillier and Jago (Hillier and Jago, 1982). All enzyme activity measurements presented are the means of at least two independent experiments. Protein concentrations were estimated by a dye-binding assay (Bradford, 1976) using bovine serum albumin as the standard.

Small-scale sugar fermentations. The *S. thermophilus* strains were grown to an OD_{600} of 1.0 in M17 broth containing 1% lactose or galactose, washed, and resuspended in a 4% β-glycerophosphate solution at a final OD_{600} of 10.0. The cells were preincubated for 2 min at 42°C, and fermentation was started by addition of 20 mM lactose. Consecutive samples were taken at regular time intervals from the primary fermentation suspension and immediately transferred to 5°C in salted ice water to prevent further uptake and metabolic conversion. The samples were centrifuged, and supernatants were analyzed by high-performance liquid chromatography (HPLC). Sugars were separated on a Polyspher CHPb18 column (Merck) with water as the eluent. Organic acids were separated on a Rezex organic acid column (Phenomenics) using 5 mM sulfuric acid as the eluent. The separations were carried out on an isocratic pumping system (M6000; Perkin-Elmer) in combination with an automatic sample injector (717+; Waters) and a refractive index detector (M410; Waters).

Western blot analysis. For CcpA detection, cells were grown to an OD_{600} of 1.0 in M17 broth containing 1% lactose or glucose. For the preparation of cell extracts, cells were disrupted with zirconium glass beads in a Bead Beater (Biospec Products) by three treatments of 1 min, interspaced by 1 min of cooling of the samples on ice; cellular debris was removed by centrifugation. For LacS analysis, portions of the cells that were used for the small-scale sugar fermentations were protoplasted by extensive treatment with a combination of lysozyme (2 mg/ml) and mutanolysin (25 U/ml) in THMS buffer (30 mM Tris [pH 8.0], 3 mM $MgCl_2$ in 25% sucrose). The protoplasts were washed once in THMS buffer and dissolved in a buffer containing 50 mM potassium phosphate (pH 8.0), 100 mM NaCl, 20% (vol/vol) glycerol, and 0.5% (wt/vol) Triton X-100 to solubilize the LacS protein (Knol *et al*., 1996). The suspensions were mixed, and after 20 min of incubation at 4°C, the insoluble material was removed by centrifugation.

Protein concentrations were estimated by a dye-binding assay (Bradford, 1976). Samples were equalized and separated by SDS-12.5% polyacrylamide gel electrophoresis. The separated proteins were transferred to Gene Screen Plus membranes (Dupont) using electroblot equipment (LKB 2051 Midget Multiblot). CcpA proteins were detected using antibodies raised against *Bacillus megaterium* CcpA that were shown to cross-react with CcpAs from various organisms (Kuster *et al*., 1996). LacS proteins were detected using antibodies raised against the COOH terminus of LacS (Poolman *et al*., 1995). These antibodies were detected using goat anti-rabbit immunoglobulin peroxidase-conjugated antibodies (Gibco-BRL) as described by the manufacturer.

Nucleotide sequence accession number. These sequence data have been submitted to the GenBank database under accession number AF231985.

RESULTS

Cloning, characterization, and disruption of the *S. thermophilus ccpA* gene.

To determine the mechanism of CR in *S. thermophilus* CNRZ302, its *ccpA* gene was identified on a 3.3-kb chromosomal fragment on basis of its hybridization with the *B. subtilis ccpA* gene and subsequently cloned, resulting in plasmid pNZ6100. Nucleotide sequence analysis showed the fragment to contain two open reading frames (ORFs). Translation of one ORF predicted a protein of 333 amino acids, corresponding to a calculated molecular mass of 36.7 kDa, which is referred to as the *S. thermophilus* CcpA, since it shared 49% amino acid identity with *B. subtilis* CcpA (Henkin *et al*., 1991). The greatest identity, however, was shared with the CcpA of *Streptococcus mutans* (80% identical amino acid residues) (Simpson and Russell, 1998). An inverted repeat structure and a stretch of five T residues, which could function as a rho-independent transcriptional terminator, followed the coding *ccpA* sequence. Primer extension experiments using total RNA from *S. thermophilus* CNRZ302 grown on glucose or lactose revealed an identical transcriptional start site located 38 bp upstream of the *ccpA* coding region (Fig. 1).

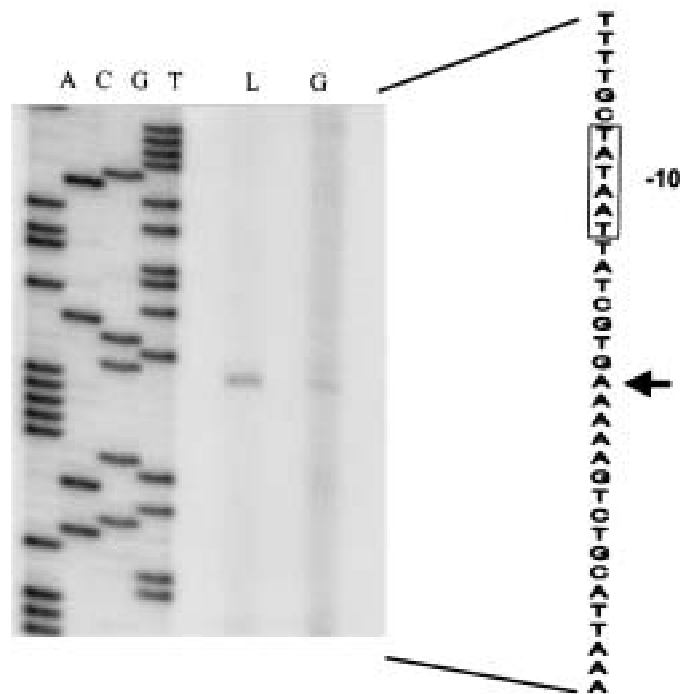


FIG. 1. Primer extension analysis of the *ccpA* promoter. The transcriptional start site is indicated with an arrow. The -10 region in the coding strand is boxed. RNA was isolated from *S. thermophilus* CNRZ302 grown on glucose (G) or lactose (L), and primer extension products were run parallel to a sequence ladder (lanes A, C, G, and T) obtained with the same primer. Approximately 15 µg of RNA was used per primer extension reaction.

Northern analysis revealed a single transcript of approximately 1.2 kb, supporting the functional role of the terminator (data not shown). The second ORF could encode a product with a high level of sequence similarity to proline peptidases (*S. mutans*, 52% identical amino acid residues; *Lactobacillus delbrueckii*, 49% identical amino acid residues) (Morel *et al*., 1999; Simpson and Russell, 1998) and was designated *pepQ*. The *ccpA* and *pepQ* genes were found in a back-to-back organization, as has been reported for several other lactic acid bacteria (*Lactobacillus pentosus*, *Lactobacillus casei*, *L. delbrükii*, *S. mutans*, and *L. lactis*)

(Mahr *et al.*, 2000). The *ccpA* gene was disrupted in strain CNRZ302 and its galactose-fermenting derivative NZ302G by a single crossing-over event of the integration vector pNZ6102, resulting in strains NZ6150 and NZ6151, respectively. Both *ccpA* disruption strains contained two truncated *ccpA* gene copies, as verified by Southern blot and PCR analysis (data not shown). The *ccpA* gene copy that is still under the control of the *ccpA* promoter encodes a CcpA that lacks the last 26 amino acids and should be nonfunctional, as was shown for similar C-terminal deletions of *B. megaterium* CcpA and its *E. coli* structural homologue, the LacI repressor (Kraus and Hillen, 1997; Luesink *et al.*, 1998). The second, promoterless *ccpA* gene (the ribosome-binding site is also missing; no expression expected) copy would encode a CcpA that lacked the first 41 amino acids, including the DNA-binding region. Neither of these truncated forms of CcpA could be detected by Western blot analysis using antibodies raised against *B. megaterium* CcpA, while the intact *S. thermophilus* CcpA, expressed in wild-type and complemented *ccpA* disruption cells, could be detected (Kuster *et al.*, 1996) (Fig.2). These results confirm that the C-terminal truncation of CcpA leads to a highly unstable form of this protein, as was shown for its structural homologue in *E. coli*, the LacI repressor (Li and Matthews, 1995). In wild-type cells, CcpA was identified as a stained band of approximately 37 kDa, and the amount of CcpA protein was at least twofold higher in cells grown on glucose relative to cells grown on lactose, indicating a form of regulation on the CcpA production. Interestingly, complementation of the *ccpA* disruption strain with pNZ6103 restored not only CcpA production but also sugar-dependent regulation of its production.

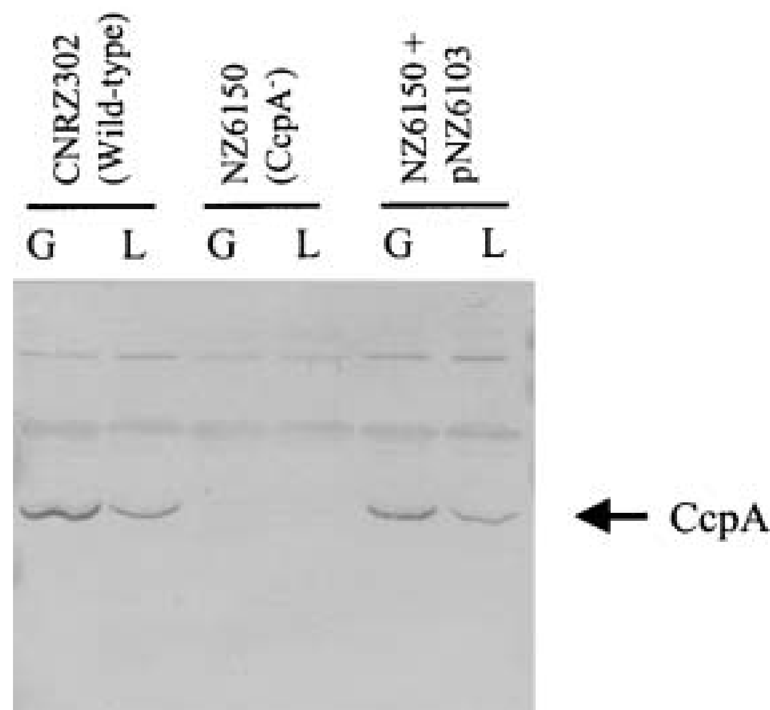


FIG. 2. Western blot analysis of total protein extracts of *S. thermophilus* strains CNRZ302 (wild-type), NZ6510 (CcpA⁻), and NZ6510 plus pNZ6103 grown on glucose (G) or lactose (L). Per sample, 10 µg of total protein was loaded, and CcpA proteins were detected using antibodies raised against *B. megaterium* CcpA. *S. thermophilus* CcpA was identified as a stained band of approximately 37 kDa. Next to the CcpA protein, several a-specific bands with a higher molecular weight were detected in every sample.

Effect of *ccpA* disruption on growth.

To analyze the physiological effects of *ccpA* disruption, *S. thermophilus* wild-type strain and NZ6150 ($CcpA^-$) were grown on M17 medium supplemented with glucose, lactose, or sucrose, while the galactose-fermenting variant NZ302G and its CcpA derivative NZ6151 were grown on M17 medium containing galactose (Fig. 3). Wild-type cells showed the highest maximal growth rate on lactose (2.48 h^{-1}) relative to glucose (1.01 h^{-1}), sucrose (1.72 h^{-1}), and NZ302G on galactose (0.67 h^{-1}). Moreover, the growth kinetics of wild-type cells grown on a combination of glucose and lactose were similar to those of lactose-grown cells (data not shown), indicating a preference for lactose as a carbon and energy source. The primary effects of *ccpA* disruption were a prolonged lag time and reduced growth rate on all sugars tested. In addition, lactose-grown NZ6150 cells reached a significantly lower OD_{600} than wild-type cells, which was not observed for growth on the other sugars (data not shown). To rule out pleiotropic effects of the insertion of the integration vector, NZ6150 was complemented with plasmid pNZ6103, expressing the *S. thermophilus ccpA* gene. Wild-type growth characteristics could be restored to NZ6150, showing that disruption of the *ccpA* gene was responsible for the observed impaired growth (data not shown).

Sugar uptake and utilization of lactose in *ccpA* disruption strains.

To obtain insight on the kinetics of lactose fermentation in the *ccpA* disruption mutant compared to the wild-type strain, small-scale fermentations were performed using resting cells in a strongly buffered system (Fig. 3). The wild-type cells show a very rapid initial uptake of lactose accompanied by the appearance of an equimolar amount of galactose in the buffer, as was observed in previous *S. thermophilus* studies (Hutkins *et al*., 1985; Luesink *et al*., 1998) (Fig. 3A). The values for lactose internalization and galactose expulsion are probably somewhat overestimated in the first 1.5 min due to the high initial transport and hydrolysis that continued for several seconds on ice during sampling. Hence, the later samples were used to calculate the rates of lactose internalization and galactose expulsion, as the overestimation decreases greatly with the decrease in transport rate. For these samples, the rates agree well with the nonlinear kinetic model for lactose uptake by the *S. thermophilus* LacS transporter (Poolman *et al*., 1989). The wild-type strain consumed half of the added amount of lactose in 10 min, whereas the *ccpA* disruption mutant achieved this in 2.5 min, consuming almost all added lactose within 20 min (Fig.3B). Remarkably, after a very short lag period (30 s), glucose appeared in the fermentation medium of the *ccpA* disruption strain and amounted after 20 min to two-thirds of the internalized lactose. This indicates that the *ccpA* disruption strain ferments only one-third of the glucose derived from the internalized lactose, whereas the wild-type strain ferments this completely. Moreover, no detectable β-galactosidase activity was found in the fermentation buffer of either strain, which rules out the possibility that the difference in lactose consumption and appearance of galactose or glucose in the fermentation buffer was caused by release of β-galactosidase due to differential lysis of the *ccpA* disruption strain. The rate and amount of lactate production agreed with the influx of lactose-derived glucose that was strongly reduced in the *ccpA* disruption strain. No end products other than lactate could be detected in the fermentation buffer, in contrast to what was found in an *L. lactis ccpA* disruption mutant, which showed a mixed acid fermentation (Luesink *et al*., 1998). In a similar experiment with NZ6151 cells fermenting galactose, no apparent differences were observed in the sugar consumption rate compared to NZ302G cells, indicating that the CcpA effect on galactoside uptake is lactose specific (data not shown).

Regulation of the *lac* operon by CcpA.

The efficiency of the *S. thermophilus lacS* promoter in CNRZ302 is induced during growth on lactose and galactose as a consequence of GalR activity (Vaughan *et al*., 2001) This *lac* promoter contains a *cre* site overlapping the -10 box and the transcriptional start site, identical in sequence and location to that previously published for strain A147 (Fig. 4) (Poolman, 1993). To study the effect of CcpA as an additional transcriptional regulator of the *lac* operon, total RNA was isolated from the *ccpA* disruption and wild-type strains grown on various carbon sources. An internal fragment of the *lacS* gene was used in Northern blots to detect the single 5.2-kb *lacSZ* messenger (Fig.5A). Lactose-grown wild-type cells showed a twofold increase in *lacSZ* expression relative to glucose-grown cells, due to GalR activity. In contrast, this increase was sevenfold in the *ccpA* disruption strain (NZ6150). Galactose-grown

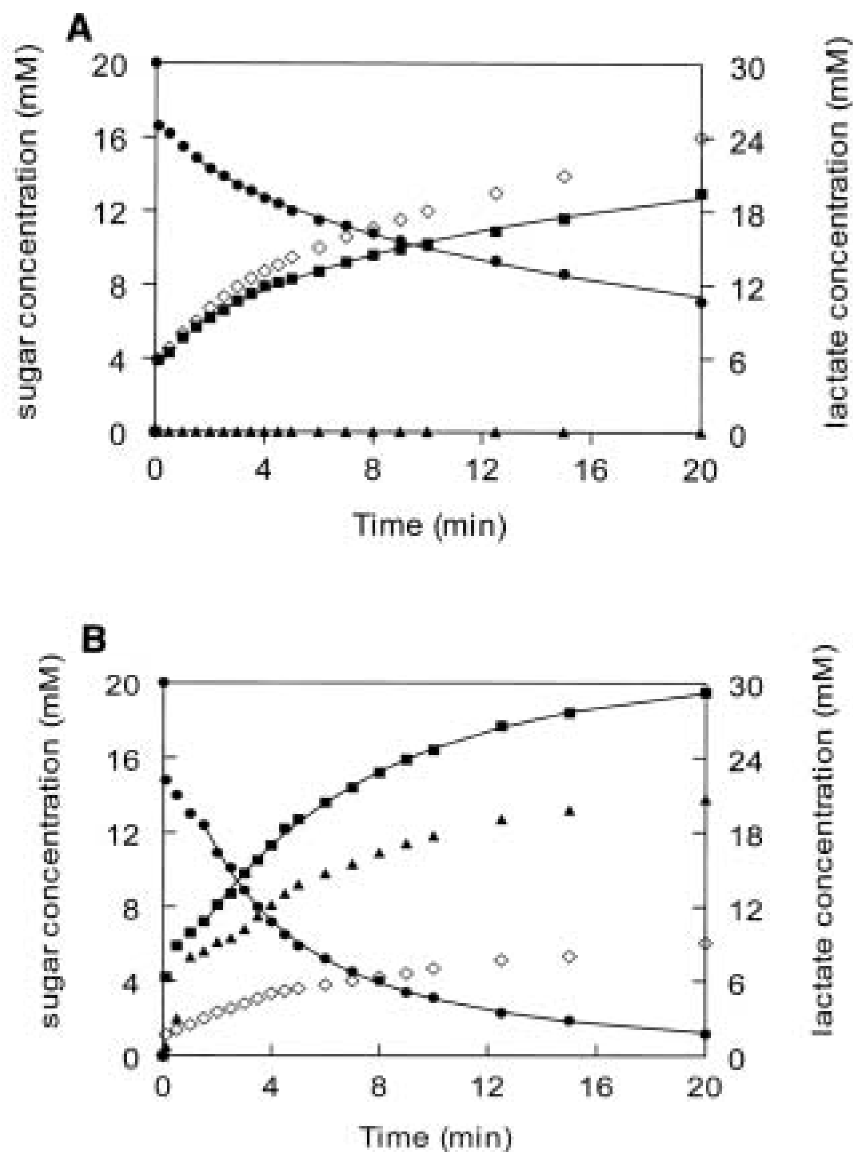


FIG. 3. Small-scale fermentation of lactose of CNRZ302 (wild-type) (A) and NZ6150 (*ccpA* disruption mutant) (B). Strains were grown to an OD_{600} of 1.0 in lactose-containing medium and resuspended in a 4% β-glycerophosphate buffer. Fermentation was started by addition of 20 mM lactose. Medium components were analyzed by HPLC. •, lactose; ■, galactose; ▲,glucose, ◊; lactate.

NZ302G cells showed a high amount of *lacSZ* transcript, even relative to lactose-grown NZ6150 cells. This did not differ significantly in strain NZ6151, indicating that CcpA-mediated repression of the *lacSZ* promoter during growth on galactose did not occur (data not shown). The basal level of *lacSZ* transcription in cells grown on glucose was not significantly affected by the loss of a functional CcpA. To further substantiate the effect of the *ccpA* gene disruption on the expression of the *lac* operon, the *lacZ* gene of this operon was used as a reporter (Fig.5B).

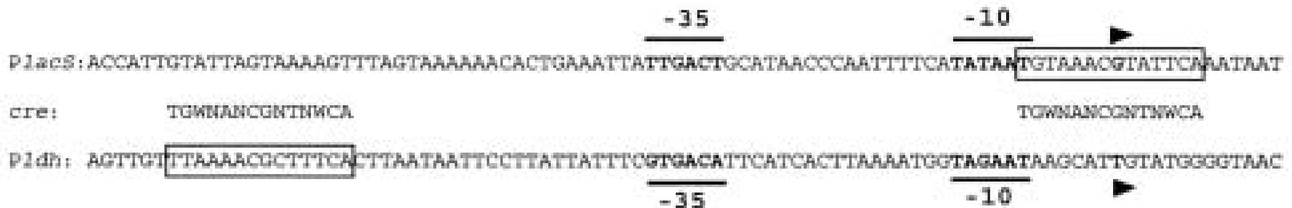


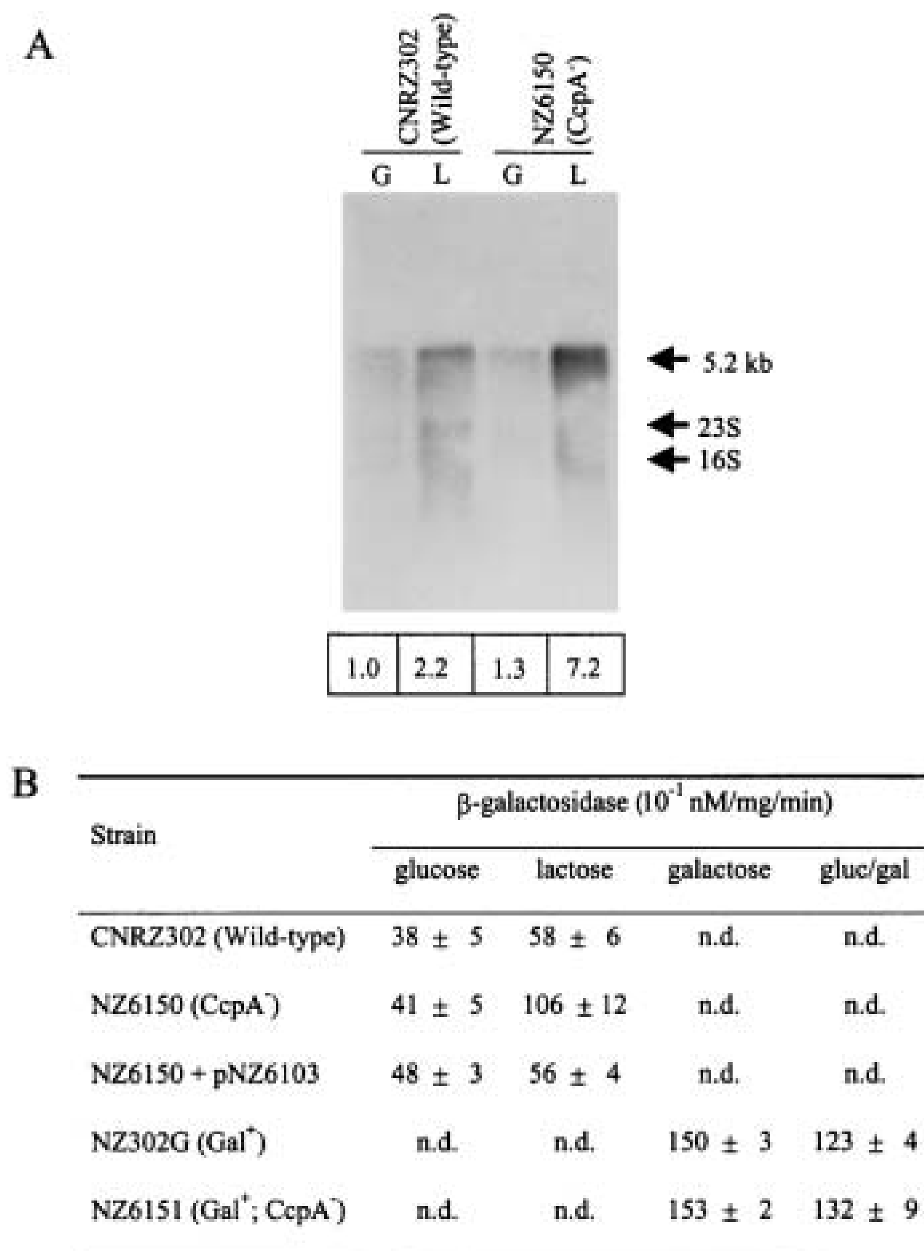
FIG. 4. Alignment of the *lacS* and *ldh* promoter regions of *S. thermophilus* CNRZ302. The -35 and -10 boxes are in bold, and the determined transcriptional start sites are indicated by arrows. The putative *cre* sites are boxed and aligned with the consensus sequence.

Lactose-grown wild-type cells showed 1.5-fold-higher β-galactosidase activity than cells grown on glucose. This induction was approximately threefold in the *ccpA* disruption mutant grown on lactose, while the β-galactosidase activity of glucose-grown cells was not significantly affected by the loss of a functional CcpA. The absolute induction values were lower relative to those of the transcriptional analysis but showed the same tendencies. Galactose-grown NZ302G cells showed a twofold-increased β-galactosidase activity relative to CNRZ302 cells grown on lactose, which was not increased further by the disruption of *ccpA* in this strain. NZ302G cells grown on a combination of galactose and glucose showed only a slightly lower β-galactosidase activity than galactose-grown NZ302G. Introduction of pNZ6103 in the *ccpA* disruption strain (NZ6150) was found to restore the wild-type β-galactosidase activity levels.

Since the *ccpA* disruption strain was able to transport lactose with a significantly higher rate than the wild-type strain, the amount of LacS protein in these strains was compared. Total protein was isolated from parent (CNRZ302 and NZ302G) and *ccpA* disruption (NZ6150 and NZ6151) strains grown on lactose or galactose. Using antibodies raised against LacS, protein bands of the expected molecular weights were detected (Knol *et al*., 1996). Significantly higher amounts of LacS protein were detected for both *ccpA* disruption strains NZ6150 and NZ6151 grown on lactose compared to their parent strains (data not shown), indicating relief of a repressing effect by CcpA on LacS production. In analogy with the β-galactosidase results, the galactose-grown NZ302G cells contained high amounts of LacS protein compared to lactose-grown wild-type cells, which was not significantly affected by the *ccpA* disruption. These results indicate that the repression of the *lacS* promoter during growth on lactose is relieved by the loss of a functional CcpA and is not occurring during growth on galactose. This strongly suggests that the glucose moiety of lactose is responsible for this CcpA-mediated repression.

Expression of the *ldh* gene.

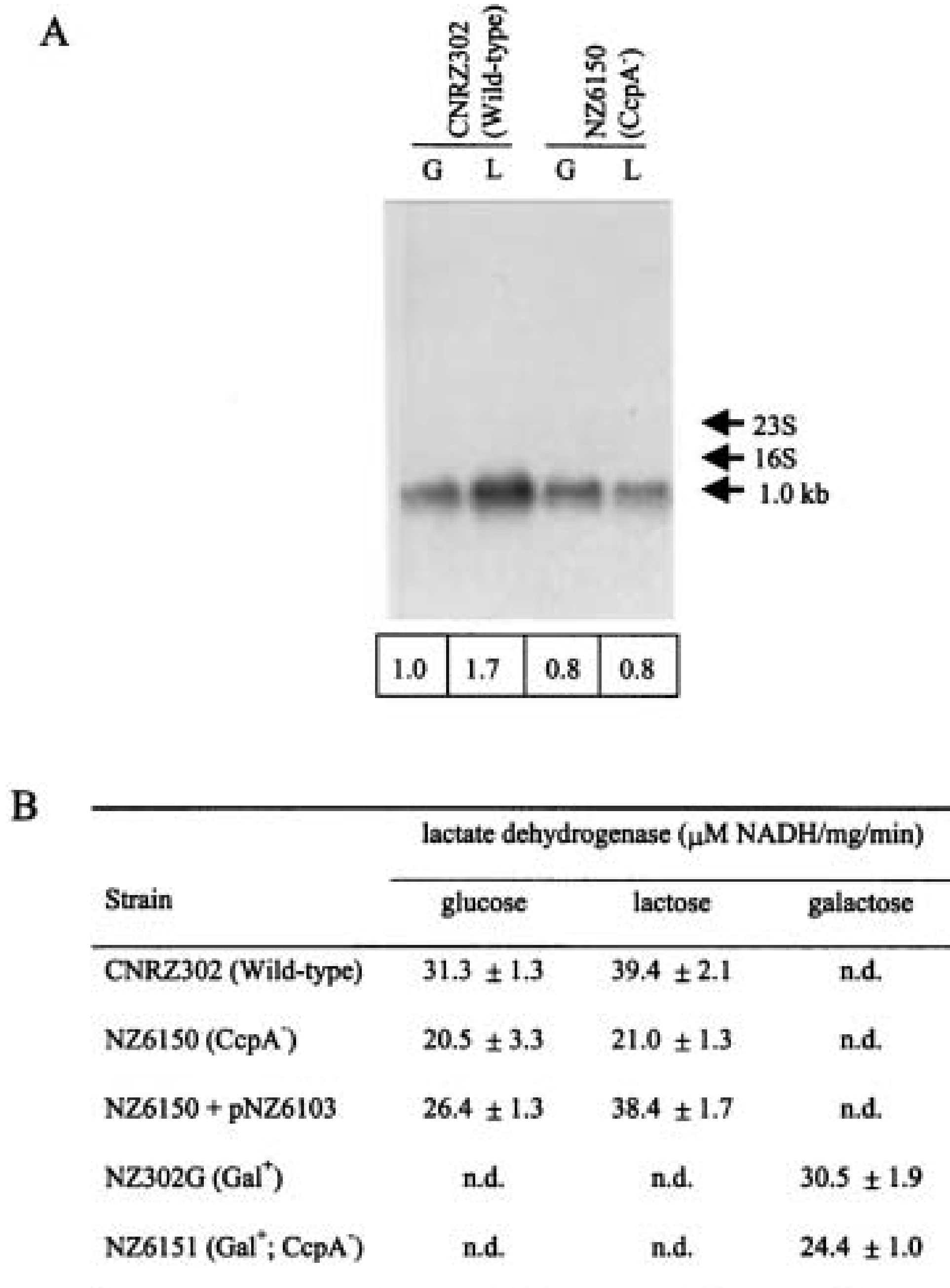
The observation that the *ccpA* disruption strain grown on lactose produced less lactate than the wild-type strain (Fig. 3) could indicate that glycolysis was affected by the disruption of the *ccpA* gene. The production of lactate from pyruvate by lactate dehydrogenase is an essential step in homofermentative lactic acid bacteria to reoxidate NADH that is generated during glycolysis. The *las* operon of *L. lactis*, comprising the *pfk*, *pyk*, and *ldh* genes, was found to be transcriptionally activated by CcpA on glucose (Luesink *et al.*, 1998). In *S. thermophilus*, the *ldh* gene (Ito and Sasaki, 1994) and the *pfk/pyk* operon (F. Crispie, J. Anba,



Strain	β-galactosidase (10^{-1} nM/mg/min)			
	glucose	lactose	galactose	gluc/gal
CNRZ302 (Wild-type)	38 ± 5	58 ± 6	n.d.	n.d.
NZ6150 (CcpA⁻)	41 ± 5	106 ± 12	n.d.	n.d.
NZ6150 + pNZ6103	48 ± 3	56 ± 4	n.d.	n.d.
NZ302G (Gal⁺)	n.d.	n.d.	150 ± 3	123 ± 4
NZ6151 (Gal⁺; CcpA⁻)	n.d.	n.d.	153 ± 2	132 ± 9

FIG. 5. CcpA regulation of the *S. thermophilus lacSZ* operon. (A) Northern blot analysis of *lacSZ* expression of strains CNRZ302 (wild-type) and NZ6510 (CcpA⁻) grown on glucose (G) or lactose (L). Below each lane, the relative amounts of the *lacSZ*-specific transcripts are given, which were obtained by phosphor image analysis of the Northern blot. These values were corrected for the total amount of RNA loaded; the *lacSZ* transcript amount of glucose-grown CNRZ302 was set at 1.0. (B) β-Galactosidase activities of the strains used in this study. Average values are presented of at least two independent experiments. n.d., not determined.

P. Renault, S. D. Ehrlich, G. F. Fitzgerald, and D. van Sinderen, unpublished data) are located at distinct chromosomal locations. Sequence analysis of the *ldh* promoter region revealed a *cre* site upstream of the -35 region (Fig. 4). This promoter was isolated from CNRZ302, and sequence analysis confirmed the presence of this *cre* site.



Strain	lactate dehydrogenase (μM NADH/mg/min)		
	glucose	lactose	galactose
CNRZ302 (Wild-type)	31.3 ± 1.3	39.4 ± 2.1	n.d.
NZ6150 (CcpA⁻)	20.5 ± 3.3	21.0 ± 1.3	n.d.
NZ6150 + pNZ6103	26.4 ± 1.3	38.4 ± 1.7	n.d.
NZ302G (Gal⁺)	n.d.	n.d.	30.5 ± 1.9
NZ6151 (Gal⁺; CcpA⁻)	n.d.	n.d.	24.4 ± 1.0

FIG. 6. CcpA regulation of the *S. thermophilus ldh* gene. (A) Northern blot analysis of *ldh* gene expression of strains CNRZ302 (wild-type) and NZ6510 (CcpA⁻) grown on glucose (G) or lactose (L). Below each lane, the relative amounts of *ldh*-specific transcripts are given, which were obtained by phosphor image analysis of the Northern blot. These values were corrected for the total amount of RNA loaded; the *ldh* transcript amount of glucose-grown CNRZ302 was set at 1.0. (B) Lactate dehydrogenase activities of the strains used in this study. Average values are presented of at least two independent experiments. n.d., not determined.

Therefore, the involvement of CcpA in the regulation of transcription of the *ldh* gene was analyzed by Northern blot analysis using an internal fragment of the *ldh* gene as a probe (Fig. 6A). The *ldh* gene showed a single transcript of 1.0 kb, of which the amount was twofold higher in lactose-grown compared to glucose-grown wild-type cells. Galactose-grown NZ302G cells showed an even lower amount of *ldh* transcript than glucose-grown wild-type cells (data not shown). This sugar-dependent regulation of *ldh* expression was completely lost in the *ccpA* disruption strains. In analogy, lactate dehydrogenase activity was highest in wild-type cells grown on lactose but significantly lower in the *ccpA* disruption strains in a sugar-independent way (Fig. 6B). Introduction of pNZ6103 in the *ccpA* disruption strain could restore lactate dehydrogenase activities towards wild-type levels. These results show that in *S. thermophilus*, CcpA is a positive regulator of the *ldh* gene and that this activation is stronger in lactose-grown cells than in glucose- or galactose-grown cells.

DISCUSSION

The role of the *S. thermophilus* catabolite control protein CcpA in fine-tuning of transport and hydrolysis of the non-PTS sugar lactose and glycolytic flux was established by the cloning, characterization, and disruption of the *ccpA* gene. CcpA was found to repress the *lacSZ* operon, encoding lactose permease and β-galactosidase, that is under positive control of the GalR activator (Vaughan *et al.*, 2001). In contrast, the gene encoding lactate dehydrogenase was found to be transcriptionally activated by CcpA. Western blot analysis showed that CcpA production was sugar source dependent, with more than a twofold-higher amount found in glucose-grown cells than in lactose-grown cells. The observed regulation of *S. thermophilus* CcpA production could be regulated at the transcriptional level as negative autoregulation, as has been found for some but not all other *ccpA* genes (Egeter and Bruckner, 1996; Mahr *et al.*, 2000; Monedero *et al.*, 1997). Inspection of the *ccpA* sequence showed the presence of two *cre*-like elements. One putative *cre* (5'-TGTAAGCGATGAAT-3') is located at -150 relative to the transcription start site of the *ccpA* promoter and is thus more closely linked to the divergently transcribed *pepQ* gene (located at -100 relative to its putative promoter), suggesting its involvement in regulation of *pepQ* expression rather than *ccpA* autoregulation. The second putative *cre* (5'-ATTCACCGGTTACA-3') is located within the coding sequence of the *ccpA* gene (+873 bp), which could suggest a role in transcriptional control. An internal *cre* site has also been found in the coding region of the *L. lactis ccpA* gene, but autoregulation could not be established in this organism (Luesink *et al.*, 1998). An alternative mechanism for the observed autoregulation involving the two *cre* sites could be that CcpA binds to these sites to form a DNA loop, thereby inhibiting *ccpA* promoter activity. The large distance found between the two sites could then explain the small magnitude of the observed regulatory effect. The restoration of sugar source-dependent regulation of CcpA production observed in the *ccpA* disruption strain when complemented with the complete *ccpA* gene in *trans* indicates that the regulation mechanism is not impaired by the presence of multiple *ccpA* gene copies, supporting the suggested autoregulation of CcpA. Nevertheless, some form of posttranslational regulation of CcpA production cannot be excluded on the basis of our experiments. Disruption of the *ccpA* gene seriously impaired the growth of *S. thermophilus*, as has also been observed for other gram-positive bacteria (Egeter and Bruckner, 1996; Hueck *et al.*, 1995; Monedero *et al.*, 1997). On both PTS (sucrose) and non-PTS (glucose, lactose, and galactose) sugars, growth of the *ccpA* disruption mutants showed a prolonged lag phase and a reduced maximum growth rate. In contrast to the other sugars tested, the *ccpA* disruption mutant NZ6150 grown on lactose reached a significantly lower

final cell density in the stationary phase compared to the wild-type cells. It is likely that growth ceased because all lactose in the medium was depleted by the high-lactose transport and hydrolysis capacity. Apparently, growth of NZ6150 does not continue on the expelled glucose.

Until now, CR by CcpA was only found for PTS substrates. In this paper, we present evidence of CcpA-mediated CR by the non-PTS substrate lactose. Northern analysis of the *lacS* promoter revealed that the negative regulation by CcpA when cells were grown on lactose occurred at the transcriptional level. The *cre* site in the *lacS* promoter is overlapping the -10 box and the transcriptional start site, in accordance with negative regulation by CcpA (Henkin, 1996). This repression was not present in cells grown on galactose which is transported by the same LacS permease. This was further substantiated by the results from β-galactosidase activity and LacS Western blot analyses. The lactose-mediated *lacSZ* repression could not be achieved by growing strain NZ302G on a combination of glucose and galactose. These results suggest that the glucose moiety derived from lactose induces CR of the *lacS* promoter. Glucose that is internalized from the growth medium is not metabolized as fast as the glucose moiety from lactose, giving virtually no CR. This indicates that glucose is not a preferred carbon source for *S. thermophilus* compared to lactose or sucrose and that uptake is probably the limiting factor for efficient glucose metabolism.

CcpA-mediated CR in low-G+C gram-positive bacteria is dependent on the intracellular amounts of FBP, as relatively high concentrations of this glycolytic intermediate stimulate the HPr kinase in *B. subtilis* to convert HPr to P-Ser-HPr (Deutscher *et al*., 1995). The LacS permease of *S. thermophilus* constitutes a very fast and efficient system for lactose uptake that facilitates high influx of glucose into glycolysis. At the maximal growth rate of *S. thermophilus*, P-Ser-HPr appears to be the dominant phosphorylated species, whereas P-His-HPr dominates in the stationary phase (Gunnewijk and Poolman, 2000). This reflects a relatively high intracellular FBP concentration that subsequently induces CR of the *lacS* promoter. Galactose metabolism by the relatively slow Leloir pathway probably yields insufficient intracellular FBP concentrations for induction of CcpA-mediated repression. CR of the *lac* operon in *S. thermophilus* may not be so much carbon source dependent as determined by the rate of glycolysis relative to sugar uptake, in which the FBP concentration may act as the intracellular indicator of this glycolytic flux. Small-scale fermentation experiments substantiated the negative regulation of CcpA on the uptake and utilization of lactose, but also showed involvement of this regulator in the central metabolism of *S. thermophilus*. In the absence of a functional CcpA, the cells not only take up lactose and expel galactose at least four times faster than the wild-type cells but also show a significant reduction in the amount of lactate produced. The increased lactose uptake by the *ccpA* disruption strain does not result in an increased growth rate. Moreover, glucose was expelled into the fermentation medium by the *ccpA* disruption mutant, and its amount correlates with the amount of internalized lactose and that of lactate produced, closing the carbon balance. Obviously, this glucose is derived from lactose, since not only the amount of LacS transporter, and hence its transport capacity, was increased in the *ccpA* disruption mutant, but also the β-galactosidase activity. Since this glucose is expelled with a short lag time, whereas galactose is expelled instantaneously and in equimolar amounts with lactose uptake, it is likely that the additional amount of glucose entering glycolysis (from the increased uptake of lactose) in the *ccpA* disruption mutant cannot be processed by glycolysis and is expelled.

S. thermophilus ldh expression is sugar regulated and mediated by CcpA. The *cre* site found in the *ldh* promoter region is situated upstream of the -35 box, agreeing with positive control by CcpA (Henkin, 1996). *ldh* induction is highest during growth on lactose and decreased during growth on glucose and galactose, the order of which correlates with the

growth rates observed. The activating effect of CcpA is presumably also mediated by P-Ser-HPr, explaining the high induction of *ldh* transcription on lactose and lower induction on glucose and galactose. However, as lactate dehydrogenase catalyzes the last step in homolactic fermentation, it is unlikely that this is the sole glycolytic step regulated by CcpA, causing the massive glucose expulsion. The *ccpA* disruption mutant of strain CNRZ302 still produces only lactate as its end product, although several strains of *S. thermophilus* have been reported to produce other end products, like acetoin, α-acetolactate, and diacetyl (Teraguchi *et al*., 1987). Apparently, no massive accumulation of the pyruvate pool occurs in this mutant, indicating that glycolysis indeed is failing at additional steps, similar to what has been reported for *L. lactis* (Luesink *et al*., 1998); E. Jamet, C. Delorme, S. D. Ehrlich, A. Bolotine, A. Sorokine, and P. Renault, Proc. 6th Symp. Lactic Acid Bacteria, abstr. H58, 1999). In the small-scale lactose fermentations, the internalization of lactose was four times faster in the *ccpA* disruption strain compared to the wild-type strain, while lactate expulsion was reduced twofold. This indicates that during exponential growth, *S. thermophilus* has a lactose transport capacity that exceeds the maximal glycolysis rate by at least twofold, suggesting that glycolysis tunes down the total lactose transport capacity to meet maximal glycolytic flux. This is in contrast to the situation in various other bacteria, where uptake of a PTS substrate is the principal rate-limiting factor in sugar metabolism (Postma *et al*., 1993). During late exponential and stationary growth, P-His-HPr becomes the predominant phosphorylated form of HPr, which indicates that lactose transport probably becomes rate limiting (Gunnewijk and Poolman, 2000).

CcpA has been studied in many low-G+C gram-positive bacteria, where it mediates CR when cells are grown on PTS carbon sources, of which glucose is the most preferred. To the best of our knowledge, no other catabolic systems have been reported in which non-PTS carbon sources induce CR at the transcriptional level. Lactose, a non-PTS sugar to which *S. thermophilus* is highly adapted for growth, causes not only repression of the *lac* operon but also activation of glycolysis, both events being mediated by CcpA. Glucose, also a non-PTS sugar for *S. thermophilus*, is not able to repress the *lac* operon, and the activation of glycolysis is not as strong as that induced by lactose. In conclusion, CcpA simultaneously tunes the uptake of lactose and the capacity for glycolysis to yield optimal glycolytic flux and growth rate of *S. thermophilus*.

ACKNOWLEDGEMENTS

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Chapter 5

Determination of Glycolytic Intermediates in *Streptococcus thermophilus* and Their Effect on Metabolic Control of Sugar Utilization

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Submitted for publication

SUMMARY

Catabolite repression in bacteria is mainly modulated by intracellular concentrations of glycolytic intermediates that are dependent on the efficiency of metabolism of a carbon source. With four sugars tested, we compared steady-state levels of glycolytic intermediates in *Streptococcus thermophilus* growing on fast-metabolizable (lactose and sucrose) and slow-metabolizable (glucose and galactose) carbon sources that are internalized via a phosphoenolpyruvate(PEP)-dependent phosphotransferase system (PTS) (sucrose) or secondary transport systems (glucose, lactose and galactose). Metabolites were isolated by extraction in boiling water and subsequent HPLC analysis allowed reproducible detection of 6 relevant glycolytic intermediates. Cells of *S. thermophilus* grown on lactose accumulated more than two times higher levels of glucose-6-phosphate and fructose-6-phosphate than cells grown on the other sugars tested. While PEP levels were low (below 1 mM), all cells were found to contain high concentrations of intracellular pyruvate that was highest (17 mM) in glucose-grown cells. Similarly, fructose-1,6-biphosphate (FBP) accumulated to high steady-state levels, notably in sucrose- and lactose-grown cells (16 mM and 5 mM, respectively). Intermediate steady-state levels for FBP were at least two-fold lower in *ccpA* mutant cells grown on these disaccharides relative to wild-type cells. ATP levels were reduced two-fold in the *ccpA* mutant strain grown on either glucose, lactose or sucrose. These results indicate that glycolytic intermediates accumulate in fast-metabolizing cells to favor catabolite control and that PTS sugar sources are preferred over permease sugars. However, *S. thermophilus* CNRZ302 grows fastest on lactose as carbon source, probably due to the kinetics of the LacS permease from which the rate of lactose uptake mimics that of a PTS without the drain of the PEP-pool.

INTRODUCTION

Bacterial carbon uptake and metabolism are closely linked to achieve maximal growth. Favorable carbohydrates are usually taken up by the phosphoenolpyruvate-dependent(PEP) sugar phosphotransferase system (PTS) that catalyzes transport and concommitant phosphorylation of the incoming carbon source (Postma *et al*., 1993). PTS uptake involves a phosphorylation cascade mediated by the general phosphocarrier proteins Enzyme I and HPr and the sugar-specific uptake complex comprising the EIIA, B and C components. At the expense of PEP, Enzyme I is autophosphorylated and donates the phosphoryl group to histidine 15 of HPr. The phosphoryl group is subsequently transferred from P-His-HPr to component IIA and finally, component IIB. Consequently, the IIC domain is activated and translocates the carbohydrate that becomes phosphorylated at the expense of P~IIB. Less favorable sugars are generally transported by non-PTS permeases and, in most cases, yield lower energy levels and slower growth rates relative to PTS sugars (Postma *et al*., 1993). Catabolite repression enables the cell to preferentially utilize the most favorable carbon source present in the growth medium, among others, by repression of operons encoding genes for uptake and metabolism of less favorable sugars. In low-G+C Gram-positive bacteria the PTS phosphocarrier HPr can be phosphorylated at an additional residue, Ser-46 (Deutscher and Saier, 1983). P-Ser-HPr acts as a corepressor to enhance the binding of the catabolite control protein A (CcpA) to catabolite response elements (*cre*) which are localized in promoters of operons under catabolite control (Henkin, 1996; Weickert and Chambliss, 1990). Intracellular levels of fructose-1,6-biphosphate (FBP), ATP and Pi modulate the activity of an ATP-dependent HPr kinase/phosphatase (HPrK/P) that is is involved in

(de)phosphorylation at residue 46. In this way glycolytic activity is linked to catabolite control (Deutscher *et al*., 1995; Jones *et al*., 1997; Kravanja *et al*., 1999).

Streptococcus thermophilus, a homofermentative thermophilic bacterium, is used in combination with other lactic acid bacteria as a starter culture for the production of yogurt and certain cheeses. Its main contribution in milk fermentations is the rapid acidification of milk by conversion of lactose to lactic acid. In contrast to what is found in most bacteria, glucose is a non-PTS carbon source for this *S. thermophilus* and is a poor substrate for growth (Poolman, 1993; van den Bogaard *et al*., 2000). Sucrose is one of the few sugars that is taken up by a PTS and allows rapid growth, that is only exceeded by growth on lactose. *S. thermophilus* is highly adapted to lactose as the primary carbon and energy source but this disaccharide, lactose is a non-PTS sugar since it is transported by the dedicated permease LacS (Foucaud and Poolman, 1992). Kinetic studies indicated that LacS mediates both galactoside exchange (e.g. lactose/galactose) and movement of galactosides and protons. However, the exchange reaction is highly favored with excess galactosides on either side of the membrane (Foucaud and Poolman, 1992). In most *S. thermophilus* strains this exchange is favored by the absence of expression of the *gal* operon involved in the Leloir pathway genes and reflects their adaptation to milk that contains high concentrations of lactose (van den Bogaard *et al*., 2002a; Vaughan *et al*., 2001). Hence, the milk-adapted *S. thermophilus* strains only ferment the glucose moiety of lactose while the galactose moiety is excreted into the medium in stoichiometric amounts with the lactose imported. Remarkably, the efficiency of the galactose/lactose exchange mode of LacS is increased by specific phosphorylation of the the LacS IIA-like domain that is mediated by P-His-HPr. Notably, this phosphoryl group is not transferred from the EIIA domain to the incoming sugar, as is the case with PTS IIA domains (Gunnewijk and Poolman, 2000; Poolman *et al*., 1995). The *S. thermophilus gal-lac* gene cluster contains the *galR* regulatory gene in opposite orientation of the *gal* operon that is followed by the genes encoding the lactose permease (*lacS*) and a β-galactosidase (*lacZ*) for lactose transport and hydrolysis. Transcription of the *lac* operon is induced during growth on lactose by the activity of the *gal* operon specific regulator protein GalR (Vaughan *et al*., 2001). Moreover, transcriptional studies of the *lac* operon showed relief of repression of the *lacS* promoter in the absence of a functional CcpA when cells were grown on lactose (van den Bogaard *et al*., 2000). Similar strains also showed the involvement of CcpA in activating expression of the *ldh* gene ensuring high concentrations of this lactate-forming enzyme during maximal growth on lactose. While the *galKTE* genes are transcriptionally silent in milk-adapted strains, galactose-fermenting mutants were isolated from *S. thermophilus* strain CNRZ302 and their molecular characterization showed these mutants all to be *galK* promoter-up mutants (Vaughan *et al*., 2001). One of these galactose-fermenting mutants, *S. thermophilus* NZ302G, was used in this study.

Intracellular levels of glycolytic intermediates serve as primary indicators of metabolic and energy status in the cell, and closely link the rates of carbon influx and central metabolism. Several methods for the isolation and measurement of intracellular metabolite levels have been reported. Due to the short half-lives of metabolites *in vivo*, accurate analysis of metabolites requires rapid extraction of cell pools and instant inactivation of metabolic enzymes (Fraenkel, 1992). Once extracted and stabilized, several methods can be employed to analyze the metabolites. A relative simple method is thin-layer chromatography, but the number of metabolites that can be separated is limited and detection limits are relatively high (Thompson and Torchia, 1984; Waring and Ziporin, 1964). Measurements of intracellular metabolite levels *in vivo* by nuclear magnetic resonance has proven to be very useful for the determination of metabolic fluxes inside living cells due to the non-invasive character of the method (Neves *et al*., 1999; Thompson and Torchia, 1984). However, a drawback of this

method is that it is not easily applied to multiple samples and high cell densities are required for detectable metabolite levels (Bhattacharya *et al*., 1995). Another commonly used method is the detection of individual metabolites with enzyme-dependent conversion reactions that can be followed spectrophotometrically. However, this is a very laborious approach, notably when multiple samples have to be handled as each individual metabolite is measured with a different assay (Babul *et al*., 1993; Thompson and Torchia, 1984). High-performance liquid chromatography (HPLC) has been used to analyze multiple metabolites in single runs but their detection by refractive index determination or ultraviolet absorption has not been not sufficiently sensitive (Fraenkel, 1992; Taha and Deits, 1994). However, sensitive detection by supressed conductivity was recently reported of virtually all glycolytic intermediates separated in a single HPLC run together with simple protocols for their extraction from *Escherichia coli* and *Lactococcus lactis* (Babul *et al*., 1993; Bhattacharya *et al*., 1995).

While the control of sugar transport and degradation in *S. thermophilus* has been studied in considerable detail, no data on the concentrations of intracellular glycolytic intermediates have been reported. Therefore, we compared the steady-state intermediate levels of wild-type and *ccpA* mutant *S. thermophilus* cells grown on PTS and non-PTS carbon sources. Metabolites were isolated by extraction in boiling water and subsequently analyzed by HPLC (Bhattacharya *et al*., 1995). With this method the majority of glycolytic intermediates from *S. thermophilus* could be isolated, stabilized and quantitatively analyzed. Our results clearly show that steady-state levels of glycolytic intermediates vary with the carbon source and appear to depend on the mode of transport and metabolism prior to glycolysis. Moreover, our presented data suggest that catabolite control operates at multiple steps of glycolysis besides the previously reported transcriptional control of lactate dehydrogenase.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions. All strains used in this study were described previously and include isogenic derivatives of *S. thermophilus* CNRZ302 (wild-type Gal^-,(Benataya ***et al.***, 1991). NZ302G is a Gal^+ derivative of CNRZ302 carrying a promoter-up mutation in the *galKTE* promoter, (Vaughan *et al*., 2001), NZ6150 is a *ccpA* disruption derivative of CNRZ302, (van den Bogaard *et al*., 2000) and NZ6151 is a *ccpA* disruption derivative of NZ302G, (van den Bogaard *et al*., 2000). These strains were routinely grown at 42°C in M17 broth (Difco, Surrey, U.K.) containing 1% (w/v) of the chosen carbon source. When appropriate, erythromycin (Em) was used for selection in at 2.5 μg/ml.

Determination of sugar uptake rates. *S. thermophilus* strains were grown to an OD_{600nm} of 1.0 in M17 broth containing 1% (w/v) of either glucose, lactose, sucrose or galactose. Cells were harvested, washed and resuspended in a 100 mM phosphate buffer (pH 6.8) at a final OD_{600nm} of 10.0. The cells were pre-incubated for 2 min at 42°C, and resting cell-fermentations were started by addition of the appropriate sugar to a final concentration of 100 mM. To measure sugar uptake rates, samples were taken from the fermentation suspension at regular time-intervals and instantly frozen in liquid nitrogen. Prior to analysis, the samples were centrifuged (20,000 x g at 4 °C for 15 min) resulting in a slow thawing and supernatants were analyzed by HPLC. The initial uptake rates were calculated from the samples taken in the first minute after addition of lactose.

Metabolite extraction and analysis. Resting cells were prepared and cell extracts were obtained by using the boiling water extraction method essentially as previously described (Thompson and Torchia, 1984). The *S. thermophilus* strains were grown to mid-logarithmic phase (OD_{600nm} of 1.0) in M17 broth containing either 1% (w/v) of either glucose, lactose, sucrose or galactose. Cells were harvested, washed and resuspended in 50 mM MES buffer (pH 6.8) at a final OD_{600nm} of 50. The cells were pre-incubated for 2 min at 42°C, and resting cell fermentations were started by addition of the appropriate sugar to a final concentration of 1% (w/v). Starved cells were prepared by incubating lactose-grown CNRZ302 and NZ6150 for 5 min at 42°C in MES buffer without a sugar source. For metabolite extraction, 1 ml samples were taken after 3 min of fermentation and quickly pipetted into 5 ml of boiling distilled water. All metabolites that were analyzed reached steady-state levels within 1 min after addition of lactose in all cultures used (data not shown). After boiling for 3 min the samples were pre-chilled on ice, followed by freezing in liquid nitrogen and lyophilization. The lyophilizate was resuspended in 500 µl of water and centrifuged (20,000 x g, 4 °C, 10 min) to eliminate cell debris and the supernatant was passed through a 0.22-µm filter before HPLC analysis. Metabolites were analyzed on a Dionex DX-300 HPLC system as described previously (Bhattacharya *et al*., 1995). A conductivity detector (Dionex, Pulsed Electrochemical Detector) was used in combination with an anion self-regenerating suppressor (Dionex) to enhance the signal-to-noise ratio. In tandem with this suppressed conductivity detector, a variable wavelength detector (Spectro-Physics UV1000) was used, set at 260 nm. Since lactate can not be detected by these detection systems, it was separately detected using a refractive index detector. All glycolytic intermediates could be resolved in a single run with the exception of pyruvate due to the buffer-system used that resulted in masking of this important intermediate by the MES buffer peak. Since this could not be prevented by the use of alternative buffers (phosphate-, TAE-, HEPES-buffers were tested), pyruvate levels were determined using a separate HPLC system. Metabolites in cell extracts were identified by cochromotography of metabolite standards. The amount of individual metabolites in the cell pool extracts were determined by interpolating integrated peak areas against a calibration curve and intracellular concentrations of metabolites were calculated from these measurements. The cell density was correlated to the cell mass at 0.455 g (dry weight)/liter of M17 medium at an OD_{600nm} of 1 and cell volume at 2.34 µl/mg (dry weight) (Hutkins and Ponne, 1991).

RESULTS

Sugar uptake-rates in *S. thermophilus*.

The initial rates of sugar uptake in *S. thermophilus* were compared in resting cells of the wild-type CNRZ302 and its isogenic derivatives carrying a deletion in the *ccpA* gene (NZ6150), a *galKTE* promoter up-mutation allowing efficient growth on galactose (NZ302G), or both (NZ6151)(Table 1). Wild-type (Gal^-; $CcpA^+$) cells showed the highest initial uptake rate for lactose followed by that for glucose, sucrose and galactose. The uptake rate for sucrose was about equal to that for galactose and two-fold lower than for glucose, although glucose and galactose are poor substrates for growth for *S. thermophilus* compared to sucrose. However, sucrose consists of two metabolizable sugar moieties per molecule transported and hence lactate formation was found to be faster on this disaccharide compared to galactose- and glucose-fermenting cells (data not shown). Lactose-grown wild-type (Gal^-; $CcpA^+$) and NZ302G (Gal^+; $CcpA^+$) cells showed an initial lactose uptake rate was that was 3-fold higher than that observed in their $CcpA^-$ derivatives as reported previously (van den Bogaard *et al*., 2000). In the galactose-fermenting strains NZ302G (Gal^+; $CcpA^+$) and NZ6151 (Gal^+; $CcpA^-$) lactose uptake

was somewhat increased compared to that in CNRZ302 (Gal$^-$; CcpA$^+$) and NZ6150 (Gal$^-$; CcpA$^-$). The glucose uptake rate did not differ significantly in the *ccpA* mutant cells (NZ6150 and NZ6151) while the uptake rates for sucrose and galactose were reduced relative to their parental CcpA$^+$ strains. Lactate production-rates were stoichiometric to the amount of sugar fermented for all CcpA$^+$ strains, which were reduced to 30-50 % in their isogenic CcpA$^-$ strains (results not shown).

Strain	Initial uptake rates (μM/min/OD_{600})			
	lactose	sucrose	glucose	galactose
CNRZ302 (Gal$^-$; CcpA$^+$)	288	76	146	n.g.
NZ6150 (Gal$^-$; CcpA$^-$)	857	40	175	n.g.
NZ302G (Gal$^+$; CcpA$^+$)	328	n.d.	n.d.	80
NZ6151 (Gal$^+$; CcpA$^-$)	1197	n.d.	n.d.	63

TABLE 1. Initial uptake rates of *S. thermophilus* CNRZ302 (Gal$^-$; CcpA$^+$) and NZ6150 (Gal$^-$; CcpA$^-$) for glucose, lactose or sucrose, and NZ302G (Gal$^+$; CcpA$^+$) and NZ6151 (Gal$^+$; CcpA$^-$) for lactose or galactose. Presented are the mean values of 2 independent experiments, errors were below 10%. n.d. indicates not determined, n.g. indicates no growth.

Metabolite isolation and detection.

Cell extraction and subsequent HPLC analysis was performed to determine the steady-state levels of the majority of glycolytic intermediates in lactose-grown cells of *S. thermophilus* CNRZ302 (Gal$^-$; CcpA$^+$) and NZ6150 (Gal$^-$; CcpA$^-$)(Fig. 1). From a mixture of standards all glycolytic intermediates could be detected in a single HPLC run except for pyruvate and the end product lactate that were determined using a separate HPLC system and detected separately. Due to the masking of several glycolytic intermediates by inorganic phosphate, the phosphate buffer used for the determination of the sugar uptake-rates was replaced by a MES buffer when metabolites were analyzed from the cells. Sugar uptake kinetics and levels of the detectable metabolites were found to be similar in both buffer systems. Furthermore, 2-phosphoglycerate and 3-phosphoglycerate could not be further separated, and hence their combined amounts were determined (2+3PG; see Fig. 1). Control experiments indicated that the metabolite standards used in this study were completely stable during the boiling and other steps of the extraction procedure. However, glyceraldehyde-3-phosphate, 1,3-diphosphoglycerate and dihydroxyacetone-phosphate were not stable under these conditions and were excluded from our analysis (Fig. 1).

The positions of the intracellular metabolites following their separation by HPLC were identical in the extracts from the wild-type CNRZ302 and its isogenic ccpA mutant NZ6150 (Fig. 1). However the amounts of these intermediates, as determined by their conductivity, generally higher in the wild-type strain than in its ccpA mutant.

To assess the reproducibility of the boiling water extraction and HPLC detection method, individual metabolites were determined from three independent extractions procedure from samples taken from a single culture of wild-type CNRZ302 (Gal$^-$; CcpA$^+$) grown on lactose. The error margin for all glycolytic intermediates was low (below 2%), indicating high reproducibility of the boiling water extraction and HPLC detection method (results not shown). Although reproducible levels for the the individual metabolites were observed, some variations were found between independently grown cultures of CNRZ302, reflecting the effect of slight differences in growth phase and culture history (see below).

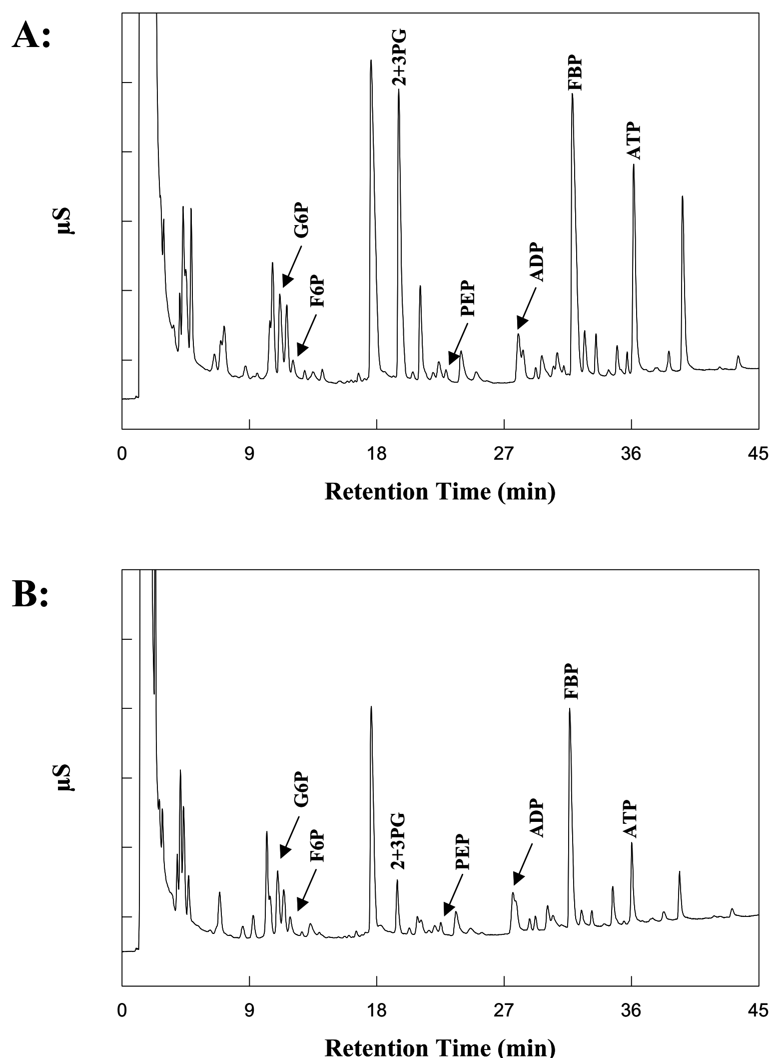

FIG. 1. HPLC analysis of steady-state cell pool metabolites in *S. thermophilus* grown on lactose. A: CNRZ302 (Gal^-; $CcpA^+$) and B: NZ6150 (Gal^-; $CcpA^-$).

Metabolite analysis in wild-type and *ccpA* mutant strains during lactose fermentation.

For the analysis of glycolytic intermediate levels, *S. thermophilus* strain CNRZ302 (Gal^-; $CcpA^+$) and strain NZ6150 (Gal^-; $CcpA^-$) were grown to mid-logarithmic phase in medium containing excess lactose. Cell extracts were prepared from resting cells (non-growing but metabolically active cells), when steady-state glycolytic intermediate levels had been reached. In lactose-fermenting *S. thermophilus* CNRZ302 cells high relative levels of FBP, 3GP and pyruvate were detected (Table 2). Glucose-6-phosphate (G6P) was detected at relatively medium level, while relative fructose-6-phosphate (F6P) and PEP levels were low. CcpA has been shown to regulate lactose uptake and hydrolysis, as well as lactate production in *S. thermophilus* (van den Bogaard *et al*., 2000). Cells of the *ccpA* mutant showed increased lactose uptake compared to wild-type cells, (Table 1), but the relative amounts of FBP and

2+3PG were 2- to 3-fold higher in wild-type cells compared to $CcpA^-$ cells, as was also the case for ATP levels There was no significant change in the other metabolites or ADP levels. In starved CNRZ302 cells only 2+3PG, PEP and pyruvate could be determined (0.079, 0.056 and 3.0 mM, respectively). In the $CcpA^-$ strain these levels were found to be equal (0.083, 0.053 and 3.0 mM, respectively), indicating that CcpA has no apparent effect on intermediate levels in starved cells (data not shown).

Metabolite	intracellular concentrations (mM)		Ratio (WT/$CcpA^-$)
	CNRZ302 (WT) (Gal^-; $CcpA^+$)	NZ6150 (Gal^-; $CcpA^-$)	
G6P	1.8 ± 0.5	1.5 ± 0.4	1.2
F6P	0.38 ± 0.05	0.35 ± 0.03	1.1
FBP	6.1 ± 1.3	3.9 ± 0.3	1.6
3PG	4.0 ± 0.8	1.5 ± 1.0	2.7
PEP	0.31 ± 0.13	0.21 ± 0.06	1.5
Pyr	5.0 ± 2.1	4.8 ± 1.5	1.0
ATP	1.95 ± 0.52	0.79 ± 0.26	2.4
ADP	0.50 ± 0.12	0.40 ± 0.12	1.2

TABLE 2. Intracellular concentrations of glycolytic intermediates in *S. thermophilus* CNRZ302 (Gal^-; $CcpA^+$) and NZ6150 (Gal^-; $CcpA^-$) during lactose fermentation.

Carbon source dependent metabolite steady-state levels. In order to determine the carbon source dependency of the glycolytic intermediate steady-state levels in *S. thermophilus,* CNRZ302 (Gal^-; $CcpA^+$) and NZ6150 (Gal^-; $CcpA^-$) cells were grown on glucose or sucrose and their isogenic galactose-fermenting derivatives NZ302G (Gal^+; $CcpA^+$) and NZ6151 (Gal^+; $CcpA^-$) were grown on galactose. Resting cells were prepared and metabolism was reinitiated by the addition of the same carbon source as was used during growth (Table 3). The carbon source clearly affects the glycolytic intermediate levels. Concentrations of the primary glycolytic intermediates (G6P and F6P) were 3- to 4-fold higher in lactose-fermenting wild-type cells compared to cells fermenting glucose, sucrose or galactose (Table 3). The steady state concentration of FBP was highest in sucrose-fermenting wild-type cells of CNRZ302 and reached values that were approximately 3-fold higher than in lactose-fermenting cells and upto 6-fold higher when compared to cells fermenting galactose or glucose. The effect on the concentrations of 2+3PG and pyruvate by the different carbon sources was very small, whereas the PEP levels appeared variable. Further in the glycolytic pathway, FBP was the first metabolite that was affected by the CcpA deficiency of strain NZ6150 (Gal^-; $CcpA^-$). Glucose-, lactose- and sucrose-fermenting *ccpA* mutant cells contained a reduced steady-state FBP level compared to the wild-type CNRZ302 strain. In contrast, the FBP level measured in galactose-fermenting cells NZ302G (Gal^+; $CcpA^+$) and NZ6151 (Gal^+; $CcpA^-$) appeared unaffected by the *ccpA* mutation.
In analogy, previous work showed that glucose, lactose and sucrose exert catabolite control in *S. thermophilus*, while galactose did not, thereby corroborating the relation between intracellular FBP concentrations and CcpA mediated catabolite control (Deutscher *et al*., 1995; van den Bogaard *et al*., 2000). Pyruvate levels were found to be high relative to the PEP levels, ranging between 10- to 20-fold in cells of all strains on tested sugars. The ATP levels were approximately two-fold reduced in the *ccpA* mutant, except for galactose-grown cells. In wild-type cells the steady-state ATP levels showed a similar sugar-dependent

relation as FBP, with highest levels for sucrose-, followed by lactose-, glucose- and galactos-fermenting cells. This reflects a similar relation between intracellular ATP concentrations and CcpA-mediated catabolite repression as was found for FBP. ADP steady-state levels were similar in all strains tested on all sugars, showing neither sugar nor CcpA dependency.

Metabolite	Intracellular concentration (mM)					
	Glucose		Sucrose		Galactose	
	CNRZ302 (Gal^-; $CcpA^+$)	NZ6150 (Gal^-; $CcpA^-$)	CNRZ302 (Gal^-; $CcpA^+$)	NZ6150 (Gal^-; $CcpA^-$)	NZ302G (Gal^+; $CcpA^+$)	NZ6150 (Gal^+; $CcpA^-$)
G6P	0.41 ± 0.10	0.53 ± 0.20	0.66 ± 0.11	0.68 ± 0.09	0.54 ± 0.19	0.67 ± 0.05
F6P	0.16 ± 0.02	0.14 ± 0.02	0.10 ± 0.03	0.09 ± 0.02	0.16 ± 0.02	0.14 ± 0.03
FBP	2.6 ± 2.3	0.82 ± 0.20	16.3 ± 2.1	6.9 ± 2.6	3.0 ± 0.2	4.2 ± 1.2
3PG	3.00 ± 0.04	2.0 ± 0.4	2.2 ± 0.8	1.5 ± 0.7	2.1 ± 0.5	1.7 ± 2.
PEP	0.57 ± 0.19	0.35 ± 0.03	0.35 ± 0.10	0.19 ± 0.03	0.17 ± 0.07	0.16 ± 0.06
Pyr	16.9 ± 1.3	4.2 ± 0.2	9.2 ± 2.1	6.3 ± 2.4	11.8 ± 3.5	7.9 ± 2.5
ATP	1.59 ± 0.44	0.73 ± 0.10	2.64 ± 0.10	1.38 ± 0.30	1.48 ± 0.07	1.11 ± 0.40
ADP	0.46 ± 0.21	0.43 ± 0.17	0.46 ± 0.29	0.37 ± 0.25	0.46 ± 0.29	0.57 ± 0.37

TABLE 3. Intracellular concentrations of glycolytic intermediates in *S. thermophilus* CNRZ302 (Gal^-;$CcpA^+$) and NZ6150 (Gal^-; $CcpA^-$) during fermentation of glucose or sucrose, and NZ302G (Gal^+; $CcpA^+$) and NZ6151 (Gal^+; $CcpA^-$) during fermentation of galactose. Presented are the mean values of 2 independent experiments.

DISCUSSION

So far, no substantial data on metabolite levels in *S. thermophilus* have been reported and metabolite analysis in lactic acid bacteria has focused mostly on *L. lactis* (Even *et al.*, 2001; Neves *et al.*, 1999; Thompson, 1978). In this study we aimed to provide insight in the concentrations of glycolytic intermediates in *S. thermophilus* in relation to its efficient use of non-PTS sugars and specific catabolite control by CcpA. Glycolytic intermediates serve as primary indicators of metabolic and energy status in the cell, and closely link the rate of carbon influx and central metabolism. We applied a straightforward method for the isolation and detection of glycolytic intermediates that was previously validated for *E. coli* and our results confirm the applicability of this method for *S. thermophilus*. We compared glycolytic intermediates steady-state levels in wild-type and *ccpA* mutant cells of *S. thermophilus* growing on fast- and slow- metabolizable carbon sources represented PTS that non-PTS sugars. The FBP levels of fast-metabolizing *S. thermophilus* cells play a central role as indicator for the rate of glycolysis and accumulate to favor catabolite control. Furthermore, FBP and ATP levels indicate that also in *S. thermophilus* PTS sugars are preferred over permease sugars. However, growth on lactose yields the highest accumulation of early glycolytic intermediates, reflecting the highest import-rate for of this preferred sugar. Moreover, starved *S. thermophilus* cells do not accumulate PEP as was found in other bacteria, indicating that they are adapted to non-PTS carbon sources.

In contrast to several other metabolite extraction methods that include the use of alcohols or organic acids and have the disadvantage of column or detector interference in HPLC analysis, no chemicals are added in the procedure used here (Bhattacharya *et al.*, 1995; Jensen *et al.*, 1993; Reiss *et al.*, 1984). Transferring the fermenting cells directly to a large volume of boiling water ensures rapid inactivation of glycolytic enzymes which is crucial for

these types of measurements. This extraction method proved to be highly reproducible as error margins were well below 2% for all relevant metabolites. Some variations in levels of individual metabolites were were found, and probably reflect the dynamic nature of central metabolism. Moreover, errors seem variable for different intermediates. This could be the result of differences in half-lives (depending on enzymatic conversion rates) of the individual intermediates as they also have different steady-state levels. Our inability to separate 2PG and 3PG is in analogy with several studies that also report difficulties separating these two components, either by HPLC, TLC or ^{31}P NMR spectroscopy (Bhattacharya *et al*., 1995; Thompson and Torchia, 1984). In *E. coli* and *L. lactis* the steady-state concentration of 3PG are 5-10 fold higher compared to 2PG so we assume that the determined amounts of 2+3PG consisted mostly of 3PG (Even *et al*., 2001; Neves *et al*., 1999).

Most absolute levels of glycolytic intermediates in sugar-fermenting *S. thermophilus* are quite similar to those found in several strains of *L. lactis*, where also high levels of FBP, intermediate levels of G6P and 2+3PG and low levels of F6P and PEP we reported (Even *et al*., 2001; Garrigues *et al*., 1997; Thompson, 1978). In starved *S. thermophilus* cells intracellular concentrations of PEP and pyruvate remained in the same order of magnitude compared with the levels observed in lactose-fermenting cells, whereas al other glycolytic intermediates were very low or undetectable. This contrasts markedly with the high concentrations of 2+3PG and PEP (more than 13 mM) that have been observed in starved *L. lactis* (Thompson and Torchia, 1984) and *E. coli* (Bhattacharya *et al*., 1995). Pyruvate concentrations remained low in starved *L. lactis* cells, more or less at the same level as sugar-fermenting cells (Thompson, 1978). It has been suggested that the PEP concentration increases in starved cells due to a partial inhibition of the pyruvate kinase by the increased free phosphate concentration and the depletion of the intracellular pyruvate kinase-activators G6P and FBP (Garrigues *et al*., 1997). By this mechanism, the cells maintain a high intracellular PEP level to allow rapid sugar intake via the PTS's upon the availability of PTS sugar sources. However, we have shown previously that lactose is the preferred carbon source for *S. thermophilus* CNRZ302 yielding the highest growth- and acidification-rate although it is not transported by a PTS but by the dedicated permease LacS. When lactose becomes limiting during growth of *S. thermophilus*, the efficiency of the LacS galactose/lactose exchange mode can be increased by specific phosphorylation of the IIA-like domain that is mediated by P-His-HPr and hence requires PEP. *S. thermophilus* apparently adapts to starvation by increasing the capacity for lactose-uptake instead of that for sugar-uptake via PTS (Gunnewijk and Poolman, 2000; Poolman *et al*., 1995). Since PEP is not consumed by the uptake of lactose it is not required to accumulate under starving conditions. However, how this is achieved is not completely clear yet. FBP is known as an activator of the lactate dehydrogenase in most lactic acid bacteria, and its depletion in starved cells also reduces lactate dehydrogenase activity (Garrigues *et al*., 1997). In contrast, FBP is not an activator of the *S. thermophilus* lactate dehydrogenase (Garvie, 1978) and other *S. thermophilus* enzymes (e.g. pyruvate kinase) may have different kinetic parameters that result in altered PEP maintenance relative to that of other LAB.

The sugar-uptake data revealed that in resting *S. thermophilus* CNRZ302 cells lactose is taken up faster than any of the other sugars tested, which reflected in the highest growth-rates on lactose. Furthermore, lactose metabolism in this strain was shown to be regulated by CcpA at the lactose uptake and lactate formation level (van den Bogaard *et al*., 2000). In contrast to lactose and other sugars, sucrose uptake by *S. thermophilus* is mediated by the sugar-specific PTS. Growth and acidification rates observed in sucrose- and lactose-growing cells are high compared to glucose- and galactose-growing cells. Therefore, metabolite steady-state levels can be compared in fast and slow metabolizing *S. thermophilus* cells. Lactose-grown cells

contained the highest levels of G6P and F6P compared to cells grown on the other sugars tested and this was found to be independent of CcpA. The LacS permease is a rapid and efficient uptake system for lactose yielding high amounts of glucose entering glycolysis via the glucokinase, which apparently leads to the accumulation of G6P and F6P. FBP also accumulates to higher steady-state concentrations in lactose-grown cells relative to glucose- or galactose-grown cells. Highest FBP steady-state levels were found in sucrose-fermenting cells, corroborating the idea that PTS sugars potentially exert stronger catabolite repression then permease sugars (Postma *et al*., 1993; Stulke and Hillen, 1999). However, in *S. thermophilus* lactose also causes catabolite control and growth on this sugar also allowed an accumulation of FBP although not as high as growth on sucrose. In lactose- and sucrose-grown cells the PEP levels were found to be about equal. Transport of sucrose by the PTS would cause a substantial drain on the PEP-pool in sucrose-grown cells, indicating that the PEP production in these cells is significantly higher than in lactose-grown cells. Since in several bacteria FBP is known to be an activator of pyruvate kinase (Fordyce *et al*., 1984; Le Bras and Garel, 1993) the high FBP levels found in sucrose-grown cells probably stimulate a high PEP production for PTS transport in concert with activation of catabolite control. In lactose-growing *S. thermophilus* cells FBP levels might only serve the activation of catabolite control for fine-tuning of lactose uptake and glycolytic activity (van den Bogaard *et al*., 2000). The steady-state levels FBP, 3PG and ATP of in *S. thermophilus* were CcpA-dependent for all catabolite control eliciting sugars (glucose, lactose and sucrose). This coincides with a reduced lactate production and probably reflects the glycolytic potential and growth rate on a given sugar. Since ATP levels are the result of various generating and consuming systems we can only conclude that CcpA indirectly influences the ATP levels, presumably by fine-tuning central metabolism. Remarkable is the observed high glucose uptake rate whereas the growth rate on glucose is low. The high pyruvate levels in glucose-grown cells might indicate an uncoupling between the glucose uptake and glycolysis that leads to a ATP-spending futile cycle. This is supported by the observation that glucose as carbon source in the growth-medium does not cause a strong CcpA-mediated catabolite repression of *lacSZ* and activation of *ldh* transcription as the glucose derived from intracellular lactose hydrolysis, (van den Bogaard *et al*., 2000). However, disruption of *ccpA* in glucose-grown cells clearly affected the level of several glycolytic intermediates (e.g. FBP, 3PG, pyruvate and ATP), indicating that a relative poor carbon source can still exert catabolite control. Galactose-grown cells are not significantly affected in any glycolytic intermediate level by mutation of the *ccpA* gene, which is in analogy with the observation that no catabolite control could be established for growth on this sugar (van den Bogaard *et al*., 2000).

Analysis of the intracellular metabolite levels can generate valuable information for the analysis of metabolic flux and are of critical importance for the construction of metabolic control models that allow to pinpoint enzymatic steps that can be targetted in effective metabolic engineering strategies (de Vos, 1996; Mendes and Kell, 1997). The data presented in this work clearly show CcpA-involvement in global regulation of the *S. thermophilus* glycolysis. Next to sugar uptake and lactate production we now have detailed information on steady-state levels of relevant glycolytic intermediates in relation to the type of carbon source. The high growth-rate and high turn-over of PEP combined with high steady-state levels of FBP and ATP in sucrose-grown cells indicate that this PTS sugar is preferred over permease sugars. This is confirmed by the growth and metabolite data from glucose- and galactose-grown cells. However, *S. thermophilus* CNRZ302 grows fastest on lactose as carbon source, probably due to the kinetics of the LacS permease that mimic that of PTS without the drain on the PEP-pool, reflecting the adaptation to growth on this sugar.

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Chapter 6

Modulation of Glycolysis by Lactose Availability in *Streptococcus thermophilus*

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SUMMARY

Lactose is the preferred carbon source for S*treptococcus thermophilus* and carbon catabolite control of lactose metabolism contributes to fine-tuning sugar uptake and glycolysis. The efficiency of glycolytic steps were determined by analysis of glycolytic intermediates in resting cells isolated from various stages of growth. The influence of catabolite repression was investigated by comparison of *S. thermophilus* CNRZ302 and its isogenic *ccpA* mutant. Wild-type cells isolated from lactose-limiting growth conditions showed decreased lactate production after the transfer to an excess of lactose. Lactose transport capacity in these cells, however, was increased creating an imbalance between lactose import and its subsequent glycolytic conversion. Indicative for this imbalance is the expulsion of the excess transported sugar in the form of glucose. Without a functional CcpA this imbalance is also manifested during growth in excess lactose conditions. Analysis of glycolytic intermediates revealed a change in flux through glycolysis at this transition in growth and indicated that the efficiency of several glycolytic steps was regulated in a growth phase-dependent way. This regulation was absent in *ccpA* mutant cells. Moreover, Northern analysis of all glycolytic genes revealed that CcpA controls the expression of both the *S. thermophilus pfk-pyk* operon and the *ldh* gene, which encode key-enzymes that confer glycolytic control. These results indicate that CcpA not only fine-tunes uptake and glycolytic activity during maximal growth rate but is also involved in derepression of transport activity and reduction of glycolytic capacity to allow cellular adaptation to conditions where lactose in growth medium becomes limiting.

INTRODUCTION

Bacteria actively regulate their metabolic capacity in response to the availability of carbon sources in the growth medium and favorable sugars often repress genes for uptake and metabolism of less favorable sugars. In enteric bacteria the enzyme IIA^{Glc} of the glucose-specific phosphoenolpyruvate(PEP)-dependent phosphotransferase system (PTS) is dephosphorylated mainly by PTS-mediated transport, hereby causing carbon catabolite repression at the transcriptional level via cAMP/CRP. Moreover, enzyme IIA^{Glc} is involved in inducer exclusion of various of enzymes involved in the metabolism of non-PTS carbon sources (Kolb *et al*., 1993; Postma *et al*., 1993). HPr is the phosphoryl-carrier in PTS transport and its phosphorylation by Enzyme I at residue His-15 is PEP-dependent and the resulting P-His-HPr can transfer its phosphoryl group to the sugar-specific PTS components (Postma *et al*., 1993). In *Escherichia coli*, dephospholrylation of enzyme IIA^{Glc} was established in the presence of non-PTS carbon sources and is likely to be determined by the PEP to pyruvate ratio (Hogema *et al*., 1998). Furthermore, P-His-HPr also contributes to catabolite control by activation of non-PTS proteins through transfer of its phosphoryl group to these proteins that include glycerol kinase, transcriptional regulators and antiterminators (Charrier *et al*., 1997; Lindner *et al*., 1999; Martin-Verstraete *et al*., 1998). In low-G+C Gram-positive bacteria, HPr can be phosphorylated at a second residue (Ser-46) by the ATP-dependent protein kinase/phosphatase (HPrK/P)(Galinier *et al*., 1998; Reizer *et al*., 1998; Stulke and Hillen, 2000). P-Ser-HPr subsequently acts as a corepressor to enhance the binding of the catabolite control protein A (CcpA) to catabolite response elements (*cre*), which are localised in promoters of operons under catabolite control (Deutscher *et al*., 1995; Gosseringer *et al*., 1997; Henkin, 1996; Jones *et al*., 1997). Furthermore, P-Ser-HPr is also suggested to be involved in inducer exclusion in (Gauthier *et al*., 1997; Ye *et al*., 1994; Ye

and Saier, 1995). High concentrations of fructose biphosphate (FBP) and ATP trigger the HPrK/P dependent phosphorylation of the Ser-46 residue of HPr, while high inorganic phosphate (Pi) concentrations promote its dephosphorylation by the same enzyme, hereby linking the levels of primary indicators for glycolytic activity and energy status to catabolite control (Deutscher *et al*., 1995; Fujita *et al*., 1995; Jones *et al*., 1997).

Streptococcus thermophilus is a homofermentative, thermophilic and Gram-positive lactic acid bacterium that is highly adapted to growth on lactose as the primary carbon and energy source. Together with other lactic acid bacteria, *S. thermophilus* is used as a starter culture for the production of yogurt and certain cheeses, where it mainly contributes to the rapid acidification of milk by conversion of lactose to lactic acid. Contrary to most bacteria, glucose is a non-PTS carbon source for *S. thermophilus* and is a poor substrate for growth (Poolman, 1993). However, lactose is taken up by a highly efficient, dedicated permease (LacS), and is a very good substrate for growth. Indicative for the adaptation to growth on lactose is the fact that the maximal growth rate on lactose is higher than that on the PTS-sugar sucrose. Most *S. thermophilus* strains only ferment the glucose moiety of lactose and excrete the galactose moiety into the medium in stoichiometric amounts to the lactose transported by the exchange-mode of the transporter LacS (Hutkins *et al*., 1985; Poolman, 1993). The *S. thermophilus* genes encoding LacS and the β-galactosidase are organized in the *lacSZ* operon, the expression of which is induced during growth on lactose (Poolman *et al*., 1989; Vaughan *et al*., 2001). Furthermore, a *cre* site was found in the *lacSZ* promoter and transcriptional analysis showed relief of repression in the absence of a functional CcpA when cells were grown on lactose, resulting in a strong increase of lactose uptake and hydrolysis (van den Bogaard *et al*., 2000). However, these cells were severely disturbed in growth and fermented only a small portion of the imported lactose while expelling the carbohydrate excess in the form of glucose. The CcpA-mediated repression of the *lacS* promoter did not occur in wild-type cells during growth on galactose, taken up by the same LacS transport system. Moreover, transcriptional analysis of the *S. thermophilus* lactate dehydrogenase-encoding *ldh* gene revealed that expression of this gene was induced at least 2-fold during growth on lactose compared to growth on glucose or galactose (van den Bogaard *et al*., 2000). This activation was absent in an isogenic *ccpA* mutant strain, indicating that CcpA is involved in fine-tuning of lactose uptake and LDH activity at maximal growth rate on lactose. In addition to regulation at the transcriptional level, lactose transport is also subject to HPr-mediated allosteric regulation. P-His-HPr can transfer its phosphoryl group to the enzyme IIA domain of the LacS permease which results in increased lactose/galactose exchange activity (Gunnewijk and Poolman, 2000; Poolman *et al*., 1995). P-Ser-HPr is the dominant phosphorylated HPr species in *S. thermophilus* when grown in excess lactose conditions, whereas P-His-HPr becomes dominant when cells encounter lactose limitation during growth (Gunnewijk and Poolman, 2000). As a consequence of this transition, catabolite control of the *lacSZ* promoter is relieved and LacS is phosphorylated, leading to increased lactose uptake capacity when lactose is limiting.

To generate more insight in modulation of the lactose metabolism in *S. thermophilus* and the role of catabolite control in response to lactose availability, we determined the concentrations of relevant glycolytic intermediates and the transcripts of the glycolytic genes as a function of the growth phase. We present evidence that glycolysis is regulated by catabolite control at several steps and show that next to the *ldh* gene also the *pfk-pyk* operon, encoding the key glycolytic enzymes phosphofructokinase and pyruvate kinase, are transcriptionaly activated by CcpA. Intracellular levels of glycolytic intermediates indicate that clear changes in glycolytic flux coincide with relieve of CcpA-mediated catabolite control upon lactose limitation at the later stages of growth. The transition in phosphorylation

state of HPr appears to cause a reduction of the glycolytic capacity, next to an increase of lactose transport capacity. Taken together, these modulations of lactose metabolism allow *S. thermophilus* to adapt effectively to the availability of lactose in the growth medium.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions. *S. thermophilus* CNRZ302 (wild-type but galactose deficient, (**Benataya *et al.***, 1991; Vaughan *et al*., 2001)), and its isogenic *ccpA* disruption derivative NZ6150 (van den Bogaard *et al*., 2000) were routinely grown at 42°C in M17 broth containing 1% (w/v) of lactose (Difco, Surrey, U.K.). If appropriate, erythromycin (Em) was used for selection in growth media at 2.5 μg/ml.

Metabolite extraction and analysis. The *S. thermophilus* strains were grown in M17 broth containing 0.25% lactose and cells were harvested during specific stages of growth during which the pH of the medium remained varied between 6.8 and 6.5 (early-log, mid-log, late-log and stationary phase). Early-log growth was identified as the fist change in OD_{600nm} after inoculation and mid-log growing cells were isolated during maximal growth rate that continued at least for 30 min. During these two growth–phases the lactose amount in the growth medium was in excess. Late-log was identified at the first sign that maximal growth was no longer maintained and stationary phase cells were isolated when growth had seized for 30 min. At these latter stages of growth the amount of lactose was low, limiting maximal growth. Cells were washed and resuspended in 50 mM MES buffer (pH 6.8). The cells were pre-incubated for 2 min at 42°C, and fermentation was started by addition of of lactose to a final concentration of 1%. At regular time-intervals consecutive samples of 1 ml were taken from the fermentation suspension, which were subjected to cell pool extraction. Metabolites were extracted and analyzed as described previously (van den Bogaard *et al*., 2002b). Glucose in the metabolite extracts was enzymatically determined using a D-glucose UV test-method (Boehringer Mannheim, Mannheim, Germany). The cell density (as measured by OD_{600}) was correlated to the cell mass to be 0.455 g dry weight/liter of M17 medium at an OD_{600} of 1 and intracellular concentrations of metabolites were calculated based on a cell volume of 2.34 μl/mg (dry weight) (Hutkins and Ponne, 1991).

NA isolation, Northern blot and primer extension analysis. *S. thermophilus* strains were grown to an OD_{600nm} of 1.0 in M17 broth (30ml) supplemented with 1% (w/v) glucose or lactose. Cells were harvested and total RNA was isolated using the Macaloid method as described previously (Kuipers *et al*., 1993) with the following adaptation: prior to bead-beating the resuspended cells were incubated on ice with lysozyme for 5 min to increase RNA yield. Subsequently, 4.5 μg of RNA was size-separated on a 1.0 % formaldehyde gel and transferred to Gene Screen Plus membranes (Dupont, Boston, Mass.) (Sambrook *et al*., 1989). RNA size markers were obtained from Bethesda Research Laboratories. Northern-blot hybridisations were performed at 65°C in a 0.5 M sodium-phosphate buffer, pH 7.2 containing 1.0% BSA (fractionV), 1.0 mM EDTA and 7.0% SDS and 1 μg of labeled gene-specific probe. The blots were washed at 55-65°C in 0.1 x SSC. Gene-specific probes were generated by PCR using *S. thermophilus* CNRZ302 chromosomal DNA as template with the primers listed in Table 1. The PCR products were purified (SephaglasTM BandPrep Kit, Amersham Pharmacia Biotech Inc., USA) and labeled by nick-translation with [α-^{32}P]dATP (Amersham International plc, UK)(Sambrook *et al*., 1989). Northern-blots were exposed to a Storage Phosphor Screen (Molecular Dynamics) and scanned using a STORM 840 Phosphor Imager (Molecular

Dynamics). Signal quantification was performed using the ImageQuant 1.2 program (Molecular Dynamics).A 16S rDNA probe was generated by PCR using primers NR7 and NR19 with *S. thermophilus* CNRZ302 chromosomal DNA as template, labeled as described above, and used to normalize the gene-specific signals to the total amount of RNA applied to the Northern blots. Primer extension was performed using 20 ng of oligonucleotide 4546 and 15 μg of total *S. thermophilus* CNRZ302 RNA as described previously (Kuipers *et al*., 1993).

Designation	Sequence (5'→3')
glcK-f	CCGTTTGGAATTGTATGG
glcK-r	GCTGTTGCAAGTCCGAGG
pgi-f	TTGCTTTCCGTGTCTTCG
pgi-r	AAGTTAGCTGAAGTTGGG
pfk-f	CGGCATTAACCGTGGATACG[a]
pfk-r	CAAGTTTGTGTGGATTGTTTACG[a]
fba-f	GTTTCCCAATCGTATTGC
fba-r	TACATCGATACGTTCTTC
gap-f	TGGATTCGAAGTTAACGG
gap-r	CGTTCACTTCATCAACAG
tpi-f	AGTTATCGGTGCACCTGC
tpi-r	AGTGAAGCTCCACCAACG
pgk-f	CACTTGCTCCTGTAGCTG
pgk-r	AGAGCTTTCGCTACATCC
pgm-f	ACAAGCTATTGACGCTGG
pgm-r	CAATGGTGGGAAGTTTGG
eno-f	TCGTTACGGTGGTCTTGG
eno-r	TCCAGCAGCTTCGATAGC
pyk-f	GTGAAGATGGTTACTGGAGTG[a]
pyk-r	TACCAGAACCACTACAAGACC[a]
stldh-f	GTCATCCTTGTTGGTGACGG[b]
stldh-r	TGCTTCATCAATGATAGCTTGC[b]
NR7	GAAGCAGCGTGG
NR19	GTCGTTATGCGGTA
4546	GTGGTTACCATGTGAGAAG

TABLE 1. Primers of the *S. thermophilus* glycolytic genes and 16S rDNA used in this study. The primers were based on *S. thermophilus* LMG18311genome sequence except for: [a] based on the *S. thermophilus* CNRZ1205 *pfk/pyk* operon sequence (Crispie *et al*., 2002); [b] based on the *S. thermophilus* M-192 *ldh* sequence (Ito and Sasaki, 1994).

Nucleotide sequence accession numbers. The sequence data of the *S. thermophilus* LMG18311 glycolytic genes have been submitted to the GenBank under the accession numbers: *glcK*, AF442549; *pgi*, AF442550; *fba*, AF442551; *gapdh*, AF442552; *tpi*, AF442553; *pgk*, AF442554; *pgm*, AF442555; *eno*, AF442556. The *pfk/pyk* sequence data of *S. thermophilus* CNRZ1205 genes have been submitted to the GenBank under the accession number AF172173.

RESULTS

Metabolic flux during lactose fermentation.

In this study we evaluated the modulation of the lactose metabolism in *S. thermophilus* and the contribution of catabolite control in response to lactose availability. *S. thermophilus* wild-type and *ccpA* mutant strains were grown in media containing a low lactose concentration (0.25%) to allow them to reach stationary phase due to carbon limitation and

under these conditions growth was considered to depend on the lactose concentration. During the first growth–phases (early-log and mid-log) the lactose amount in the growth medium was in excess while at the later stages of growth (late-log and stationary phase) the lactose amount was at low concentrations, limiting maximal growth. Cells were harvested during these specific stages of growth and were used to determine the rate of sugar import and lactate production as well as the fluxes of glycolytic intermediates in the presence of excess lactose (1%)(Fig. 1). Lactose import rate was increased in resting cells of the wild-type strain grown on low lactose concentration (late-log and stationary phase) relative to cells isolated from lactose excess conditions (early-log and mid-log)(Fig. 1A). However, a clear transition in the efficiency of carbon utilization can be deduced from the expulsion of glucose by late-log and stationary phase cells, which was absent in early- and mid-log cells. This growth phase-dependent modulation of lactose metabolism is reflected by the glycolytic potential of these cells (Fig. 1B). The early glycolytic intertermediates glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) accumulated faster and reached higher steady state levels in wild-type *S. thermophilus* cells grown in lactose excess compared to cells grown at low lactose concentration. The initial FBP accumulation rate was higher when cells were grown under lactose limitation relative to cells grown in lactose excess. However, the initial accumulation rates of 2-phospho- and 3-phosphoglycerate (2+3PG) were very low in cells grown in lactose excess compared to cells grown at low lactose concentration. Similar results were obtained for PEP (data not shown). The initial accumulation rate of ATP was higher in cells glycolysing excess lactose compared to cells isolated under lactose limitation (data not shown). Apparently, the glycolytic capacity in cells growing on at a low lactose concentration was reduced, while transport capacity remained high, leading to the glucose accumulation when these cells were transferred to an excess of lactose. These observations were confirmed by the reduced rate of lactate production in these cells relative to those grown in lactose excess (Fig. 1A). Clearly, wild-type *S. thermophilus* cells grown in an excess of lactose have a higher glycolytic potential than cells grown at a low lactose concentration.

During all growth phases the *ccpA* mutant cells displayed increased lactose uptake rates and reduced lactate production rates as compared to the parental wild-type strain, while substantial amounts of glucose were found during all growth phases (data not shown). These results comply with previous data on lactose fermentation by this *ccpA* mutant, including glucose expulsion in the growth medium and decreased lactate production rates, indicating that CcpA plays an important role in lactose availability dependent regulation of lactose metabolism (van den Bogaard *et al*., 2000). The initial accumulation rates and steady-state levels of G6P and F6P in *ccpA* mutant cells were similar to the wild-type cells indicating that CcpA is not involved in growth phase-dependent modulation of the upper part of glycolysis in response to lactose availability (Fig. 1B).

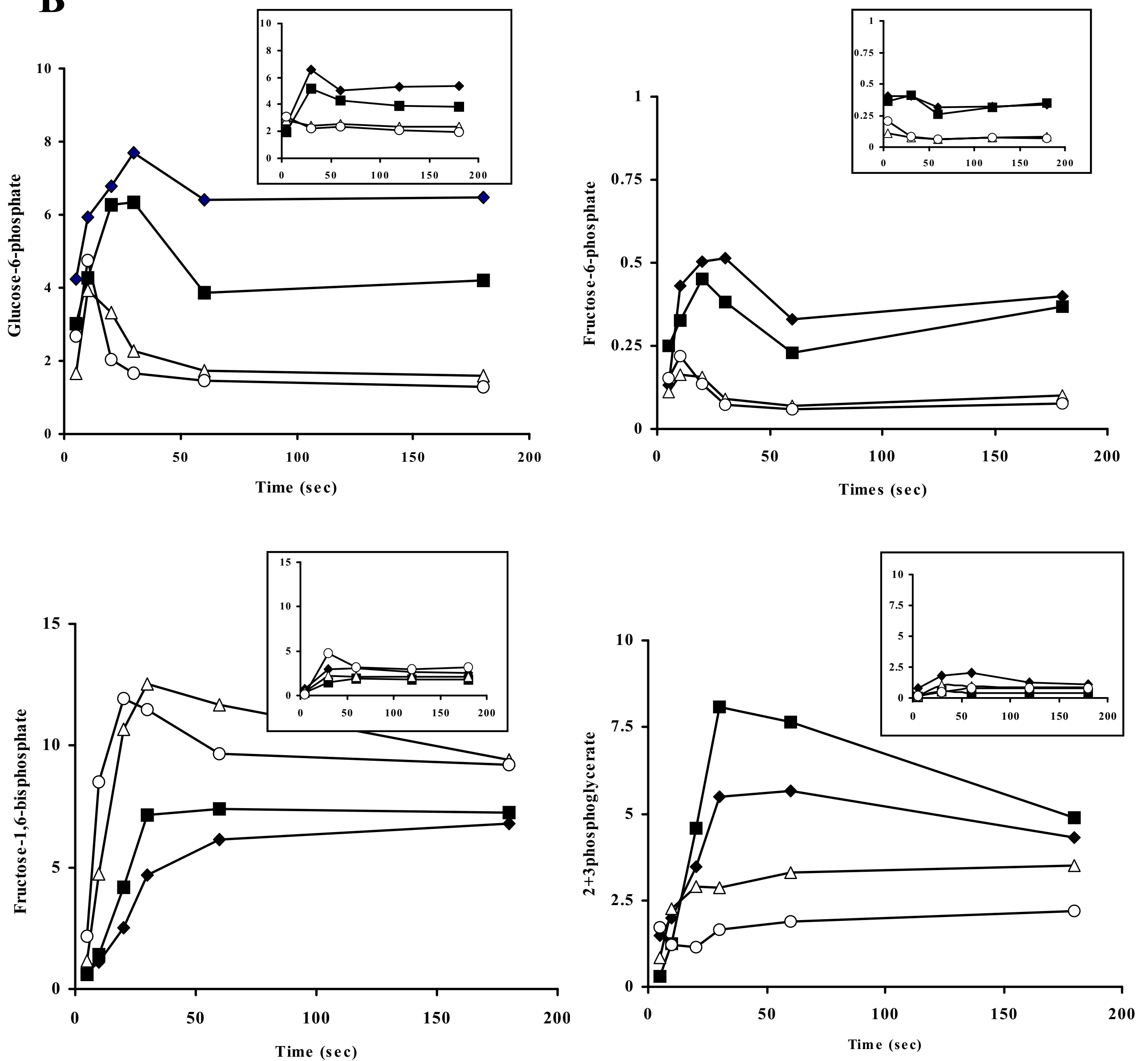
►

FIG. 1. Metabolic flux during lactose fermentation. Lactose-grown cells of *S. thermophilus* were harvested during specific stages of growth (early-log, mid-log, late-log and stationary phase), resuspended in MES buffer containing excess lactose and samples were taken for further analysis of metabolites. Errors were found to be well within 10%, except for the values of the early-log harvested cells of which the errors were within 25%, due to the low OD_{600nm} of the cultures at the moment of harvesting. A: Specific rates of substrate consumption and product formation during lactose fermentation of *S. thermophilus* CNRZ302 (wild-type) isolated from different stages of growth. B: Kinetics of glycolytic intermediates G6P, F6P, FBP and 3PG in *S. thermophilus* CNRZ302 (wild-type) isolated fom different stages of growth; ◆, early-log; ■, mid-log; Δ, late-log; O, stationary phase. The insets for show the kinetics of these intermediates in NZ6150 ($CcpA^-$).

A

Growth phase	Rate of lactose consumption ($mM \cdot OD_{600}^{-1} \cdot min^{-1}$)	Rate of lactate production ($mM \cdot OD_{600}^{-1} \cdot min^{-1}$)	Rate of glucose production ($\mu M \cdot OD_{600}^{-1} \cdot min^{-1}$)
Early-log	0.17	0.32	0.01
Mid-log	0.14	0.31	0.01
Late-log	0.20	0.26	0.42
Stationary	0.20	0.21	0.42

B



Similar findings have been reported for cells grown on other carbon sources than lactose, including glucose, sucrose and galactose (van den Bogaard *et al*., 2002b). However, the initial accumulation rate of FBP and its final steady state level was reduced in the *ccpA* mutant cells and the growth-phase dependency observed in the wild-type cells was lost. The same results were found for the levels and accumulation of 2+3PG, PEP and ATP (Fig 1B and data not shown). ADP levels seemed not significantly altered during growth or dependent on a functional CcpA (data not shown). Apparently, CcpA regulates several steps of glycolysis besides the conversion of pyruvate to lacate and this control of glycolytic capacity appears to be in response to lactose availability. The strongest regulatory effect of CcpA was observed in early-log and mid-log isolated cells when lactose was in excess.

CcpA mediated regulation of glycolysis genes.

The results on the sugar import rate and concentrations of glycolytic intermediate implied that several glycolytic genes were regulated by lactose availability and that CcpA plays an important role in this process. To evaluate the predicted differential expression of the *S. thermophilus* glycolysis genes, mRNA was isolated from wild-type and *ccpA* mutant cells grown in an excess of lactose and used to generate Northern blots. The glycolytic genes were identified in the genome of *S. thermophilus* LMG18311, the sequence of which has been completed (Hols *et al*., 2002), based on the homology to the known genes of *B. subtilis* and *L. lactis*. Primers were designed to generate internal fragments by PCR that were labeled and used as probes in hybridizations to the Northern blots (Fig. 2). Although in some cases additional bands with low intensity were observed, each of the glycolytic gene probes, except for those from the *pfk* and *pyk* genes, generated one major band with the size expected for a single transcript. This indicates that these glycolytic genes of *S. thermophilus* were transcribed into a monocistronic mRNA. This is compatible with the genome sequence data that indicate that the glycolytic genes are not genetically linked and, apart for the *pfk* and *pyk* genes, showed no apparent operon structures (unpublished observations). The juxtaposed *pfk* and *pyk* genes of *S. thermophilus* were found to generate three transcripts; a dicistronic transcript of 2.7 kb containing *pfk* and *pyk*, a monocistronic *pfk* mRNA of 1.3 kb, and a monocistronic *pyk* mRNA of 1.6 kb.

The Northern signals obtained using RNA isolated from wild-type and *ccpA* mutant cells were quantified and compared to identify additional glycolytic genes that were regulated by CcpA (Fig. 3) The levels of the mRNA's transcribed from the *pfk* promoter (P*pfk*) were two-fold reduced in the *ccpA* mutant strain compared to the wild-type cells. The messenger transcribed from the *pyk* promoter (P*pyk*) was only reduced slightly in the *ccpA* mutant cells. Besides the two-fold transcriptional activation of the *ldh* gene and the *pfk-pyk* operon by CcpA, no substantial catabolic regulation of other *S. thermophilus* glycolytic genes could be established in lactose-grown cells. Sequence analysis of the putative promoter regions of all other glycolytic genes of *S. thermophilus* strain LMG18311 led to the identification of possible –10 and –35 sequences in the promoter regions of *tpi*, *pgk* and *pgm*. In contrast, no consensus promoter sequences were found upstream of the *glcK*, *pgi*, *fba*, *gap* and *eno* genes. The *pfk* promoter was mapped previously (Crispie *et al*., 2002) and the *pyk* transcription initiation site was mapped by primer extension to a single position located 47 bp upstream of the *pyk* coding sequence (data not shown). It was preceded by a consensus –10 (ACTAAT) and –35 (TTGAAA) sequences. The promoter regions of the glycolytic genes were analysed for the presence of putative *cre* sequences to corroborate CcpA regulation. In a previous study, a consensus *cre* sequence was found upstream of the *S. thermophilus ldh* promoter and CcpA regulation at the transcriptional level was confirmed (van den Bogaard *et al*., 2000). Two *cre* sequences were identified upstream of the P*pfk* –35 sequence (at position -192 and –

73 relative to the transcriptional start site) and one putative *cre* was found upstream of the P*pyk* –35 sequence (at position -98 relative to the transcriptional start site) that were identical in sequence and position in the *pfk-pyk* operon of strain CNRZ1205. Moreover, only in the putative promoter region of *glcK*, a possible *cre* sequence was identified. However, this *cre* sequence did not appear to be functional under these conditions. These results clearly establish the functionality of these *cre* sequences in the *pfk* and *ldh* promoter.

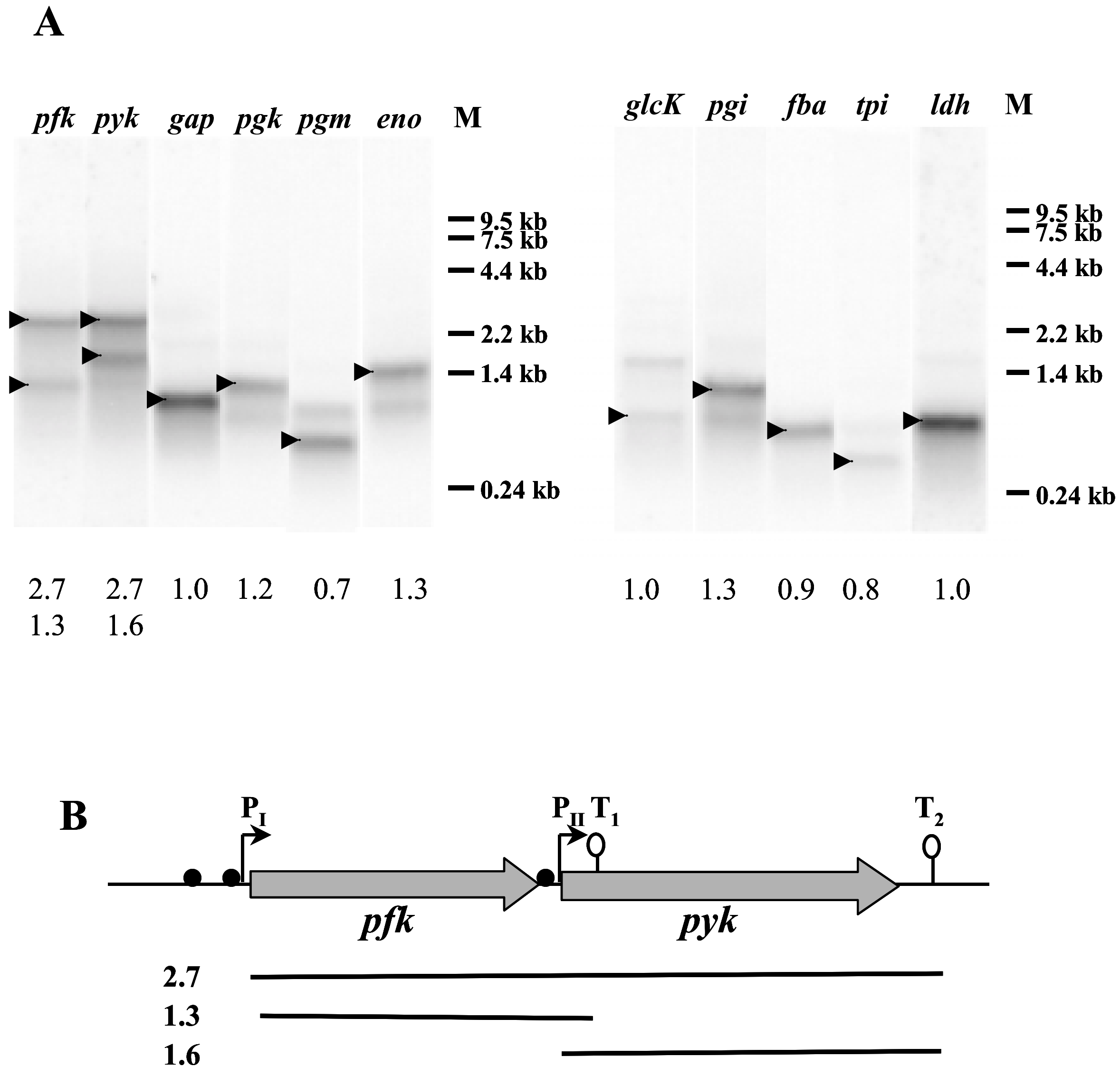


FIG. 2. Northern analysis of the *S. thermophilus* genes encoding glycolytic enzymes. A: Total RNA from *S. thermophilus* CNRZ302 grown on excess lactose (1%) was hybridized with probes specific for the *S. thermophilus* glycolytic genes. The ORF sizes are given below each lane, gene-specific signals are indicated by an arrow head, and transcript sizes are related to the marker (M). B: Depiction of the *S. thermophilus pfk/pyk* operon. The *pfk* and *pyk* promoters are indicated by arrows P_{pfk} and P_{pyk}, respectively. The black lines below the genes represent the transcript derived from these promoters. The *pfk* and *pyk* terminators are indicated by T1 and T2, respectively. The closed circles indicate the putative *cre* sequences.

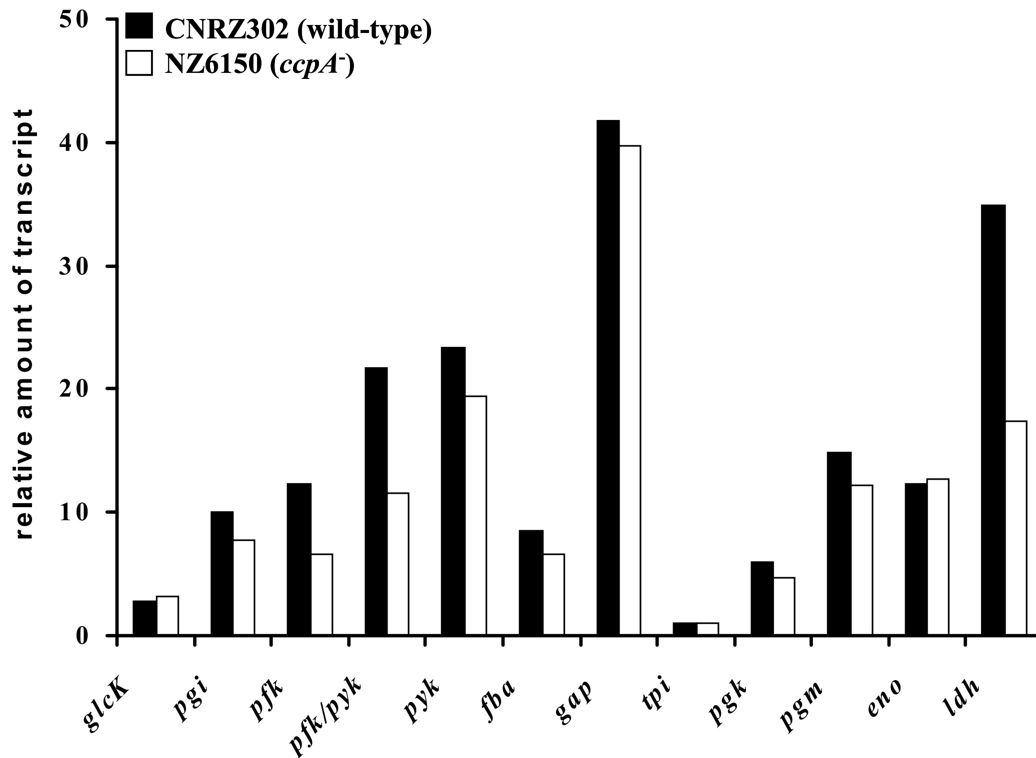


FIG. 3. Quantitative analysis of mRNAs of glycolytic enzymes. Total RNA was isolated from excess lactose (1%) grown *S. thermophilus* CNRZ302 (wild-type) and NZ6150 (CcpA$^-$). Relative amounts of the specific transcripts were quantified by phosphor image analysis of the Northern blot. As differentially labeled probes were used for the individual genes, only the transcript amounts could be compared between the two strains CNRZ302 (wild-type) and NZ6150 (CcpA$^-$) for each gene. A 16S rDNA probe was used to normalize the gene-specific signals to the total amount of RNA applied to the Northern blots. The *tpi* transcript amount measured in wild-type cells was arbitrarily set to 1.0.

DISCUSSION

In this study we investigated catabolite control of the *S. thermophilus* lactose metabolism and confirmed that CcpA is a global regulator of carbon metabolism mediating control at the level of transport and glycolytic conversion. A clear relation was found between CcpA activation of specific glycolytic genes and high glycolytic flux-rates in cells grown in early stages of growth during which lactose was in excess. In late growth phase cells that experience low lactose concentration, the rate of glycolysis is decreased and less lactate is produced compared to cells grown in excess lactose in the early growth phase. However, lactose transport capacity in these cells was increased, leading to an imbalance between lactose import and subsequent conversion in glycolysis when these cells were confronted with lactose excess. Indicative for this imbalance is the expulsion of glucose, resulting from an excessive carbohydrate import rate. Without a functional CcpA, this imbalance is also observed during growth in excess lactose indicating the involvement of catabolite control in this carbon availability-dependent modulation of glycolytic capacity.

The steady-state levels of G6P and F6P were found to be growth-phase dependent. However, these levels seem not to be dependent on a functional CcpA and the initial accumulation rate of these metabolites appeared to be equal in cells at all growth phases

independent of the lactose concentration. Moreover, Northern analysis of the *glcK* and *pgi* genes showed that CcpA did not regulate expression of these genes in cells at the growth phases in which they experience lactose excess. Previous work showed that the steady-state levels of these intermediates were carbon source dependent (van den Bogaard *et al*., 2002b). It appears that total glycolytic potential determines the flux through the upper part of glycolysis, rather than direct catabolite repression by CcpA. Moreover, the steady state levels of G6P and F6P in cells at the end of the growth phase when grown at low lactose concentration were at least 3-fold lower than were found in early growth phase cells grown in an excess of lactose, although lactose import exceeded glycolytic capacity. Glucose was found to be produced in cells grown at low lactose concentrations and we assume this to be extracellular since similar results were obtained with *ccpA* mutant cells that were found to expell the excess glucose into the medium (van den Bogaard *et al*., 2000). Still, the question remains to answered if this glucose is expelled by passive diffusion or by an active system of expulsion that transports excess carbohydrates out of the cell. High intracellular glucose concentrations (up to 100 mM) were observed in a mutant strain of *L. lactis* defective in glucose transport and glucokinase that was grown on lactose (Thompson and Chassy, 1985). It is very likely that the observed expulsion of glucose in *S. thermophilus* is independent of a functional CcpA.

Downstream of F6P in the glycolytic pathway, the first differences between wild-type and CcpA deficient cells were found for the initial FBP accumulation rates and steady-state levels. In cells growing at the end of their growth phase at low lactose concentration FBP accumulated much more rapidly than in cells grown in lactose excess. This glycolytic modulation in response to lactose availability was lost in *ccpA* mutant cells. It was found that in *S. thermophilus* not only the *ldh* gene (van den Bogaard *et al*., 2000), but also the *pfk-pyk* operon is subjected to CcpA-mediated activation during grown on an excess of lactose (Table 3). In *L. lactis* these genes comprise the *las* operon together with the *ldh* gene and are also activated by CcpA (Llanos *et al*., 1993; Luesink *et al*., 1998). The reactions catalyzed by phosphofructokinase, pyruvate kinase and lactate dehydrogenase require activation and are predominantly unidirectional steps in glycolysis and obvious points for catabolite control. Downstream of FBP in the glycolytic pathway, 2+3PG and PEP accumulation rates appeared to depend on the lactose availability, which was absent in *ccpA* mutant cells. In a previous study, the transcriptional repression of the *S. thermophilus lacSZ* operon and activation of the *ldh* gene by CcpA was already established (van den Bogaard *et al*., 2000). Besides the CcpA-mediated transcriptional activation of the *pfk-pyk* operon no transcriptional regulation of the other glycolytic genes was found. Taken together, these results imply that lactose import and fermentation is regulated by lactose availability and that CcpA plays an important role in this process, concommitantly with repression of *lacSZ*. In *L. lactis* the glycolytic genes *fba*, *tpi* and *glcK* also appeared to be sugar-regulated, although CcpA involvement is still inconclusive (Even *et al*., 2001). In *B. subtilis*, many of the glycolytic genes are organized in operons. The *pgk-tpi-pgm-eno* operon and the *yvbQ-gap* operon in this bacterium are induced by glucose in a CcpA-dependent manner (Tobisch *et al*., 1999). However, in the same studie expression was found to be constitutive for the *pfk-pyk* operon as wel as the other glycolytic genes. These data indicate that Gram-positive bacteria have adapted different variations of catabolic activation of the glycolytic potential. Finally, in cells growing at the late-log and stationary phase at low lactose concentration, ATP accumulated more rapidly that in cells grown at the early growth phases at lactose excess. However, as flux through glycolysis and lactate production was slower in cells grown at low lactose it seems likely that this is not due to an increase in ATP-generating steps but is the result of a decrease of total ATP consuming activity. While proton-coupled lactate efflux has been reported for *L. lactis* the molecular

mechanism of lactate-export from lactic acid bacteria has not yet been established (Otto *et al.*, 1982). However, the observed ATP-increase in cells isolated from the later stages of growth, when introduced to an excess of lactose, coincided with a decrease in lactate production. This observation could agree with the model that lactate is activally transported out of the cell at the expense of ATP. Alternatively, F1F0-ATPases are involved in maintenance of pH homeostasis in the cell and constitute a large part of the ATP consuming activities in lactic acid bacteria (Kullen and Klaenhammer, 1999). They are usually activated under low intracellular pH conditions (Suzuki *et al.*, 1988) but could also be uncoupled due to the metabolic shift. This could partially explain the lower ATP accumulation in the cells grown in lactose excess that displayed rapid lactate production relative to that of lactose-limited cells.

The regulation of *S. thermophilus* genes by the abundance of lactose is probably not only mediated by CcpA but also by HPr. Under similar conditions as described in this study, a direct relation between the phosphorylation state of HPr and the expression of *lacS* in *S. thermophilus* was reported by Gunnewijk (Gunnewijk and Poolman, 2000). In early- and mid-log growing cells (lactose excess) P-Ser-HPr was dominant in whereas in late-log and stationary phase growing cells (low lactose concentration) P-His-HPr was the dominant species. This apparently relieves the *lacS* promoter from catabolite repression and concommitantly allowed the LacS permease to become phosphorylated, increasing total lactose transport capacity (Gunnewijk *et al.*, 2001; van den Bogaard *et al.*, 2000). The glycolysis data presented in this paper corroborates the reported shift in dominant P~HPr species in response to lactose availability. *S. thermophilus* cells growing in lactose excess repress the *lacS* promoter and activate *pfk, pyk* and *ldh* by the concerted action of the dominant P-Ser-HPr species and CcpA. When the cells enter late-logarithmic growth phase and lactose becomes limiting the shift from P-Ser-HPr to P-His-HPr relieves catabolite control by CcpA. Hereby *lacS* transcription increases, as does the phosphorylation of the IIA domain of LacS while activation of glycolytic genes seizes. When these cells are transferred to the fermentation medium containing an excess of lactose, transport and glycolysis are uncoupled resulting in lower lactate production and efflux of the excess carbon transported. Remarkably, a similar uncoupling is observed in *ccpA* mutant cells.

The strong accumulation in FBP in cells at the later stages of growth at low lactose concentrations compared to cells at the early stages of growth at lactose excess is likely to be due to the decrease of glycolytic activity downstream of FBP. Influx of carbon into glycolysis was low in these latter cells and Northern analysis showed that *pfk* is activated by CcpA under high-energy conditions. However, the regulatory processes like modulation of the phosphorylation state of HPr and allosteric control of enzymes are likely to be active in wild-type cells. The FBP accumulation could again cause a shift in the phosphorylated HPr species back from P-His-HPr to P-Ser-HPr resulting in a loss of LacS phosphorylation and consequently a reduction of lactose/galactose exchange capacity of this transporter. Resting cells represent a snapshot of cellular state and alterations in transcription patterns are unlikely. A limited influx of lactose into glycolysis is observed in *ccpA* mutant cells throughout growth and FBP concentrations appear to be low, suggesting that P-His-HPr is the dominant P~HPr species throughout growth. Consequently, besides the lack of activation of the glycolytic genes and the relieve of catabolite repression of the *lacS* promoter in the absence of a functional CcpA, the LacS transporter is permanently in its phosphorylated state which strongly increases lactose transport. Following this reasoning, it is likely that the phosphorylation state of LacS in these experiments contributes at least equally as strong to the total lactose transport capacity as the total amount of LacS protein that is affected by its expression modulation by CcpA.

In *S. thermophilus* CcpA and HPr are the key factors in balancing import and glycolytic potential during lactose growth. The activity of these proteins is modulated by glycolytic intermediates that may be seen as the primary indicators of energy status of the cell. During growth in excess lactose, glycolytic activity increases by the activation of highly unidirectional steps and, consequently, lactose import rate is modulated to achieve maximal carbon flux.

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Chapter 7

Characterization of the *Streptococcus thermophilus ptsHI* operon: trans-complementation and functional analysis in *Lactococcus lactis*

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SUMMARY

We cloned and characterized the single chromosomal *Streptococcus thermophilus* *ptsHI* operon and expressed *S. thermophilus* wild-type and mutant *ptsH* alleles in *Lactococcus lactis*. Transcription analysis in *S. thermophilus* indicated that two different transcripts are generated from the single strong promoter upstream of *ptsH*, one encompassing *ptsHI* (2.0 kb) and one specific for *ptsH* (0.6 kb). The *ptsH* gene appeared to be essential in *S. thermophilus*, since it could not be inactivated or deleted. In the absence of a homologous *S. thermophilus ptsH* deletion strain, the functional role of specific residues of *S. thermophilus* HPr was assessed in a *ptsH* deficient mutant of *L. lactis* that could be complemented by the wild-type *S. thermophilus* HPr. To study the role of specific amino acid residues of HPr in sugar transport and its enzymatic and transcriptional control, site-specific mutations were introduced in *ptsH* that affected residues His-15, Arg-17, Ser-46 and Ile-47. Residues His-15 and Arg-17 but not Ile-47 were found to be essential for the transport of glucose and sucrose that are transported via a phosphotransferase system (PTS) in *L. lactis*. Production of HPr-S46A had little effect on growth while that of HPr-S46D resulted in severe retarded growth on PTS and non-PTS sugars, probably due to permanent catabolite repression and inducer exclusion. Utilization of maltose that is transported via an ABC transporter, was hardly affected by the other mutations in the *S. thermophilus ptsH* gene. Remarkably, metabolism of galactose that is transported in *L. lactis* by the permease GalA, was inhibited by the same mutations in HPr that affected PTS sugar utilization but to a different extent. This was not caused by a reduction of *galA* transcription but was a result of reduced galactose transport rates. These results suggest that in *L. lactis* there is an interaction between HPr and GalA that directly modulates the efficiency of GalA, which is phosphotransfer-independent.

INTRODUCTION

HPr is the histidine-containing phosphocarrier protein of the bacterial phosphotransferase system (PTS) that controls sugar uptake and carbon utilization in Gram-negative and low-G+C Gram-positive bacteria. PTS transport of carbohydrates requires P-His-HPr that is generated by enzyme I (EI) at the expense of phosphoenolpyruvate (Postma *et al*., 1993). The phosphoryl group is transferred via the sugar-specific PTS enzymes IIA and IIB to the imported sugar, which is transported by enzyme IIC. In addition to its function in the PTS, P-His-HPr is also involved in regulation of sugar metabolism by modulating activities of enzymes or transcription factors. Regulation by P-His-HPr generally mediates secondary catabolite repression by phosphoryl-transfer to non-PTS proteins in low-G+C Gram-positive bacteria (Deutscher *et al*., 1993; Martin-Verstraete *et al*., 1998). Phosphoryl-acceptor proteins comprise anti-terminators and transcriptional activators with a PTS regulation domain containing multiple phosphorylation sites that are recognized by P-His-HPr (Lindner *et al*., 1999; Stulke *et al*., 1998). Moreover, the activities of several enzymes are also modulated by P-His-HPr and in several enterococci phosphoryl-transfer controls the activity of glycerol kinase (Charrier *et al*., 1997), while in *Streptococcus thermophilus* it modulates transport activity of the lactose permease LacS (Gunnewijk and Poolman, 2000; Poolman *et al*., 1995). In low-G+C Gram-positive bacteria, HPr contains a second phosphorylation site, residue Ser-46 that may be phosphorylated by the bifunctional HPr kinase-phosphatase. The formation of P-Ser-HPr is stimulated by high intracellular fructose-1,6-biphosphate (FBP), whereas inorganic phosphate causes its dephosphorylation (Galinier

et al., 1998; Reizer *et al*., 1998; Stulke and Hillen, 2000). P-Ser-HPr acts as a corepressor to enhance the binding of the catabolite control protein A (CcpA) to catabolite response elements (*cre*) that are localized in promoters of operons under catabolite control (Deutscher *et al*., 1995; Gosseringer *et al*., 1997; Henkin, 1996; Jones *et al*., 1997). The position of the *cre* box with respect to the promoter region may vary, suggesting different modes of action in activation or inhibition of transcription by binding of the P-Ser-HPr/CcpA complex (Henkin, 1996). This may lead to differential effects on sugar transport and metabolism as was first reported for *Lactococcus lactis*, where CcpA was found to repress transcription of the *galA* gene coding for the galactose permease GalA and activate transcription of the *las* operon that includes the glycolytic *pfk*, *pyk* and *ldh* genes (Luesink *et al*., 1998). Similarly, in *S. thermophilus*, catabolite control by CcpA represses expression of the *lacSZ* genes for lactose transport and hydrolysis, while it activates that of *ldh* and the *pfk-pyk* genes encoding key enzymes of the glycolysis (van den Bogaard *et al*., 2002c; van den Bogaard *et al*., 2000). Furthermore, P-Ser-HPr is also suggested to be involved in inducer exclusion in *L. lactis* (Dossonnet *et al*., 2000; Gauthier *et al*., 1997; Viana *et al*., 2000). In *Lactobacillus brevis*, interaction of P-Ser-HPr with non-PTS permeases inhibits transporters by uncoupling them from proton symport (Djordjevic *et al*., 2001; Ye *et al*., 1994; Ye and Saier, 1995).

The genes for HPr and EI are usually located in the *ptsHI* operon that is very well conserved among bacteria and ensures production of significant levels of the phosphocarrier to fulfill all necessary functions (Deutscher *et al*., 1994; Lai and Ingram, 1995; Vadeboncoeur *et al*., 2000). Furthermore, the amino acid sequence and structural conformation of HPr are highly conserved, which is reflected by functional heterologous complementation of *ptsH* mutations in several bacteria (Djordjevic *et al*., 2001; Luesink *et al*., 1999).

Here we discribe the characterization the *S. thermophilus ptsHI* operon and study the role of specific amino acid residues of HPr in sugar transport and its control. Unexpectedly, we were not able to functionally delete the *S. thermophilus ptsH* gene, suggesting a different role of HPr in *S. thermophilus* compared to that in other bacteria where such deletions have been realized. Hence, we characterized the effect of specific *S. thermophilus ptsH* mutant alleles in a *L. lactis ptsH* deletion strain and describe here the role of specific conserved amino acids in transport and catabolite control of PTS and non-PTS sugars.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and culture conditions. *Escherichia coli* strain DH5α was grown in TY broth (Sambrook *et al*., 1989) with aeration at 37°C and used in in transformation with the plasmids pBlueScript (Stratagene) and pGEM-T (Promega). Other strains and plasmids used in this study are listed in Table 1. *S. thermophilus* was routinely grown at 42°C in M17 broth (Difco, Surrey, U.K.) containing 1% (w/v) of lactose. *L. lactis* was routinely grown at 30°C in M17 broth (Difco, Surrey, U.K.) containing 1% (w/v) of the chosen carbon source unless stated otherwise. The expression of the Sth *ptsH* alleles was induced by the addition of nisin A to the growth medium to a final concentration of 1 ng/ml. The antibiotics used for selection in growth media were chloramphenicol (4 μg/ml for *S. thermophilus*, 10 μg/ml for *L. lactis*), erythromycin (2.5 μg/ml for *S. thermophilus*, and 5 μg/ml for *L. lactis*) and ampicillin (50 μg/ml for *E. coli*).

DNA manipulations and transformations. Transfer to and isolation of plasmid DNA from *E. coli* was performed using established protocols (Sambrook *et al*., 1989). Plasmid and chromosomal DNA from *S. thermophilus* was isolated as described previously for *L. lactis* (Vos *et al*., 1989). Electroporation of *S. thermophilus* was performed by the procedure described by (Mollet *et al*., 1993) with the modification that the harvested cells were incubated in the electroporation buffer at 4°C for at least 4h prior to electroporation. *L. lactis* was transformed by electroporation as described by Holo and Nes (Holo and Nes, 1995). Restriction enzymes, T4 DNA ligase and other DNA-modifying enzymes were used as recommended by the suppliers (Gibco/BRL or Boehringer Mannheim). DNA fragments were recovered from agarose gels using the Glass Matrix DNA Isolation System (Gibco/BRL).

Strains	Relevant features	Reference
S. thermophilus CNRZ302	Wild-type Gal$^-$ strain	(Benataya *et al*., 1991)
S. thermophilus NZ302G	Gal$^+$ derivative of CNRZ302	(Vaughan *et al*., 2001)
S. thermophilus NZ6150	CcpA$^-$ derivative of CNRZ302	(van den Bogaard *et al*., 2000)
S. thermophilus NZ6151	CcpA$^-$ derivative of NZ302G	(van den Bogaard *et al*., 2000)
L. lactis NZ9800	NICE expression host	(Kuipers *et al*., 1993)
L. lactis NZ9880	NZ9800 derivative; Δ*ptsH*	(Luesink *et al*., 1999)
Plasmids		
pG$^+$host8	Temperature-sensitive shuttle vector	(Maguin *et al*., 1996)
pNZ6110	pGEM-T containing 0.8-kb *S. thermophilus ptsHI* PCR fragment	This study
pNZ6111	pGEM-T containing 3.5-kb *S. thermophilus Hin*dIII inverse-PCR fragment	This study
pNZ6112	pGEM-T containing 1.4-kb *S. thermophilus ptsI* PCR fragment	This study
pNZ6113	pNZ8010 containing *L. lactis ptsI* gene	This study
pNZ6114	pNZ6113 containing *S. thermophilus* wild-type *ptsH* gene fused to *nisA* promoter	This study
pNZ6115	pNZ6113 containing *S. thermophilus* H15A *ptsH* gene transcriptionally fused to *nisA* promoter	This study
pNZ6116	pNZ6113 containing *S. thermophilus* S46A *ptsH* gene transcriptionally fused to *nisA* promoter	This study
pNZ6117	pNZ6113 containing *S. thermophilus* S46D *ptsH* gene transcriptionally fused to *nisA* promoter	This study
pNZ6118	pNZ6113 containing *S. thermophilus* R17A *ptsH* gene transcriptionally fused to *nisA* promoter	This study
pNZ6119	pNZ6113 containing *S. thermophilus* R17E *ptsH* gene transcriptionally fused to *nisA* promoter	This study
pNZ6120	pNZ6113 containing *S. thermophilus* R17K *ptsH* gene transcriptionally fused to *nisA* promoter	This study
pNZ6121	pNZ6113 containing *S. thermophilus* I47A *ptsH* gene transcriptionally fused to *nisA* promoter	This study
pNZ6122	pNZ6113 containing *S. thermophilus* I47D *ptsH* gene transcriptionally fused to *nisA* promoter	This study
pNZ8010	Lactococcal cloning and expression vector	(de Ruyter *et al*. 1996)

Table 1. Bacterial strains and plasmids used in this study.

Cloning of the *S. thermophilus ptsHI* operon. Degenerated oligonucleotides PTS1 and PTS2 (Table 2) were used in a PCR reaction with *S. thermophilus* CNRZ302 chromosomal DNA as a template to amplify part of the *ptsHI* operon (Luesink *et al*., 1999). The 0.8-kb PCR fragment obtained was cloned in the pGEM-T vector (Promega) yielding pNZ6110 and the identity of the

cloned fragment was confirmed by sequence analysis. Based on this fragment two outward primers PTS3 and PTS4 were designed in order to clone the complete *ptsHI* operon by inverse PCR (Xu *et al*., 1997). A *S. thermophilus* CNRZ302 chromosomal DNA *Hin*dIII digest was self-ligated and used as template for a PCR with primers PTS3 and PTS4 (Advantage Genomic Polymerase kit, Clontech Laboratories). An inversed PCR product of 3.5 kb was cloned in the pGEM-T vector (Promega) yielding pNZ6111 (Table 1). Sequence analysis confirmed that the insert contained the *ptsH* gene and upstream region, and a 3' truncated *ptsI* gene. As the *S. thermophilus ptsI* sequence displayed high identity with that of *S. salivarius* (95 %), the reverse primer PTS7 was based on the latter sequence and used in a PCR reaction with PTS4 and *S. thermophilus* CNRZ302 chromosomal DNA as a template (Advantage Genomic Polymerase kit, Clontech Laboratories). The PCR product of 1.4 kb was cloned in the pGEM-T vector (Promega) yielding pNZ6112 (Table 1). Sequence analysis confirmed that the PCR product contained the 3' region of the *S. thermophilus ptsI* gene.

Oligo nucleotides	Sequence (5'-3')	Reference
PTS1	AACWGGWATTCATGCWMGWCCWGC	(Luesink *et al*., 1999)
PTS2	GGTACCWCCAATATTWGTWACAAAWGC	(Luesink *et al*., 1999)
PTS3	ATTCAAGAGTGATATCTGAAGC	This study
PTS4	GTTGCTCATGACTTGACGCC	This study
PTS7	CTATACAAAGGCTATCAATGA	This study
PTS-ECO	GGACGAATTCGACTGGTGTTTGATTATGGTTTG	This study
PTS-HIN	CCTGAAGCTTAGCAACTGCAACACCATCAGATG	This study
ST-H15A	GAAACTGGTATTGCCGCACGACCAGCTAC	This study
ST-S46A	GTTAACCTTAAAGCTATCATGGGTGTTATGAG	This study
ST-S46D	GTTAACCTTAAAGATATCATGGGTGTTATGAG	This study
ST-R17A	GTATTCACGCAGCACCAGCTACTTTGC	This study
ST-R17E	GTATTCACGCAGAACCAGCTACTTTGC	This study
ST-R17K	GTATTCACGCAAAACCAGCTACTTTGC	This study
ST-I47A	CCTTAAATCTGCCATGGGTGTTATGAG	This study
ST-I47D	CCTTAAATCTGACATGGGTGTTATGAG	This study
PTS-XHO	CGACCTCGAGCAATGACTAAAGAAGGACTCG	This study
PTS-SAL	CAGCAGGTCGACCGAAACTAATTGAAAATATTAT	This study
GALAF	GGATATTGCTTTCTGGTCAATGATTCC	This study
GALAR	ACCGTATTCTACAGAGTCGG	This study

Table 2. Oligonucleotides used in this study.

The wild-type *S. thermophilus ptsH* gene was amplified using flanking primers PTS-ECO and PTS-HIN (Advantage Genomic Polymerase kit, Clontech Laboratories). Site-directed mutagenesis of the *S. thermophilus ptsH* gene was performed as described previously (Kuipers *et al*., 1992) with flanking primers PTS-ECO and PTS-HIN, and with the following mutagenic primers: ST-H15A, ST-S46A, ST-S46D, ST-R17A, ST-R17E, ST-R17K, ST-I47A and ST-I47D (Table 2). All *ptsH* alleles were digested with *Sst*I and *Hin*dIII, cloned in *Eco*RV-*Hin*dIII digested pBlueScriptSK and anticipated mutations were verified by sequence analysis. The *L. lactis ptsI* gene was amplified using primers PTS-XHO and PTS-SAL and *L. lactis* NZ9800 chromosomal DNA as a template. The PCR product was digested with *Xho*I and *Sal*I and cloned in *Xho*I-digested pNZ8010 yielding plasmid pNZ6113. For the construction of a transcriptional fusion between the *nisA* promoter and the *L. lactis ptsI* gene the *S. thermophilus ptsH* alleles were isolated as *Bam*HI-*Xho*I fragments from the corresponding pBluescriptSK constructs and cloned in *Bam*HI-*Xho*I digested pNZ6113 yielding plasmids pNZ6114 to pNZ6122 (Table 1).

RNA isolation and Northern blot analysis. *S. thermophilus* strains were grown in M17 broth (30 ml) containing either 1 % (w/v) glucose or lactose to an OD_{600nm} of 1.0. *L. lactis* strains were grown in M17 broth (50 ml) containing 1 % (w/v) of the appropriate sugar to an OD_{600nm} of 1.0. Total RNA was isolated from 20 ml of these cultures using the Macaloid method as described previously (Kuipers *et al*., 1993) with the following adaptation for *S. thermophilus*: prior to bead-beating the resuspended cells were incubated with lysozyme for 5 minutes on ice to increase RNA yield. Per sample 4.5 µg of RNA was size separated on a 1.0 % formaldehyde gel (Sambrook *et al*., 1989) and transferred to Gene Screen Plus membranes (Dupont, Boston, Mass.) according to the protocols provided by the manufacturers. RNA size markers were obtained from Bethesda Research Laboratories. Hybridisations were performed at 65°C in a 0.5 M sodium-phosphate buffer, pH 7.2 containing 1.0 % BSA (fractionV), 1.0 mM EDTA and 7.0 % SDS, and 1 µg of labeled probe. Subsequently, blots were washed at 65°C in 0.1 x SSC. For probing the *ptsHI*-specific transcripts, a fragment including the 3' end of the *ptsH* and the 5' end of the *ptsI* fragment was generated by PCR using PTS1 and PTS2 primers and *S. thermophilus* CNRZ302 chromosomal DNA as a template. For probing the *galA*-specific transcripts an internal fragment was generated by PCR using GALAF and GALAR primers and *L. lactis* NZ9800 chromosomal DNA as template. These fragments were Glass Matrix purified and labeled by nick translation with [α-^{32}P]dATP (Amersham International plc, UK) and were subsequently used as hybridisation probes (Sambrook *et al*., 1989). Slot-blots were exposed to a Storage Phosphor Screen (Molecular Dynamics) and scanned using a STORM 840 Phosphor Imager (Molecular Dynamics). Signal quantification was performed using the ImageQuant 1.2 program (Molecular Dynamics). Hybridization to a α-^{32}P-labelled 16S rDNA probe (generated by PCR using primers NR7 and NR19 with *L. lactis* NZ9800 chromosomal DNA as template) was used to correct the gene-specific signals for the total amount of RNA loaded per sample.

Galactose uptake assay. From the 50 ml *L. lactis* cultures used for Northern blot analysis the residual 30 ml was centrifuged, washed and resuspended in 100 mM phosphate buffer pH 6.8 to a final OD_{600} of 5. Cells were preincubated for 5 min at 30ºC and galactose uptake assays were started by addition of 50 mM galactose containing 1.35 µM Ci D-[U-^{14}C]galactose (Amersham International plc, UK). Consecutive samples were taken at regular time-intervals from the cell suspension and immediately filtered (0.45µm; NC 45; Schleicher & Schuell, Dassel Germany) to separate cells from the buffer to prevent further uptake. The filters were washed two times with redestilled water and air-dried. The radioactivity present on the membranes was analyzed and used as a measure for the amount of galactose taken up (Peckard Tri-Carb 2300TR liquid scintillation analyzer).

Nucleotide sequence accession numbers. The *ptsHI* sequence data have been submitted to the GenBank database under accession number AY064171.

RESULTS

Cloning, characterization and transcriptional analysis of the of the *S. thermophilus ptsHI* operon.

To study the functionality of HPr in *S. thermophilus* strain CNRZ302 its *ptsH* and *ptsI* genes were characterized at the nucleotide and transcriptional level. The degenerated primers PTS1 and PTS2, based on conserved regions in HPr (residue 15 to residue 19) and EI (residue 178 to residue 185), respectively, were used to amplify an 0.8-kb internal fragment of the *ptsHI*

operon. Sequence analysis of this fragment showed it to contain two truncated ORFs, the products of which shared a high degree of homology with the C-terminal end of HPr and the N-terminal end of EI. With a subsequent inverse and heterologous PCR approach the complete *S. thermophilus* CNRZ302 *ptsHI* operon was cloned and sequenced (Fig. 1). The first open reading frame in the operon could encode a protein of 87 amino acids with highest identity (96%) to *S. salivarius* HPr (Gagnon *et al*., 1995). The second open reading frame could encode a 577 amino acid protein with 97 % identity to *S. salivarius* EI (Gagnon *et al*., 1995). The *S. thermophilus* CNRZ302 *ptsHI* operon is highly similar to that of *S. salivarius*, sharing an identity of 93 % at the nucleotide level, and contains a sequence that is identical to that of the P_I promoter recently reported for a *ptsHI* operon from another *S. thermophilus* strain (Vadeboncoeur *et al*., 2000). Furthermore, the *S. thermophilus ptsH* and *ptsI* genes were immediately followed by terminator-like inverted repeat sequences with ΔG values of –9.7 kcal mol^{-1} and –11.4 kcal mol^{-1}, respectively (Fig. 1). Northern blot analysis of the *ptsHI* operon in *S. thermophilus* CNRZ302 using a 0.8-kb insert from pNZ6110 as a probe revealed two *ptsHI*-specific transcripts (Fig. 1A). One transcript of 0.6 kb corresponded to *ptsH*, while the other transcript of 2.0 kb corresponded to *ptsHI*. The length of these transcripts agrees with a transcription start at the P_I promoter and termination at either of the observed terminators. No *ptsI*-specific transcript was found, similar to the situation in *S. salivarius* and *L. lactis* (Gagnon *et al*., 1995; Luesink *et al*., 1999).

Transcription analysis of RNA isolated from *S. thermophilus* CNRZ302 grown on glucose, lactose and galactose showed that the amounts of *ptsHI*-specific transcript did not change with the use of different sugars in the growth medium (Fig. 1B). In contrast, the amounts of *ptsH*-specific transcript were 2-fold higher in glucose-grown cells compared to lactose- or galactose-grown cells. Similar analysis in an isogenic *ccpA* mutants of strain CNRZ302 and a galactose-utilizing derivative revealed a 2-fold reduction of the *ptsH*-specific transcript relative to wild-type cells (Fig. 1B). This suggests CcpA-mediated activation of *ptsH* although no consensus *cre* sequence was found in the P_I promoter.

Inactivation of the *S. thermophilus ptsH* gene.

To analyze the functional role of the *S. thermophilus* HPr in carbon metabolism several attempts were made to delete the *ptsH* gene by replacement with an erythromycin-resistance gene. An integration vector was constructed based on $pG^{+}host8$ with a terminatorless erythromycin-resistance gene cassette flanked by upstream and downstream sequences of *ptsH* (of aproximately 900 bp each) (Maguin *et al*., 1996). Single-cross over integration in either of the flanking regions the *ptsH* gene could be readily obtained with approximately equal frequency. However, when each of these single cross-over integrants was further grown, it was found that all erythromycin-sensitive cells that were generated as a result of a double cross-over event appeared to be wild-type cells (data not shown). Moreover, no *ptsH::ery* gene replacement mutants could be obtained following direct selection of a double cross-over event despite the fact that these experiments were performed at different temperatures and conditions of variable concentration and nature of carbon source, such as glucose, lactose and galactose (data not shown). For each method, several hundreds of colonies were screened but under none of the tested conditions the *ptsH* gene could be activated. These results suggest that the *ptsH* gene is either essential or that its deletion results in severe growth rate reduction in *S. thermophilus*.

Overproduction of *S. thermophilus* mutant HPr in a *L. lactis ptsH* deletion strain.

To analyze the regulatory role of HPr in sugar metabolism, a series of mutated *S. thermophilus ptsH* genes was constructed. These mutated *ptsH* genes were to be characterized in a *ptsH*-deficient derivative of *S. thermophilus* CNRZ302. However, the failure to construct such

a strain made us decide to test the ability of these mutants to complement the *ptsH*-deficient strain *L. lactis* NZ9880 (Luesink *et al*., 1999). Transcriptional fusions of the wild-type and mutated *S. thermophilus ptsH* genes under control of the *nisA* promoter resulted in plasmids pNZ6114 to pNZ6122 (see Table 1). In *L. lactis* NZ9880 the *ptsI* gene is still transcribed by read-through of the erythromycin-resistance gene that was used for the replacement of the *ptsH* gene.

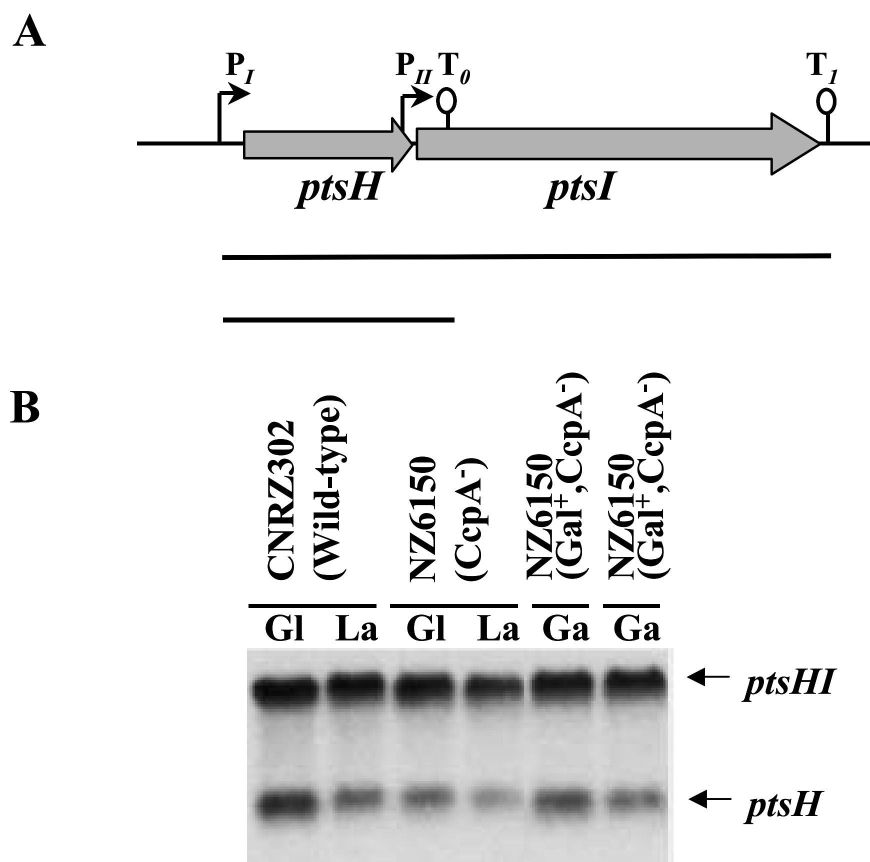

Fig 1. Transcriptional organization of the *S. thermophilus ptsHI* operon. A: Open arrows indicate genes, P_I and P_{II} promoters denoted by black arrows, and putative terminators T_0 and T_1 are indicated. The transcripts obtained by Northern analysis are denoted underneath as lines.
B: Northern analysis of glucose- and lactose-grown CNRZ302 (wild-type) and NZ6150 (CcpA$^-$) and galactose-grown NZ302G (Gal$^+$) and NZ6151 (Gal$^+$, CcpA$^-$). The blot was hybridized with the 0.8-kb *ptsHI* fragment as a probe. Transcript species are indicated by arrows.

However, it was reported that the *ptsI* expression level in this strain was probably too low to allow full complementation by HPr *in trans* (Luesink *et al*., 1999). Therefore the *L. lactis ptsI* was included in the overexpressing plasmids as a transcriptional fusion to the *S. thermophilus ptsH* genes (Table 1). Overexpression of wild-type *S. thermophilus ptsH* in combination with *L. lactis ptsI* led to the complementation of growth of the *L. lactis ptsH* deficient strain NZ9880 with maximal specific growth-rates that reached 65 % of that of the wild-type on glucose or sucrose but up to 80 % on galactose and maltose (Table 3). This indicates that the *S. thermophilus ptsH* gene is functionally expressed in *L. lactis*.

L. lactis strain and plasmid complement	Maximal growth rate (h^{-1})			
	Glucose	Sucrose	Galactose	Maltose
NZ9800	1.45	1.24	0.67	0.70
NZ9880 (Δ*ptsH*)	0.11	n.g.	0.27	0.30
NZ9880+pNZ6114 (Sth HPr)	0.87	0.86	0.62	0.65
NZ9880+pNZ6115 (Sth HPr-H15A)	n.g.	n.g.	0.46	0.45
NZ9880+pNZ6116 (Sth HPr-S46A)	0.77	0.77	0.72	0.72
NZ9880+pNZ6117 (Sth HPr-S46D)	0.11	0.08	0.07	0.10
NZ9880+pNZ6118 (Sth HPr-R17A)	n.g.	n.g.	0.28	0.39
NZ9880+pNZ6119 (Sth HPr-R17E)	0.14	0.09	0.09	0.38
NZ9880+pNZ6120 (Sth HPr-R17K)	0.43	n.g.	0.59	0.50
NZ9880+pNZ6121 (Sth HPr-I47A)	0.59	0.56	0.25	0.39
NZ9880+pNZ6122 (Sth HPr-I47D)	n.g.	n.g.	n.g.	0.45

Table 3. Maximal growth rate of *L. lactis* N9800 and derivatives in medium containing 1% (w/v) of the indicated sugar. n.g. indicates no growth.

The mutated *ptsH* alleles were created in such a way that HPr was affected in its possible phosphorylation at residues histidine 15 and serine 46. Histidine at position 15 was replaced by an alanine residue that can no longer be phosphorylated. Serine 46 was replaced by an aspartic acid residue that mimics a phosphate group on the seryl residue, or by an alanine residue that can not be phosphorylated (Deutscher *et al.*, 1994). In *L. lactis* NZ9880 producing HPr-H15A, growth was abolished on the PTS sugars glucose and sucrose. Growth of this mutant strain on galactose was only slightly hampered and no effect of growth on maltose was observed. The replacement of Ser-46 by alanine had no effect on growth of any of the sugars tested, but by its conversion to aspartic acid growth on all sugars was significantly retarded. This contrasts to previous findings for a similar *L. lactis* HPr mutant but this was overproduced in wild-type *L. lactis* and not in the *ptsH* deficient strain (Luesink *et al.*, 1999).

Residue arginine at position 17 was identified as crucial in the phosphoryl transfer between from P-His-HPr to the $EIIA^{glc}$ of *B. subtilis* probably by stabilizing the intermediary HPr-P-$EIIA^{glc}$ complex (Herzberg *et al.*, 1992). By site-directed mutagenesis Arg-17 was replaced by alanine (non reactive residue), glutamic acid (introduction of negative charge) or lysine (re-introduction of positive charge). Overproduction of HPr-R17A and HPr-R17E completely abolished growth on glucose and sucrose. In contrast, these mutations had no effect on maltose growth. Remarkably, growth on galactose was reduced significantly or almost completely in *L. lactis* NZ9880 overproducing in HPr-R17A or HPr-R17E, respectively. The *L. lactis* NZ9880 strain overproducing HPr-R17K was able to grow normally on galactose and almost normally on glucose, but could not grow at all on sucrose.

The residue isoleucine 47 was identified as an important residue in catabolite control and in interaction of HPr and $EIIA^{glc}$ of *B. subtilis* (Chen *et al.*, 1993; Gauthier *et al.*, 1997). By site directed mutagenesis Ile-47 was replaced by alanine (non-reactive residue) or an aspartic acid (introduction of negative charge). The replacement of Ile-47 by alanine had no effect on growth on glucose, sucrose or maltose, but, maximal growth on galactose was about 2-fold reduced. Replacing Ile-47 by aspartic acid abolished growth on glucose, sucrose and galactose but had no effect on maltose growth. The results derived from production of the HPr alanine-scanning mutants clearly indicate that residues His-15 and Arg-17 but not Ile-47 are crucial in PTS

transport. In the additional HPr mutant strains, however, the introduction of a disruptive residue at position 47 also disrupted PTS transport. Remarkably, the introduction of disruptive residues at positions 17 and 47 resulted in reduced growth on galactose implying a role for HPr in galactose uptake by the GalA permease but not in uptake by the maltose-specific ABC transporter.

The role of HPr mutants in transcription of the *gal* operon and galactose uptake.

Expressing of the mutated *ptsH* genes gave unexpected results on galactose growth. Two possible points of control by HPr in galactose metabolism are catabolite repression of the *galA* promoter (Luesink *et al*., 1998) and allosteric control of the galactose permease GalA. The *L. lactis gal* operon consists of five genes with the order *galAMKTE* and the involvement of CcpA in the regulation of the expression of these genes has been reported (Luesink *et al*., 1998). As not all the mutant strains grew on galactose, maltose was used as a carbon source as no apparent catabolite control was found for this sugar. To induce the *gal* operon, galactose was added during growth, and exponentailally grown cultures were used in a galactose transport assays and Northern analysis using a *galA* specific-probe.

The relative amounts of *galA* transcript found in galactose-induced *L. lactis* NZ9880 cells expressing the various *S. thermophilus ptsH* alleles was 4 to 5-fold higher compared to wild-type *ptsH* expressing NZ9880 cells grown only on glucose or NZ9800 (wild-type *L. lactis*) cells grown on maltose (Fig. 2). This confirms the induction of the *gal* operon by the addition of galactose to the growth medium. In cells producing the R17K HPr mutant, *galA* expression levels were 2-fold lower relative to the amounts found in induced cells of NZ9880, overexpressing the wild-type *S. thermophilus ptsH*. To investigate the contribution of the mutations in *ptsH* on CcpA-mediated catabolite repression of the GalA permease, transcriptional analysis of the *galA* gene and important glycolytic genes was assessed in Northern analysis using probes specific for *galA, ldh* and *pgi* (Fig. 2). No significant differences in transcript amounts were found between strains expressing wild-type *ptsH* (either *L. lactis* or *S. thermophilus*). These results indicate that the mutant HPr's did not have a significant impact on the expression of several important glycolytic genes, from which at least the *ldh* gene has been found to be regulated by CcpA (Luesink *et al*., 1998). Moreover, these results indicate that differences found in galactose uptake rates in the HPr mutant strains expressing different *ptsH* genes (see below) can not be attributed to differences in expression of the *galA* gene.

Galactose-induced cells of *L. lactis* NZ9880 producing the wild-type *S. thermophilus* HPr showed a 10-fold higher initial galactose transport rate compared to cells grown on glucose or NZ9800 (wild-type *L. lactis*) cells grown on maltose (Table 4). The galactose-induced cells expressing the *ptsH* alleles encoding HPr-R17A and HPr-R17K displayed initial galactose transport rates that were 2.5-fold lower relative to the wild-type *ptsH* expressing cells. The mutant HPr-R17E overproducing strain displayed low galactose uptake rates, which were comparable to wild-type *ptsH* expressing cells in which the *gal*-operon was not induced. Cells producing mutant HPr-I47A displayed a 1.5-fold increased galactose uptake rate relative to wild-type *ptsH* expressing cells, while cells expressing mutant HPr-I47D showed an uptake rate that was comparable to wild-type *ptsH* expressing cells in which the *gal*-operon was not induced. These results indicate that HPr is involved in galactose uptake in *L. lactis* and that amino acid residues R17 and I47 play an important part herein. This involvement of HPr in regulating galactose metabolism occurs probably at the level of transport and not by CcpA-mediated catabolite repression.

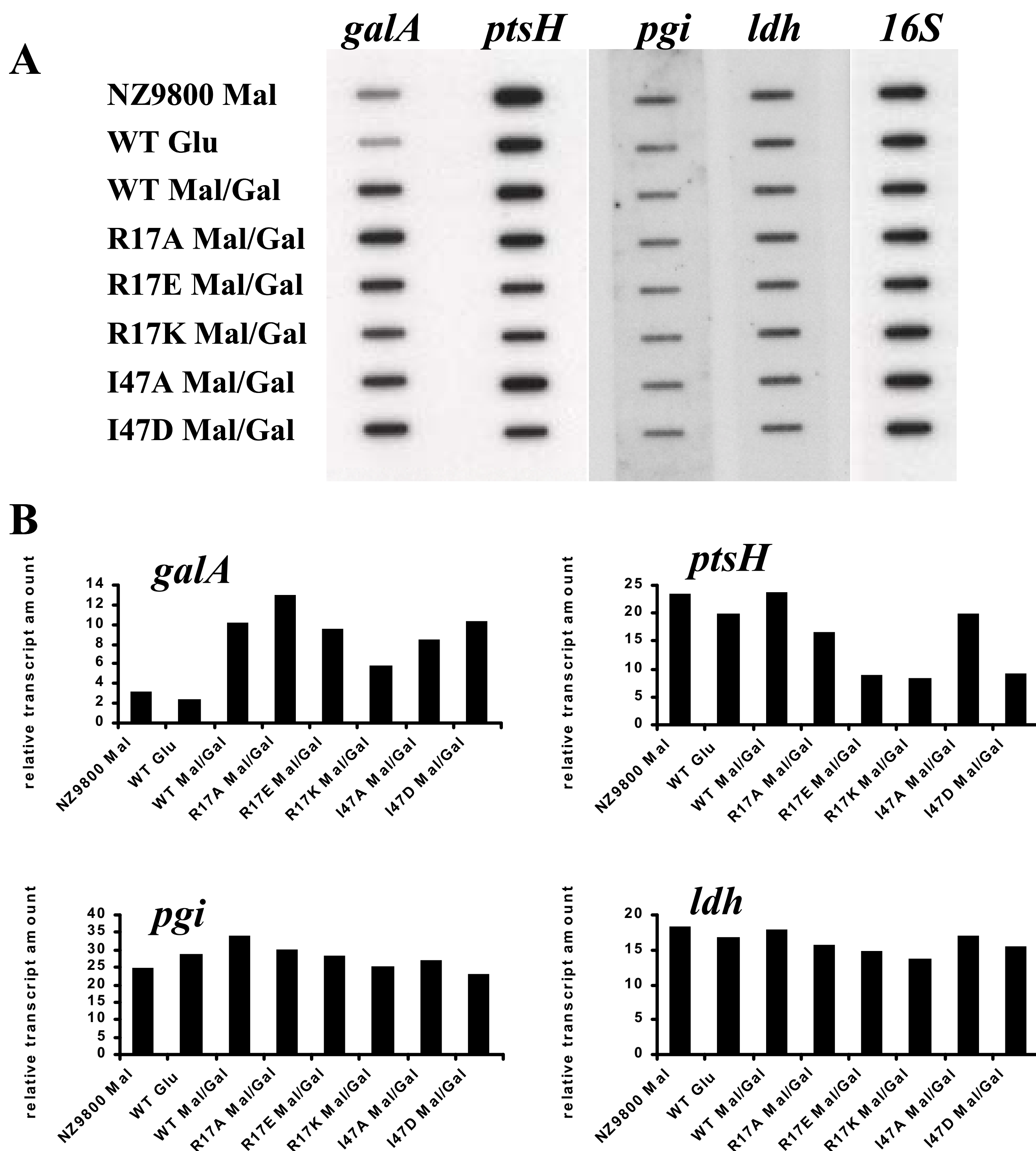

Fig. 2. Transcriptional analysis of genes involved in galactose utilization. *L. lactis* strains were grown in M17 broth (50 ml) containing the appropriate sugar to an OD_{600nm} of 1.0, and total RNA was isolated from 20 ml of these cultures. Slot blot analysis of 20 µg of total RNA isolated from of *L. lactis* N9800 and derivatives grown in medium containing either glucose or maltose with or without galactose. A: Slot blots probed with *galA, ptsH, pgi, ldh* or *16S* rDNA specific probes. B: Relative amounts of the indicated transcripts, obtained by phosphor image analysis of the slot blots. These values were corrected for the total amount of RNA loaded using the 16S rDNA-specific hybridization.

L. lactis strain and plasmid complement	Sugar	Initial galactose uptake rate ($mM \cdot sec^{-1} \cdot OD_{600}^{-1}$)
NZ9800	Galactose/Maltose	80.6
	Glucose	4.2
	Maltose	5.8
NZ9880+pNZ6114 (Sth HPr)	Galactose/Maltose	59.3
	Glucose	7.2
NZ9880+pNZ6118 (Sth HPr-R17A)	Galactose/Maltose	21.7
NZ9880+pNZ6119 (Sth HPr-R17E)	Galactose/Maltose	7.4
NZ9880+pNZ6120 (Sth HPr-R17K)	Galactose/Maltose	23.1
NZ9880+pNZ6121 (Sth HPr-I47A)	Galactose/Maltose	92.3
NZ9880+pNZ6122 (Sth HPr-I47D)	Galactose/Maltose	11.9

Table 4. Galactose uptake assay. Initial galactose uptake rates of *L. lactis* N9800 and derivatives grown in medium containing either glucose or maltose with or without galactose. From the *L. lactis* cultures used for transcriptional analysis (Fig. 2), the residual 30 ml was centrifuged, washed and resuspended in 100 mM phosphate buffer pH 6.8 at a final OD_{600} of 5. Cells were preincubated for 5 min at 30ºC and galactose uptake assays were started by addition of 50 mM galactose containing 1.35 μM Ci D-[U-^{14}C]galactose. The radioactivity present on the membranes was analyzed and used as a measure for the amount of galactose taken up.

DISCUSSION

HPr is the central protein component in low G+C Gram-positive bacteria that regulates the optimal utilization of carbon and energy sources and provides an appropriate balance between carbon uptake and metabolic conversion rates to ensure optimal growth (Luesink *et al*., 1999; Vadeboncoeur *et al*., 2000). In this study we cloned, sequenced and characterized the *S. thermophilus pts* operon and probed the functionality of specific amino acid residues in HPr. The *S. thermophilus pts* operon is composed of two genes, *ptsH* and *ptsI*, which encode the non-sugar specific PTS proteins HPr and EI, respectively. With few exceptions the organization of the *ptsHI* operon is highly conserved among bacteria (Deutscher *et al*., 1994; Lai and Ingram, 1995). Transcription data generated in this study indicated that the P_I promoter upstream of the *ptsH* gene is active and that two different transcripts are generated, one encompassing *ptsH* and *ptsI*, and one specific for only *ptsH*. This is also found for the transcription of the *pts* genes of several other low G+C Gram-positive bacteria that share a high degree of sequence identity in their promoter regions (Luesink *et al*., 1999; Vadeboncoeur *et al*., 2000). Small (upto two-fold) differences in expression of the *S. thermophilus pts* operon were detected when cells were grown on different carbon sources. These appeared to be CcpA-independent but in the tested *S. thermophilus ccpA* mutants transcription of the *pts* operon was two-fold reduced on all sugars tested compared to wild-type cells. Streptococcal *pts* promoters were reported to contain a functional *cre* sequence that harbors only one mismatch relative to the consensus sequence WWTGNAARCGNWWWCAWW (Miwa *et al*., 2000; Vadeboncoeur *et al*., 2000). However, in the *S. thermophilus* promoter sequence, like in the other streptococcal *pts* promoters, this mismatch is a C to G substitution in the highly conserved core (at position 9) of the *cre* consensus sequence, suggesting that this element is not functional. Moreover, the position of this *cre* sequence, directly upstream of the –35 sequence of the P_I promoter predicts negative regulation of the *pts* transcription instead of a positive regulatory role (Henkin, 1996). Therefore, it seems more likely that CcpA is not directly involved in

transcription of the streptococcal *pts* operon and that environmental conditions (e.g. pH, growth rate or carbon source and its availability) dictate the relatively minor differences in mRNA amounts that are generally found for this operon. The slightly reduced *ptsH* promoter activity in the *ccpA* mutant cells could be the result of the growth retardation generally found in these mutants.

Overall, the *S. thermophilus pts* operon seems quite similar in organization and regulation compared to the *pts* operons of other low G+C Gram-positive bacteria. The same strategy that was successfully applied to generate a functional *ptsH* deletion strain in *L. lactis* was also employed in *S. thermophilus* (Luesink *et al*., 1999). However, despite various attempts a *ptsH*::*ery* gene replacement could never be obtained. Single cross-over events occured with the same frequency in either of the flanking regions, but the desired double-cross over event leading to the replacement in *ptsH* was never obtained, independent of the carbon source provided. This lack of success to inactivate the *ptsH* gene suggests that the functional role of HPr in *S. thermophilus* is somewhat different than that in other low G+C Gram-positive bacteria. This possibility is supported by several unique features of sugar metabolism in *S. thermophilus*. Remarkably, glucose, the paradigm carbon source, is not taken up by *S. thermophilus* via a PTS and is a poor substrate for growth (Poolman, 1993). In contrast, lactose is the preferred carbon and energy source of *S. thermophilus* and the galactoside transporter LacS is the sole transporter for this sugar. Several reports have shown that P-His-HPr is involved in modulation of the transport activity of LacS by phosphorylation of its EIIA like domain (Gunnewijk and Poolman, 2000; Poolman *et al*., 1995). Moreover, a *ccpA* mutant in *S. thermophilus* is completely deregulated in lactose uptake and glycolysis (van den Bogaard *et al*., 2000). Next to lactose and glucose, *S. thermophilus* can only grow on sucrose and fructose that are both taken up via PTS (van den Bogaard *et al*., 2002a). Therefore, a hypothetical *S. thermophilus ptsH* mutant is predicted to be still capable of transporting glucose and lactose. However, because of the role of HPr in both the regulation of *lacSZ* transcription (via CcpA) and the activity modulation of the LacS transporter, it is difficult to predict the consequences of a *ptsH* mutation for the lactose metabolism. The mechanism of the glucose transport, a poor carbon source for *S. thermophilus*, is still unknown and the consequences of a *ptsH* mutation for the glucose metabolism is difficult to predict.

In the absence of a *S. thermophilus ptsH* deletion strain, the functional role of specific residues of *S. thermophilus* HPr was assessed by heterologous complementation in a lactococcal *ptsH* negative background (Luesink *et al*., 1999). First, it was established that wild-type *S. thermophilus* HPr can complement this *L. lactis* mutant ((Table 3). Similar complementation of *ptsH*-deficient strains has been reported previously. The *L. lactis ptsH*-mutation was succesfully complemented with the *B. subtilis ptsH* gene and the *Lactobacillus brevis ptsH* gene was shown to complement the *ptsH*-mutation in *B. subtilis* (Luesink *et al*., 1999) (Djordjevic *et al*., 2001). Transcription data showed that all mutants were expressed under the conditions tested and that the *ptsH* transcript levels were in the same order of magnitude for all *ptsH* alleles. Furthermore, our observations indicate that all HPr mutants were properly produced since all variants gave a distinct phenotype compared to the *L. lactis ptsH* mutant. Histidine 15 is clearly an essential residue for the transport of PTS sugars as it is the acceptor for the phosphoryl group but its substitution by an alanine residue is not affecting growth on galactose or maltose, that are transported by a GPH family or ABC transporter, respectively. The HPr-S46A is apparently not active in catabolite control of metabolism as was shown in *L. lactis* and *B. subtilis* (Deutscher *et al*., 1994; Luesink *et al*., 1999), while the introduction of an aspartate residue at this position (HPr-S46D) resulted in growth retardation on all sugars tested. This observation is probably explained by permanent

catabolite repression and inducer exclusion, leading to a reduction of non-PTS metabolism. On the other hand, PTS transport is probably severely hampered due to the inefficient formation of P-His-HPr-S46D. It was previously reported that wild-type *L. lactis* cells producing HPr-S46D displayed severe impairment of growth on galactose, while growth was not affected for the PTS sugars glucose and sucrose. However, in contrast to the studies presented here, those cells contained also wild-type HPr next to its mutant HPr-S46D thereby allowing normal PTS transport rates (Luesink *et al*., 1999).

Previous studies predicted that specific amino acid residues of HPr were involved in the interaction with PTS EIIA. Residue Arg-17 was identified as potentially crucial in the phosphoryl transfer between from P-His-HPr to the EIIA^{glc} of *B. subtilis* probably by stabilizing the intermediary HPr-P-EIIA^{glc} complex (Herzberg *et al*., 1992). Mutation of residue Arg-17 to alanine or glutamic acid in *S. thermophilus* HPr substantiates the essential role of this residue for PTS sugar utilization since these mutations abolished growth on PTS sugars. In contrast, glucose utilization was not affected by the replacement of Arg-17 by a lysine residue that is apparently able to mimick the role of arginine. While maltose utilization was not affected, galactose growth was retarded or abolished by the replacement with alanine or glutamic acid, respectively, and was again restored by the replacement with lysine. Replacement of residue Ile-47 with alanine had no significant effect on utilization of any of the sugars tested, confirming that the residue itself is not catalytically active. However, when replaced with aspartic acid only maltose utilization remained. The results in this study show that residue Ile-47 is not directly involved in phosphoryl transfer of PTS transport but more in the interaction of HPr with EIIA as was suggested for *B. subtilis* (Chen *et al*., 1993). In *S. salivarius* HPr Ile-47 was identified as an important residue in catabolite control since its replacement by threonine rendered the strain insensitive to catabolite repression while it the phosphorylation of the His-15 and Ser-46 residues was still allowed indicating that phosphoryl-transfer is not hindered by the replacement of the Ile-47 residue (Gauthier *et al*., 1997; Viana *et al*., 2000).

The effects on galactose metabolism of HPr mutations that were anticipated to only interfere with PTS IIA interaction or phosphotransfer are quite remarkable. The *L. lactis* GalA belongs to the same GPH family of galactose transporters as the *S. thermophilus* galactoside transporter LacS (Grossiord *et al*., 1998). However, GalA does not harbor the IIA domain found in the LacS transporter that significantly increases exchange-activity upon phosphorylation by P-His-HPr (Grossiord *et al*., 2002; Gunnewijk and Poolman, 2000). Nevertheless, galactose transport in *L. lactis* is inhibited by the same mutations in HPr that also affect PTS transport. Previously, the *galA* promoter was shown to be under catabolite control by CcpA (Luesink *et al*., 1999). Moreover, for HPr in *S. salivarius* and *L. casei* it has been proposed that residue Ile-47 might be important also for the interaction of P-Ser-HPr with CcpA (Gauthier *et al*., 1997; Viana *et al*., 2000). Therefore, to assess whether the observed effects were the result of *galA* transcription or GalA transport capacity variation, we determined *galA* transcription levels and GalA initial transport rates (Table 4). The results clearly show that the substitutions in HPr at residue Arg-17 and Ile-47 that abolished PTS sugar utilization also impaired growth on galactose at the transport level. In contrast, the induction of the *gal* operon was not dramatically altered due to the expression of the different mutant HPr's and thus cannot account for the observed differences in galactose transport. Transcription analysis of several *L. lactis* glycolytic genes, including the *ldh* gene that is part of the CcpA-regulated *las* operon, showed that there were no differences in the expression due to the mutations in the *ptsH* gene. Analysis of the His-15 and Arg-17 residues of HPr showed phosphoryl transfer of P-His-HPr is not directly involved in galactose metabolism. P-Ser-HPr is involved in inducer exclusion of non-PTS carbohydrates in Gram-positive bacteria. (Dossonnet *et al*., 2000; Viana *et al*., 2000; Ye *et al*., 1994). This mechanism was

described for the *L. brevis* galactose permease GalP, which is very homologous to GalA of *L. lactis*, where galactose transport was uncoupled from H^+ symport by binding of P-Ser-HPr (Djordjevic *et al*., 2001). Recently, inducer exclusion in *L. lactis* via P-Ser-HPr was reported for maltose uptake (Monedero *et al*., 2001). Although the mechanism of inducer exclusion for maltose and galactose are probably both mediated by binding of P-Ser-HPr to the transporter, the mechanism is apparently different since maltose utilization was not affected by substitution of Arg-17 and Ile-47 whereas galactose transport was.

Clearly, the *S. thermophilus pts* operon is very conserved in organization and regulation compared to the *pts* operons of other low G+C Gram-positive bacteria. By overexpressing wild-type *S. thermophilus ptsH* and specific mutant alleles of *ptsH* in a *ptsH* deletion strain of *L. lactis*, the functional role of *S. thermophilus* HPr was confirmed and specific amino acid residues were identified that are essential for its function in utilization of PTS and non-PTS carbohydrates. Moreover, our results suggest that in *L. lactis* there is a interaction between HPr and GalA that directly affects the functionality of GalA, which is phosphotransfer-independent. Since GalA of *L. lactis* and LacS of *S. thermophilus* belong to the same GPH family of transporters one could consider the possibility of a similar modulation for LacS functionality in *S. thermophilus* without the phosphotransfer by P-His-HPr.

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Chapter 8

Concluding remarks and future perspectives

Streptococcus thermophilus is one of the most important lactic acid bacteria in the dairy industry. It is applied as a starter culture for the production of various cheeses and yogurt. Characteristic for *S. thermophilus* is its adaptation to growth on lactose, which is the primary carbon and energy source in milk. A detailed understanding of the *S. thermophilus* lactose metabolism would provide the knowledge-base for improving or modulating the performance of this microbe in industrial fermentations by metabolic pathway engineering. Although the fundamentals of lactose metabolism in *S. thermophilus* are known for quite some time, detailed information on the specific and global control of the *gal-lac* operon is limited. Only recently, studies on lactose transport regulation generated more insight in the factors involved in lactose metabolism in *S. thermophilus* (for a review see Gunnewijk *et al*., 2001). This thesis describes the molecular mechanisms involved in specific and global regulation of the *S. thermophilus* lactose metabolism.

The *S. thermophilus gal-lac* gene cluster

Streptococcus thermophilus strain CNRZ302, like most other dairy strains of *S. thermophilus*, is unable to ferment galactose that is generated intracellularly by lactose hydrolysis or present as free sugar. Nevertheless, sequence analysis and complementation studies in *Escherichia coli* demonstrated that strain CNRZ302 contained structurally intact genes for the Leloir pathway enzymes (Vaughan *et al*., 2001). These were organized in an operon with the order *galKTE*, which was preceded by a divergently transcribed regulator gene *galR,* and followed by a *galM* gene and the lactose operon *lacSZ* (Fig. 1). The *S. thermophilus gal-lac* gene cluster is very conserved in sequence, organization and flanking regions among strains isolated from various fermented products (van den Bogaard *et al*., 2002a). This gene order was found to be conserved in *S. salivarius*, which reflects the evolutionary relationship between these bacteria (Schleifer *et al*., 1991; Vaillancourt *et al*., 2002). Although the *S. salivarius gal-lac* gene cluster appeared to be nearly identical to that of *S. thermophilus*, *S. salivarius* grows well on galactose. More general, in streptococci the organization of *galR* and *galKT* seems to be conserved, while variations are observed for the *galE* and *galM* genes location. In various organisms the *galK*, *galT* and *galE* genes which encode the Leloir pathway for galactose utilization are frequently clustered or organized in a single operon. While the similarity between the deduced primary sequences of the Leloir enzymes is very high among lactic acid bacteria, the genomic organization of the *gal* clusters and gene order appears species specific (Grossiord *et al*., 1998). Conservation of the location of the *lac* operon downstream of the *gal* cluster seems to be restricted to *S. thermophilus* and *S. salivarius.* A potential transcriptional regulatory gene, *galR*, has also been identified for *Lactobacillus casei* and is transcribed withinin the *L. casei gal* operon *galKETRM* (Bettenbrock and Alpert, 1998). Recently, upstream of the *galA* gene in *L. lactis* ATCC 7962 (GenBank Accession no. U60828 (lee *et al*., 1997)) and IL1403, an open reading frame was found, that can encode for a putative GalR of the SIS family (Vaughan *et al*., 2002). In contrast, the putative *galR* homologues in *Escherichia coli*, *galR* and *galS*, are not genetically linked to the *gal* operon (Weickert and Adhya, 1993), and *galR*-like regulatory genes have not been identified to date in the *gal* operons of other lactic acid bacteria, including as *Leuconostoc lactis* and *Lactobacillus helveticus* (Grossiord *et al*., 1998; Mollet and Pilloud, 1991).

Regulation of the *gal-lac* gene cluster by GalR

The efficiency of the *S. thermophilus galK* and *lacS* promoter in CNRZ302 is improved during growth on lactose and galactose as a consequence of GalR activity (Vaughan *et al.*, 2001) (Fig. 1A). Moreover, disruption of *galR* indicated that it negatively regulated its own expression. While in *S. mutans* the *galR* gene is also oriented in the opposite direction relative to

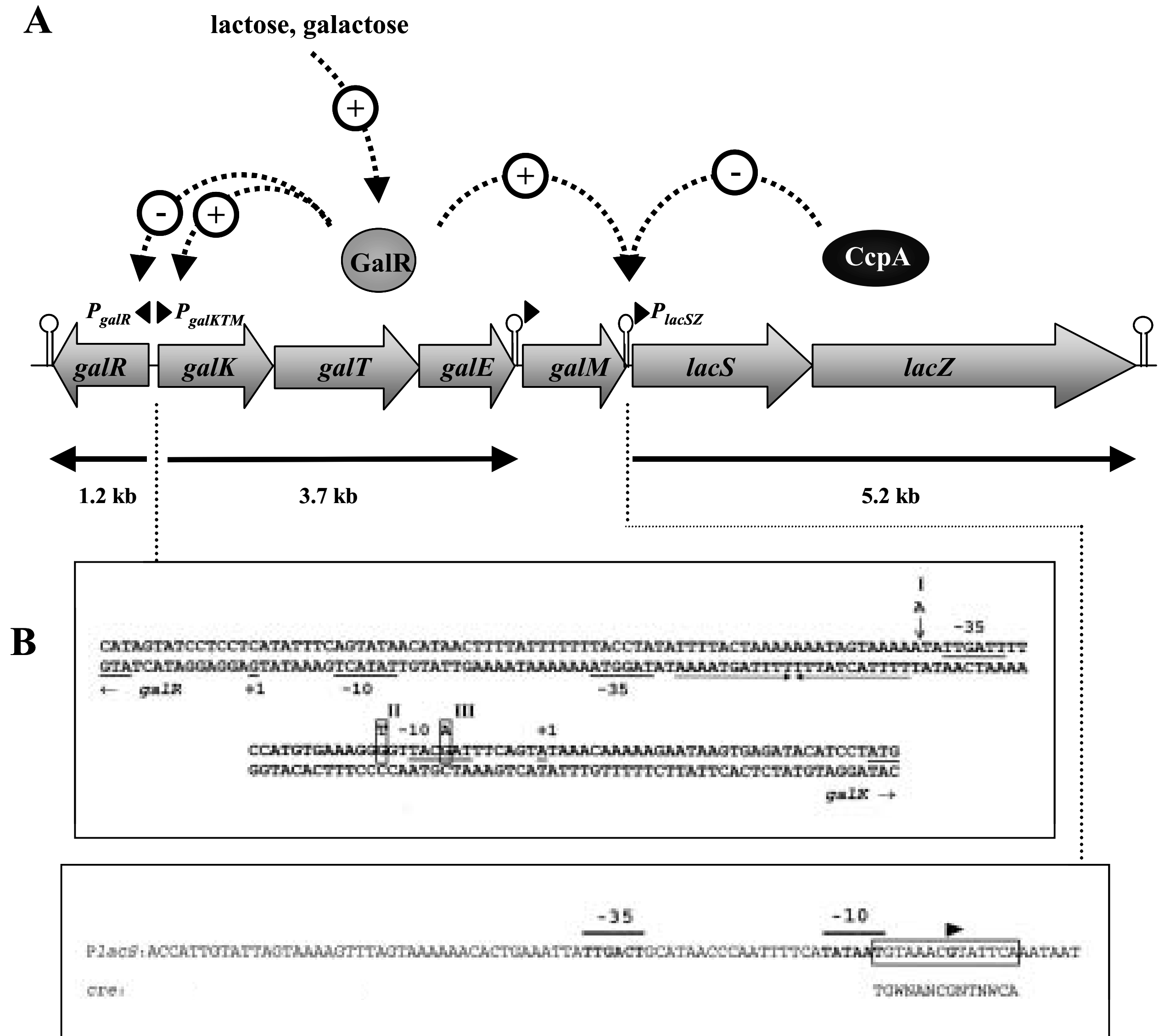

Figure 1. Proposed model for the regulation of the lactose-specific regulation of the *gal-lac* gene cluster in *Streptococcus thermophilus*. A: the presence of lactose or galactose in the growth medium activates the operon-specific regulator GalR that induces the *galKTE* promoter as well as the *lacSZ* promoter. Most *S. thermophilus* strains, however, do not grow on galactose probably due to the inefficiency of the *galKTE* promoter. GalR has been shown to negatively autoregulate its own transcription. B: molecular characterization of galactose fermenting mutants of Gal$^-$ strain CNRZ302 as well as naturally galactose fermenting isolates showed them all to contain *galKTE* promoter-up mutations. These mutations all involved single base pair insertions or substitutions that could be devided into three classes depending on the location of the mutations in the promoter region. The *lacSZ* promoter was found to countain a *cre* site that was shown to be involved in CcpA mediated regulation on this promoter.

the *galKTE* operon, disruption of the *galR* gene resulted in constitutive expression of galactokinase, suggesting that GalR functions as a repressor of the *gal* operon in this species (Ajdic and Ferretti, 1997; Ajdic and Ferretti, 1998). Upon lactose- or galactose-mediated induction, the structural *galKTE* genes in *S. thermophilus* CNRZ302 were found to be transcribed very weakly, indicating that insufficient transcription of these genes could account for the galactose-negative phenotype of this strain (Vaughan *et al*., 2001). This is supported by the characterization of independently isolated galactose-fermenting mutants. These mutants showed high activity of the Leloir enzymes relative to their parent strains. Transcriptional study of one of these spontaneous galactose-fermenting mutants, designated NZ302G, showed that the *galKTE* genes were expressed at high levels in cells grown on lactose or galactose. Sequence analysis of the *gal* promoter regions of NZ302G and other independently isolated Gal^+ mutants revealed so-called promoter-up mutations at three positions in the *galK* promoter region, which included substitutions in the extended –10 sequence as well as a single base pair insertion at position –36 (Fig. 1B). The latter mutation results in an altered spacing between the *galK* promoter and an inverted repeat, representing the postulated binding site for GalR. In *S. thermophilus* strains isolated from various fermented products little sequence variation was found among the *galR-galK* intergenic regions compared to that of CNRZ302. Only in naturally galactose-fermenting strains single base pair changes in the –10 sequence were found that can be considered *galK* promoter-up mutations (van den Bogaard *et al*., 2002a). The up-regulation of the *galKTE* promoter seems to suffice for a galactose fermenting phenotype of *S. thermophilus.* These results provide insight into the mechanism of down-regulation of the *galKTE* expression in the galactose-negative *S. thermophilus* CNRZ302 via mutations in the *galK* promoter. The loss of the ability to ferment galactose can be attributed to the adaptation to milk in which the lactose levels are in excess. A recent report suggested that relatively poor translation efficiency of the *galK* gene of *S. thermophilus* strain SMQ-301 compared to that of *S. salivarius* strain ATCC 25975 could account for the difference between these species regarding growth on galactose. Supportive for this suggestion was the observation that the *galKTE* transcript was present in both species in similar amounts (Vaillancourt *et al*., 2002). Nevertheless, the sequence of the *S. salivarius galK* promoter is not entirely identical to that of *S. thermophilus* and these sequences differ in the –10 sequence of the *galK* promoter, which could have a major impact on the promoter activity. Moreover, the RNA from *S. salivarius* was isolated at a very different growth phase than that from *S. thermophilus* that can have consequences for operon induction and RNA steady state concentrations, which could explain the similar transcript amounts while promoter activity might be different. Furthermore, the same study also reported a catabolite response element (*cre*) in the *galK* promoters of *S. thermophilus* and *S. salivarius*. However, no catabolite control by CcpA was observed for the *S. thermophilus gal* operon (van den Bogaard *et al*., 2000). Moreover, these putative *cre* sequences harbor at least one mismatch (C to G substitution in the core at position 9) in the highly conserved core region of the consensus *cre* sequence postulated by Weickert and Chambliss and recently adapted by Miwa *et al*., WWTGNAARCGNWWWCAWW (Miwa *et al*., 2000; Weickert and Chambliss, 1990). The galactose-fermenting capacity of *S. salivarius* is probably explained by the fact that this species is found mostly in oral cavities and most likely experiences a whole array of carbon sources in this niche whereas *S. thermophilus* is almost solely found in dairy fermentations where lactose is abundant. Apparently, the additional fermentation the galactose moiety via the relative slow Leloir pathway by a Gal^+ mutant is energetically less favorable, yielding 1.2-fold lower specific growth-rates than that of the Gal^- parent (Levander *et al*., 2002). At the molecular level this is also reflected by the highly efficient lactose/galactose exchange activity of the galactoside transporter LacS.

The *S. thermophilus* CcpA

The identification of a catabolite responsive element (*cre*) in the *LacS* promoter implicated a role for CcpA-mediated regulation of the *S. thermophilus* lactose metabolism (Fig. 1B). To assess the role of CcpA in global regulation of carbon metabolism in *S. thermophilus,* the *ccpA* gene was cloned and characterized (van den Bogaard *et al*., 2000). Western blot analysis showed that the CcpA production itself was sugar-source dependent with more than two-fold higher amount found in glucose-grown cells relative to lactose-grown cells. In a Northern analysis the abundance of *ccpA*-specific transcripts was analysed in wild-type and *ccpA* disruption cells that still expressed truncated and inactive *ccpA* transcripts using a labeled internal *ccpA* fragment. Although this experiment is not conclusive due to the probe used, it strongly suggests that the *ccpA* promoter is negatively autoregulated. Negative autoregulation at the transcriptional level has been found for some but not all other *ccpA* genes (Egeter and Bruckner, 1996; Monedero *et al*., 1997). Inspection of the *ccpA* sequence showed the *ccpA* gene to be in a back-to-back organization with a *pepQ* gene as has been reported for several other lactic acid bacteria including *Lactobacillus pentosus*, *Lactobacillus casei*, *L. delbrückii*, *S. mutans*, and *L. lactis* (Mahr *et al*., 2000), Remarkably, in *Lactobacillus plantarum* the *ccpA* and *pepQ* genes are also linked but in a tandem organization (Muscariello *et al*., 2001). Furthermore, two putative *cre* were located in the *S. thermopilus ccpA* gene, of which one was found upstream of the transcription start site of the *ccpA* promoter but more closely linked to the divergently transcribed *pepQ* gene, suggesting its involvement in regulation of *pepQ* expression rather than *ccpA* autoregulation. In *L. lactis* a weak CcpA-mediated regulation was observed of the *pepQ* transription (Hellendoorn *et al*., 1999). The other putative *cre* is located within the coding sequence of the *ccpA* gene, which could suggest a role in transcriptional control. An internal *cre* site has also been found in the coding region of the *L. lactis ccpA* gene, but autoregulation could not be established in this organism (Luesink *et al*., 1998). An alternative mechanism for the observed autoregulation involving the two *cre* sites could be that CcpA binds to these sites to form a DNA loop, thereby inhibiting *ccpA* promoter activity.

CcpA mediated regulation of lactose uptake

CcpA has been studied in many low G+C Gram-positive bacteria where it mediates catabolite repression when cells are grown on PTS carbon sources, of which glucose is generally the most preferred. *S. thermophilus*, unlike many other Gram-positive bacteria, prefers lactose over glucose as the primary carbon and energy source. Notably, lactose is not taken up by PTS but by the dedicated transporter LacS (Poolman *et al*., 1989).

The position of the *cre* relative to the *lacS* promoter predicted transcriptional repression by CcpA (Fig 1B) (Poolman, 1993) (Henkin, 1996). This prediction was confirmed since the functional disruption of the *S. thermophilus ccpA* gene resulted in relieve of repression of this promoter when cells were grown on lactose. This observation was remarkable since, to date, CcpA-mediated repression was only described for growth on PTS sugars (for a review see Stulke and Hillen, 2000). From the strongly reduced growth-rate of the CcpA$^-$ strain on lactose it could be deduced that global regulation plays an important role in *S. thermophilus* lactose metabolism. At the metabolic level this was reflected in the increased lactose uptake and hydrolysis capacity, the massive expulsion of glucose and the reduced lactate production. *S. thermophilus* possesses a very high capacity for lactose uptake and hydrolysis. The role of CcpA in *S. thermophilus* appeared to include the fine-tuning of

lactose uptake and hydrolysis rate to the overall glycolytic capacity. This activity apparently includes an antagonizing activity of CcpA on the GalR-mediated activation of the *lacSZ* operon. It appears that CcpA, in concert with GalR, acts more like a fine-tuning regulator of lactose metabolism rather than its classical role of catabolite repressor of sugar hierarchy in other low G+C gram-positive bacteria (van den Bogaard *et al*., 2002c; van den Bogaard *et al*., 2000). The adaptation to lactose is reflected in a complex regulation of lactose metabolisme in *S. thermophilus.* In contrast, *L. delbrückii* subsp. *bulgaricus* has been shown to adapt to lactose growth by a completely different mechanism after selective pressure in milk (Lapierre *et al*., 2002). A complex of insertion sequence (IS) elements inserted in the *lac* promoter led to the constitutive high expression of the *lac* genes in this species. This is a rather simple and fast adaptation resulting in the fermention of lactose at a higher rate. In contrast, the complex adaptation *in S. thermophilus* allows fast and delicate tuning of the metabolism to the modulations in the lactose availability level and has probably developed over a long period of time.

Regulation of central metabolism

The main system for energy generation LAB is the glycolytic or Embden-Meyerhoff pathway. Global control in bacteria is mainly modulated by intracellular concentrations of glycolytic intermediates. The levels of these intermediates are dependent on the efficiency of metabolism of a carbon source. Primarily, the observation of glucose expulsion and reduced lactate production in CcpA$^-$ cells grown on lactose led to the investigation of CcpA-mediated control of the *S. thermophilus ldh* gene. The conversion of pyruvate to lactate by lactate dehydrogenase is an essential step in homofermentative lactic acid bacteria to reoxidize NADH that is generated during glycolysis. Transcriptional analysis of the *S. thermophilus ldh* gene showed that expression was induced in a CcpA-dependent manner during growth on lactose compared to glucose or galactose. The *cre* site found in the *ldh* promoter region is situated upstream of the –35 box, which is in agreement with positive control by CcpA (Henkin, 1996). Northern analysis showed that the *S. thermophilus pfk-pyk* operon is subjected to CcpA-dependent regulation. The reactions catalyzed by their gene-products PFK, PYK and LDH are highly unidirectional steps in glycolysis and effective points for catabolite control. In analogy, no CcpA dependent regulation for the other glycolytic genes was observed. Table 1 shows the *cre* sequences and their positions in the genes that were found to be under CcpA-mediated control in *S. thermophilus*. In analogy, the *las* operon of *L. lactis*, comprising the *pfk, pyk* and *ldh* genes, was found to be transcriptionally activated by CcpA on glucose (Fig 2A) (Luesink *et al*., 1998). In *L. lactis* the glycolytic genes *fba*, *tpi* and *glcK* also appeared to be sugar regulated, although CcpA involvement is still inconclusive (Even *et al*., 2001). In *B. subtilis*, many of the glycolytic genes are organized in operons. The *pgk-tpi-pgm-eno* operon and the *yvbQ-gap* operon in this bacterium are induced by glucose in a CcpA-dependent manner. However, expression was found to be constitutive for the *pfk-pyk* operon as well as the other glycolytic genes indicating that a microbe with a more complex metabolism than LAB, including a complete TCA cycle, has different points for catabolite control of central metabolism (Tobisch *et al*., 1999). DNA micro-array technology and genomics have been used to explore the genome of *B. subtilis* in the expression analysis to investigate CcpA regulation (Moreno *et al*., 2001). This powerful approach revealed novel regulation modes but could not conclusively distinguish between the primary or secondary effect of a CcpA mutation. The interpretation of the vast amount of data obtained from DNA micro-array technology is often difficult. Interestingly, this approach led to the discovery of

genes that were regulated by CcpA in a glucose-independent manner, and many of these genes were involved in nitrogen metabolism. A similar study of CcpA regulation will soon be possible for *S. thermophilus* with the completion of the genome sequence of strain LMG18311 (Hols *et al*., 2002).

Promoter	*cre* sequence	Position relative to the transcriptional start	Type of CcpA-mediated control
P*lacSZ*	TGTAAACGTATTCA	-7	-
P*ldh*	TTAAAACGCTTTCA	-71	+
P*pfk*	AAAAACCGATAAAA	-73	+
	GTTATTCGTTATCA	-192	
P*pyk*	AATTATCGTAAACA	-98	+

Table 1. the *cre* sequences and their positions (from the first nucleotide of the *cre*) in the genes that were found to be under CcpA-mediated control in *S. thermophilus*. Mismatches with the consensus *cre* sequence (TGTAAACGTATTCA) are underlined (Weickert and Chambliss, 1990). Type of CcpA-mediated control; - indicates reprression and + indicates activation of the promoter.

Glycolytic intermediate levels are the primary indicators of energy status in the cell

Catabolite control in bacteria is mainly modulated by intracellular concentrations of glycolytic intermediates that are dependent on the efficiency of metabolism of a carbon source. In addition, carbon flux through glycolysis is subject to allosteric glycolytic-intermediate control at least at two reactions, catalyzed by phosphofructokinase (PFK) and pyruvate kinase (PYK) (Fothergill-Gilmore and Michels, 1993). In *L. lactis* and *Lactobacillus bulgaricus* PFK is allosterically activated by phosphoenolpyruvate (PEP) (Fordyce *et al*., 1982), while allosteric control of PYK activity includes activation by glucose-6P (G6P), fructose-6P (F6P) and/or fructose-1,6-bisP (FBP) and inhibition by high levels of free phosphate (Pi) (Fordyce *et al*., 1984; Le Bras and Garel, 1993). Lactate dehydrogenase (LDH) is also activated by FBP, although under normal growth conditions the concentration is sufficiently high to ensure full activation (Garrigues *et al*., 1997). These observations indicate that intracellular levels of glycolytic intermediates serve as primary indicators of the metabolic and energy status in the cell, and closely link the rate of carbon influx to the central metabolism. Therefore, metabolite level determination can generate valuable information for the analysis of metabolic flux and are of critical importance for the construction of metabolic control models that allow to pinpoint enzymatic steps that can be targeted to achieve effective metabolic engineering (de Vos,1996; Mendes and Kell, 1997). So far no substantial data on metabolite levels in *S. thermophilus* have been reported and metabolite analysis in lactic acid bacteria has focussed mostly on *L. lactis* (Even *et al*., 2001; Neves *et al*., 1999; Thompson, 1978). To study the correlation between the carbon source in the growth medium and catabolite control in *S. thermophilus* a straightforward method for isolation of glycolytic intermediates was applied that was previously validated for *E. coli*. The boiling water cell pool extraction method for glycolytic intermediates isolation and subsequent HPLC analysis proved to be very reproducible while some errors observed in intermediate levels seem to be introduced by growth phase variations and culture history. Alternatively, it is possible that some glycolytic enzymes are more abundantly present than others. In many LAB, PYK and LDH are present in high concentrations in the cell (Garrigues *et al*., 1997; Garvie, 1978) and minute differences during inactivation of these high level activities may have consequences

on the intermediate levels. In analogy with the highest growth-rates of *S. thermophilus* CNRZ302 on lactose, the uptake data revealed that in resting cells lactose is taken up faster than the other sugars tested. Furthermore, lactose metabolism in this strain was shown to be regulated by CcpA at the lactose uptake and lactate formation level (van den Bogaard *et al*., 2000).

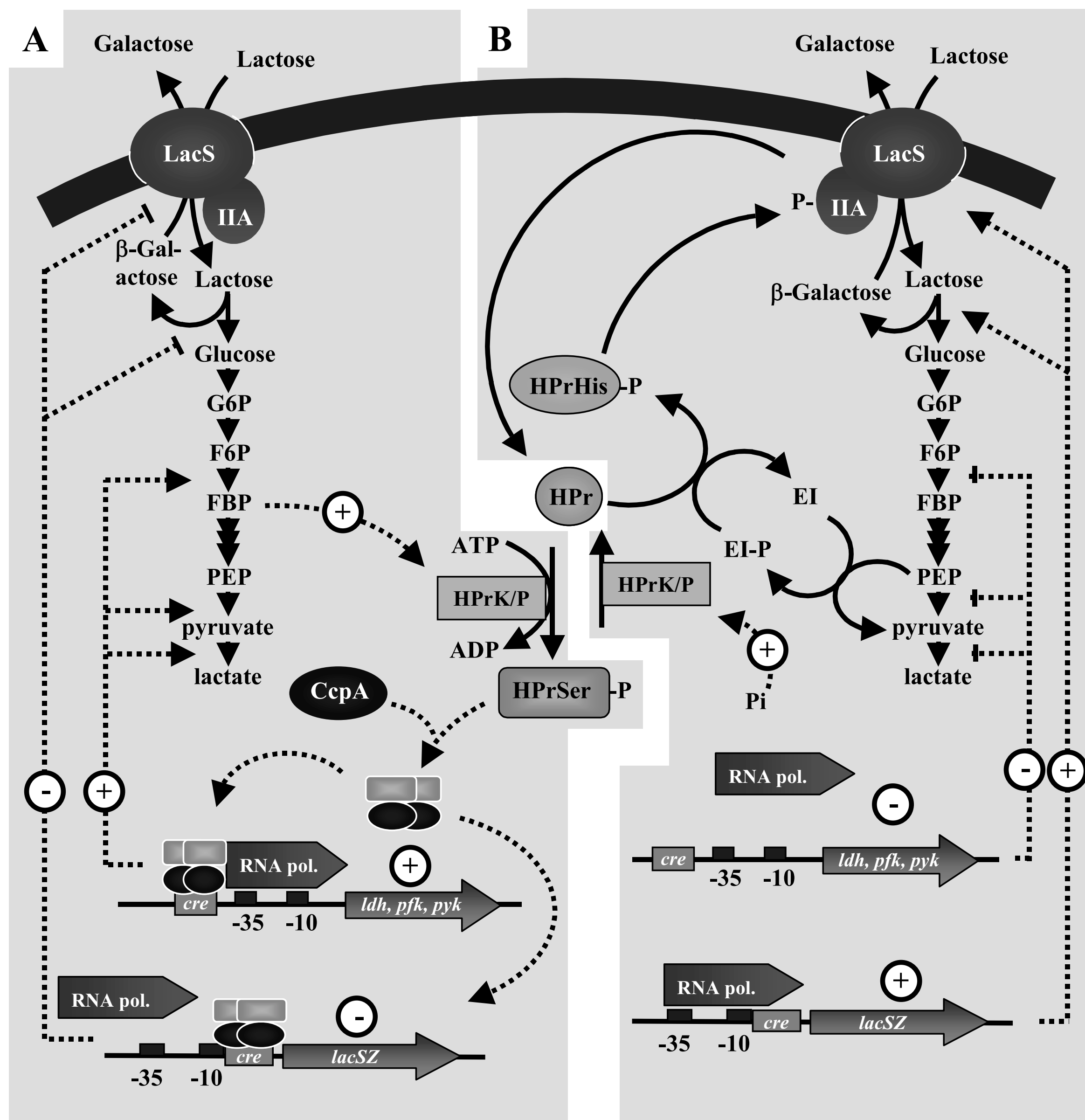

Figure 2. Proposed model for the regulation of lactose metabolism in *Streptococcus thermophilus*. A: when lactose is present in excess in the growth medium the high influx of glucose into glycolysis leads to accumulation of fructose-1,6-bisphosphate (FBP), which activates the HPrK to form P-Ser-HPr. The P-Ser-HPr/CcpA complex subsequently increases glycolytic activity and decreases lactose import and hydrolysis, at the transcriptional level. When lactose concentration in the growth medium becomes low the intracellular concentration of FBP decreases and is relieved. Moreover, under these conditions P-His-HPr becomes dominant, which leads to the phosphoryl transfer from P-His-HPr to the IIA doas a consequence catabolite repression of the *lacSZ* promoter main of LacS and thereby to the increase in lactose uptake activity by this transporter (Gunnewijk and Poolman, 2000). Taken together these modulations of lactose metabolism allow *S. thermophilus* to adapt effectively to the availability of lactose in the growth medium.

Uptake of sucrose in *S. thermophilus* is mediated by the sucrose-PTS. Uptake of lactose, galactose and glucose on the other hand, are transported via permeases. Growth and acidification rates observed for sucrose- and lactose-growing cells are high compared to cells growing glucose and galactose. Thereby, this study presents the comparison of metabolite steady-state levels in fast and slow metabolizing cells for PTS and permease sugars in *S. thermophilus*. Lactose-grown cells contained the highest levels of G6P and F6P, which seemed independent of CcpA. The rapid and efficient lactose uptake by LacS yields high amounts of intracellular glucose that enter glycolysis following phosphorylation by glucokinase, which apparently leads to the accumulation of G6P and F6P. FBP also accumulates to higher steady-state concentrations in lactose-grown cells when compared to glucose- or galactose-grown cells. However, the highest FBP steady-state levels were found in sucrose-fermenting cells, corroborating the idea that PTS sugars potentially exert stronger catabolite repression then permease sugars (Postma *et al*., 1993; Stulke and Hillen, 1999). In starved *S. thermophilus* cells intracellular concentrations of PEP and pyruvate remained in the same order of magnitude compared with the levels observed in lactose-fermenting cells, whereas al other glycolytic intermediates were very low or undetectable. This contrasts markedly with the high concentrations of 2- and 3-phosphoglycerate (2+3PG) and PEP that have been observed in starved *L. lactis* (Thompson and Torchia, 1984) and *E. coli* (Bhattacharya *et al*., 1995). Pyruvate concentrations remained low in starved *L. lactis* cells, more or less at the same level as sugar-fermenting cells (Thompson, 1978). It has been suggested that the PEP concentration increases in starved cells due to a partial inhibition of the pyruvate kinase by the increased free phosphate concentration and the depletion of the intracellular pyruvate kinase-activators G6P and FBP (Garrigues *et al*., 1997). By this mechanism, the cells maintain a high intracellular PEP level to allow rapid sugar intake via the PTS's upon the availability of PTS sugar sources. However, since *S. thermophilus* is highly adapted to growth on lactose, the capacity for lactose-uptake instead via LacS is apparently increased rather than that for sugar-uptake via PTS under starvation conditions (Gunnewijk and Poolman, 2000; Poolman *et al*., 1995). Since PEP is not consumed by the uptake of lactose it is not required to accumulate under starving conditions.

Northern analysis of the expression of the *pfk-pyk* operon suggested that the CcpA-mediated activation of the *pfk* promoter is highest in sucrose-grown cells, followed by lactose-grown cells (van den Bogaard *et al*., 2002c). In analogy with the observed sugar-dependent activation of the *S. thermophilus ldh* gene (van den Bogaard *et al*., 2000), the activation of the *pfk* promoter was low in glucose- and galactose-grown cells (van den Bogaard, unpublished results). The high growth-rate and high turn-over of PEP combined with high steady-state levels of FBP and ATP in sucrose-grown cells indicate that this PTS sugar is preferred over permease sugars. However, *S. thermophilus* CNRZ302 grows fastest on lactose as carbon source, probably due to the kinetics of the LacS permease, for which the rate of lactose uptake mimics that of PTS's without the drain of the PEP-pool.

The pleiotropic role of HPr

HPr, the histidine-containing phosphocarrier protein of the bacterial phosphotransferase system (PTS) controls sugar uptake and carbon utilization in low-G+C Gram-positive and in Gram-negative bacteria. HPr has been described in many studies as the key factor in global control of sugar metabolism in bacteria. With few exceptions the organisation of the *ptsHI* operon is well conserved among bacteria and intracellular HPr and EI amounts always seem to be kept at relatively high levels (Deutscher *et al*., 1994; Lai and Ingram, 1995;

Vadeboncoeur *et al*., 2000). Furthermore, the amino acid sequence and structural conformation are highly conserved among HPr proteins, which is reflected by functional heterologous complemention of *ptsH* mutations in several bacteria (Djordjevic *et al*., 2001; Luesink *et al*., 1999). This does not necessarily imply that the role for HPr in sugar metabolism in each bacterium is identical. In *S. thermophilus* for example, HPr in PTS transport is restricted to two sugars (sucrose and fructose) that are not readily found in dairy fermentations, the most prominent environmental niche for this organism (Poolman, 1993). However, next to PTS transport P-His-HPr is involved in modulation of the activity of the lactose transporter LacS, which indicates that the function of this protein in sugar regulation may include the adaptation to lactose metabolism in this microbe (Gunnewijk *et al*., 2001).

The lack of success to inactivate the *ptsH* gene in *S. thermophilus* hampered the charaterization of the role of HPr in this organism (van den Bogaard *et al*., 2002d). By overexpressing wild-type *ptsH* and specific mutated *ptsH* alleles in a *ptsH* deletion strain of *L. lactis* the functional role of *S. thermophilus* HPr was confirmed and specific amino acid residues were identified that are essential for its function in utilization of PTS and non-PTS carbohydrates. Moreover, the results suggest that in *L. lactis* there is an interaction between HPr and GalA that directly modulates the functionality of GalA, which is likely to be phosphotransfer independent. Since GalA of *L. lactis* and LacS of *S. thermophilus* belong to the same GPH family of transporters one could consider the possibility of a same modulation for LacS functionality besides the phosphotransfer by P-His-HPr. It was also shown that HPr has little to no effect on the utilization of maltose, which is transported by an ABC transport ATPase in *L. lactis*. This sugar would make a good candidate sugar for the selection of a *ptsH* deletion strain in *S. thermophilus*. Unfortunately, to our knowledge no carbon source is known that can be utilized by *S. thermophilus* via an ABC transport system and no homologue for a sugar transporting ABC transport ATPase encoding gene was found in the genome sequence of *S. thermophilus* LMG18311 (Hols *et al*., 2002; van den Bogaard *et al*., 2002a). The construction of a *S. thermophilus* strain carrying the *L. plantarum* maltose ABC transporter was initiated but was not completed due to limitations in selectable markers for the construction of such a strain. Another option to study the interaction between HPr and LacS is to express the (mutated) *S. thermophilus ptsH* allele and the *S. thermophilus lacSZ* operon in the *ptsH* deletion strain of *L. lactis*. In *L. lactis* a copy of a lactose transporter gene and a β-galactosidase gene are present but not active (De Vos and Simons, 1988).

Lactose metabolism in *S. thermophilus* is regulated by lactose availability

CcpA, in concert with HPr, not only fine-tunes uptake and glycolytic activity during maximal growth rate but is also involved in derepression of transport activity and reduces glycolytic capacity to allow cellular adaptation to conditions where lactose in the growth medium becomes limiting. Excess lactose in the growth-medium of *S. thermophilus* leads to high lactose transport and hydrolysis activity, the result of upregulation of the *lacS* promoter by the activity of GalR, which is counteracted by CcpA (Fig. 2) (van den Bogaard *et al*., 2000; Vaughan *et al*., 2001). The question is, however, why this organism requires such a tightly regulated and highly adaptable mechanism to respond to changes in lactose concentrations in the growth medium since the lactose levels in is milk are in excess. The steady state concentration of glycolytic intermediates in the cell is directly dependent on the import rate of sugar and rate of glycolysis (van den Bogaard *et al*., 2002b). In many low-G+C Gram-positive bacteria the intracellular level of FBP, the primary indicator of energy level, is increased when cells are grown on favorable sugars, leading to the activation of HPrK

resulting in the seryl phosphorylated HPr (Kravanja *et al*., 1999; Reizer *et al*., 1998). In *S. thermophilus* a simlar situation in encountered. However, not only growth on a PTS sugar but also growth on excess lactose leads to increased intracellular FBP, which coincides with the presence of P-Ser-HPr as the dominant HPr~P (Fig. 2A) (Gunnewijk *et al*., 2001; van den Bogaard *et al*., 2002b). P-Ser-HPr subsequently activates CcpA, leading to increased glycolytic activity and decreased lactose import and hydrolysis, at the transcriptional level. By these modulations of lactose metabolism *S. thermophilus* simultaneously fine-tunes the uptake of lactose and the capacity for glycolysis to yield optimal glycolytic flux and growth rate. P-Ser-HPr is the dominant phosphorylated HPr species in *S. thermophilus* when grown in excess lactose, however, P-His-HPr becomes dominant when cells encounter lactose limitation (Fig 2B) (Gunnewijk and Poolman, 2000). This transition in HPr phosphorylation state appears to coincide with a reduction of glycolytic flux, probably due to the decrease in FBP concentrations in the cell (van den Bogaard *et al*., 2002b). As a consequence, the IIA domain of LacS is phosphorylated by P-His-HPr leading to increased lactose/galactose exchange and increased total transport activity. In this way P-His-HPr in *S. thermophilus* has a double function in regulation of lactose metabolism as compared to that in most other low-G+C Gram-positive bacteria. This can be considered as the “fast” response to the decrease in lactose in the growth medium. In the “slow” response catabolite repression of the *lacSZ* promoter is relieved, thereby increasing the total lactose uptake capacity when lactose is limiting (van den Bogaard *et al*., 2002c). Moreover, the genes encoding key-steps in glycolysis are no longer activated. Taken together these modulations of lactose metabolism allow *S. thermophilus* to adapt effectively to the availability of lactose in the growth medium.

Perspectives

Streptococcus thermophilus is used, in concert with other LAB, in various industrial dairy fermentations that require processing of milk at elevated temperatures such as yogurt, Mozzarella, Gruyère-type and Emmental cheeses. Its primary function in these fermentations is the rapid conversion of lactose to lactate, while it also contributes to delicate sensory qualities. It is unlikely that lactose metabolism and lactate production itself can effectively be optimized since *S. thermophilus* is already highly adapted to lactose as the primary carbon and energy source. However, there are other aspects of the lactose metabolism that can be considered for improvement. One undesired side-reaction of *S. thermophilus* lactose metabolism in dairy fermentations is the massive excretion of galactose in the fermentation medium. This provides an ample carbon source for food spoilage microorganisms and causes “browning” of the fermented product when heated (Kaanane and Labuza, 1989). Furthermore, high concentrations of ingested galactose can cause cataract (Couet *et al*., 1991). By engineering the lactose metabolism of *S. thermophilus* it is possible to develop strains that do not excrete the galactose moiety of lactose or actively utilize solely the excreted galactose from the fermentation medium. However, the use of one of the galactose-fermenting strains does not suffice to stop the excretion of galactose (van den Bogaard *et al*., 2000; Vaughan *et al*., 2001). These strains still excrete the majority of the galactose moiety from the internalized lactose since the exchange reaction of the LacS transporter is highly favored when galactosides are in excess on either side of the membrane (Foucaud and Poolman, 1992; Vaughan *et al*., 2001). However, when in such a Gal^+ strain the LacS transporter would be replaced by a galactose-specific transporter that does not have the exchange activity, e.g. the GalA transporter of *L. lactis* (Grossiord *et al*., 1998), full fermentation of galactose could be achieved. However, such a strain would have lost the capacity to ferment lactose since the

GalA transporter of *L. lactis* solely transports galactose. Hence, it should be used as an adjunct starter, next to a regular *S. thermophilus* strain or *L. bulgaricus*. Alternatively, a *S. thermophilus* strain that utilizes only the galactose moiety of lactose could possibly be constructed by deletion of the glucokinase gene in a Gal^+ strain. Moreover, by simply over-expression of the *galKTE(M)* and α-*pgm* genes, the flux toward galactose degradation could exceed the exchange activity of LacS and efficient galactose utilization might already be achieved.

Modulation of catabolite control could be used to get improved flavor or texture component production. In the CcpA-negative strain of *S.thermophilus* sugar transport and central metabolism have been shown to be completely deregulated. This strain can be used to produce vast amounts of glucose from lactose in a fermented product but a more subtle approach would be needed to efficiently alter CcpA-regulation of specific genes without disrupting growth. One option would be to generate CcpA mutants that still excert general catabolite repression but are specifically affected in regulation of certain operons as was shown in *B. subtilis* (Turinsky *et al*., 2000). Another elegant option would be to introduce or remove *cre*-sites in genes to allow or relieve CcpA-mediated regulation and shift metabolic fluxes in the cell.

Because of its simple metabolism, *S. thermophils* could be a suitable "plug and play" organism by simply introducing new metabolic routes for the production of specific metabolites or endproducts. As proof of concept, alanine was successfully produced in a pilot experiment where an alanine dehydrogenase gene from *Bacillus sphaericus* was expressed in *S. thermophilus* CNRZ302 by use of the implemented NICE system (van den Bogaard, unpublished results) (Hols *et al*., 1999). The alanine levels produced were not sufficiently high to envisage relevant industrial application, which could be attributed to the very low K_m of the endogenous lactate dehydrogenase enzyme for pyruvate thereby competing strongly for the available pyruvate with the heterologous alanine dehydrogenase. *S. thermophilus* could be a suitable host as a production organism for alanine or other metabolic components provided that an adapted strain is choosen or created. An example of such a strain for the production of alanine is a *S. thermophilus* strain that contained an LDH with a high K_m for pyruvate.

In conclusion, the detailed insight in the global control and diversity of the sugar utilization by *S, thermophilus* as described here, allows for understanding its adaptation to the milk sugar lactose, underlines the complexity of the important glycolytic pathway, and provides the framework for further metabolic engineering strategies.

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Chapter 9

Summary

Summary

Streptococcus thermophilus is used in many industrial dairy fermentations that require processing of milk at elevated temperatures, including yogurt and Mozzarella, Gruyère-type and Emmental cheeses. Its primary function in these fermentations is the rapid conversion of lactose to lactate, while it also contributes to important sensory qualities. CcpA has been studied in many low G+C Gram-positive bacteria where it mediates catabolite repression when cells are grown on phosphoenolpyruvate(PEP)-dependent phosphotransferase system (PTS) carbon sources, of which glucose is generally the most preferred sugar. *S. thermophilus*, unlike many other Gram-positive bacteria, prefers lactose to glucose as the primary carbon and energy source. Moreover, lactose is not taken up by a PTS but by the galactoside transporter LacS.

S. thermophilus strain CNRZ302 is unable to ferment galactose, neither the free sugar, nor when it is generated intracellularly by lactose hydrolysis. Nevertheless, sequence analysis and complementation studies in *Escherichia coli* demonstrated that strain CNRZ302 contained structurally intact genes for the Leloir pathway enzymes (Chapter 2). These genes appeared to be organized in an operon with the order *galKTE*, which was preceded by a divergently transcribed regulator gene *galR*, and followed by a *galM* gene and the lactose operon *lacSZ*. This *S. thermophilus gal-lac* gene cluster is very conserved in sequence, organization and flanking regions among strains isolated from various fermented products (Chapter 3). Similarly, this gene order is identical to that of *S. salivarius*, which reflects the evolutionary relationship between these bacteria. Results of Northern blot analysis showed that the structural *gal* genes were transcribed weakly by strain CNRZ302, and only in medium containing lactose. However, in a spontaneous galactose-fermenting mutant, designated NZ302G, the *galKTE* genes were well expressed in cells grown on lactose or galactose. In both CNRZ302 and the Gal^+ mutant NZ302G, the transcription of the *galR* gene was induced by growth on lactose. Disruption of *galR* indicated that it functioned as a transcriptional activator of both the *gal* and *lac* operons while negatively regulating its own expression. Sequence analysis of the *gal* promoter regions of NZ302G and nine other independently isolated Gal^+ mutants of CNRZ302 revealed mutations at three positions in the *galK* promoter region (Chapter 2). Moreover, among strains isolated from various fermented products little sequence variation was found between the complete *galR-galK* intergenic regions compared to that of CNRZ302. Only in naturally galactose-fermenting strains single base pair changes in the –10 sequence were found that can be considered *galK* promoter-up mutations (Chapter 3). The upregulation of the *galKTE* promoter seems to suffice for a galactose-fermenting phenotype of *S. thermophilus*. This loss of the ability to ferment galactose can be attributed to the fact that *S. thermophilus* does not need to ferment the galactose moiety via the relative slow Leloir pathway since lactose is always available in excess in milk.

A catabolite responsive element (*cre*) was identified in the promoter of the *lacSZ* operon indicating a possible role for CcpA in regulation of transcription of this operon. To assess the role of CcpA mediated global regulation in *S. thermophilus* the *ccpA* gene was cloned and sequenced (Chapter 4). Western blot analysis showed that the CcpA production itself was sugar-source dependent as indicated by the two-fold higher amount found in glucose-grown cells relative to lactose-grown cells. The observed regulation of the *S. thermophilus* CcpA production appeared to be achieved at the transcriptional level by negative autoregulation as has been found for some but not all other *ccpA* genes in other Gram-positive bacteria. Transcription analysis of the *lacSZ* operon showed relief of repression in the absence of a functional CcpA when cells were grown on lactose. This CcpA-mediated repression did not occur in wild-type cells during growth on galactose, taken up by the same LacS transport system. In strains carrying a disrupted *ccpA* gene lactose transport was increased significantly while lactate production was reduced relative to wild-

type cells. Moreover, a *ccpA*-disruption strain was found to release substantial amounts of glucose into the medium when grown on lactose. Transcription analysis of the *S. thermophilus ldh* gene showed that expression was induced during growth on lactose compared to glucose or growth on galactose, in a CcpA-dependent manner. The *cre* site found in the *ldh* promoter region is situated upstream of the –35 box, which is in agreement with positive control by CcpA. Clearly, CcpA regulated not only lactose uptake and hydrolysis in *S. thermophilus* but also affected the flux to lactate.

The main system for energy generation in lactic acid bacteria is the glycolytic or Embden-Meyerhoff pathway. Global control of carbon metabolism in bacteria is primarily modulated by intracellular concentrations of glycolytic intermediates. The levels of these intermediates depend on the efficiency of metabolic conversion of a certain carbon source. With four sugars tested, glycolytic intermediate steady-state levels were compared in *S. thermophilus* growing on fast (lactose and sucrose) and slow (glucose and galactose) metabolizable carbon sources and for PTS (sucrose) versus non-PTS (glucose, lactose and galactose) sugars (Chapter 5). The boiling water cell pool extraction method for glycolytic intermediates isolation and subsequent HPLC analysis proved to be very reproducible. Nevertheless, some variations were found, reflecting the effect of slight differences in growth phase and history. *S. thermophilus* cells grown on lactose accumulated G6P and F6P with respect to cells grown on the other sugars tested. However, FBP, which is regarded as a strong effector of catabolite repression in Gram-positive bacteria, accumulated to highest steady-state levels in sucrose-grown cells. Lactose-grown cells accumulated FBP to a lesser extent but clear catabolite control was established for cells grown on this sugar. Following glycolysis downward, the first indications of CcpA involvement on intermediate steady-state levels were found for FBP, showing a two-fold reduction in *ccpA* mutant cells grown on lactose or sucrose relative to wild-type cells. Furthermore, glycolytic intermediates accumulate in fast metabolizing cells to favor catabolite control and result in a preference for PTS sugars over permease sugars. However, *S. thermophilus* CNRZ302 grows fastest on lactose as carbon source, probably due to the kinetics of the LacS permease, for which the rate of lactose uptake has been shown to mimic that of PTS's without the drain of the PEP-pool.

The efficiency of glycolytic steps were determined by glycolytic intermediate dynamics analysis in resting cells isolated from various stages of growth (Chapter 6). The influence of catabolite repression was investigated by comparison of the wild-type strain versus a *ccpA* mutant. Wild-type cells isolated from lactose-limiting growth conditions showed decreased lactate production following the transfer to an excess of lactose. However, lactose transport capacity in these cells was unchanged creating an imbalance between lactose import and subsequent conversion in glycolysis. Indicative for this imbalance is the expulsion of the excess of imported carbon in the form of glucose. Without a functional CcpA this imbalance is manifested also during growth in excess lactose conditions. Glycolytic intermediate analysis showed a change in flux through glycolysis coinciding with this transition in growth by the lactose availability level indicated that the efficiency of several glycolytic steps were growth phase dependently regulated. This regulation was lost in *ccpA* mutant cells. Moreover, Northern analysis showed that CcpA acts as a activator of the *pfk-pyk* operon as well as of the *ldh* gene in *S. thermophilus.* These genes encode key-enzymes of glycolysis, thereby establishing the role of CcpA in glycolytic control. These results comply well with previous studies that indicate that lactose uptake and utilization is subject to HPr mediated control that is modulated by the availability of lactose. From FBP downstream in glycolysis, 3PG and PEP accumulation was dependent on the lactose availability, which was lost in *ccpA* mutant cells. However, no CcpA dependent regulation of the other glycolytic

genes was found. In conclusion, CcpA not only fine-tunes lactose uptake and conversion when cells are grown in excess lactose, but is also involved in derepression of transport activity and reduction of glycolytic capacity to allow cellular adaptation to conditions where lactose becomes limiting.

HPr has in many studies been described as the key factor in global control of sugar metabolism in bacteria. The *S. thermophilus pts* operon, encoding HPr and EI, is very conserved in organization and regulation compared to the *pts* operons of other low G+C Gram-positive bacteria (Chapter 7). By over-expressing wild-type *ptsH* and specifically mutated *ptsH* alleles in a *ptsH* deletion strain of *L. lactis* the functional role of *S. thermophilus* HPr was confirmed and specific amino acid residues were identified that are essential for its function in utilization of PTS and non-PTS carbohydrates. However, we hypothesize that the role of HPr in *S. thermophilus* may have been adapted to the preferential lactose metabolism similar to what was found for CcpA in this organism.

In Chapter 8 the results of the previous chapters are discussed to provide more knowledge and insight into the global and specific regulation of lactose metabolism in *S. thermophilus*. The work described in this thesis can provide the basis for metabolic engineering of fermentation properties of this lactic acid bacterium, which is used in many dairy fermentations and is of great commercial importance.

Chapter 10

Nederlandse Samenvatting

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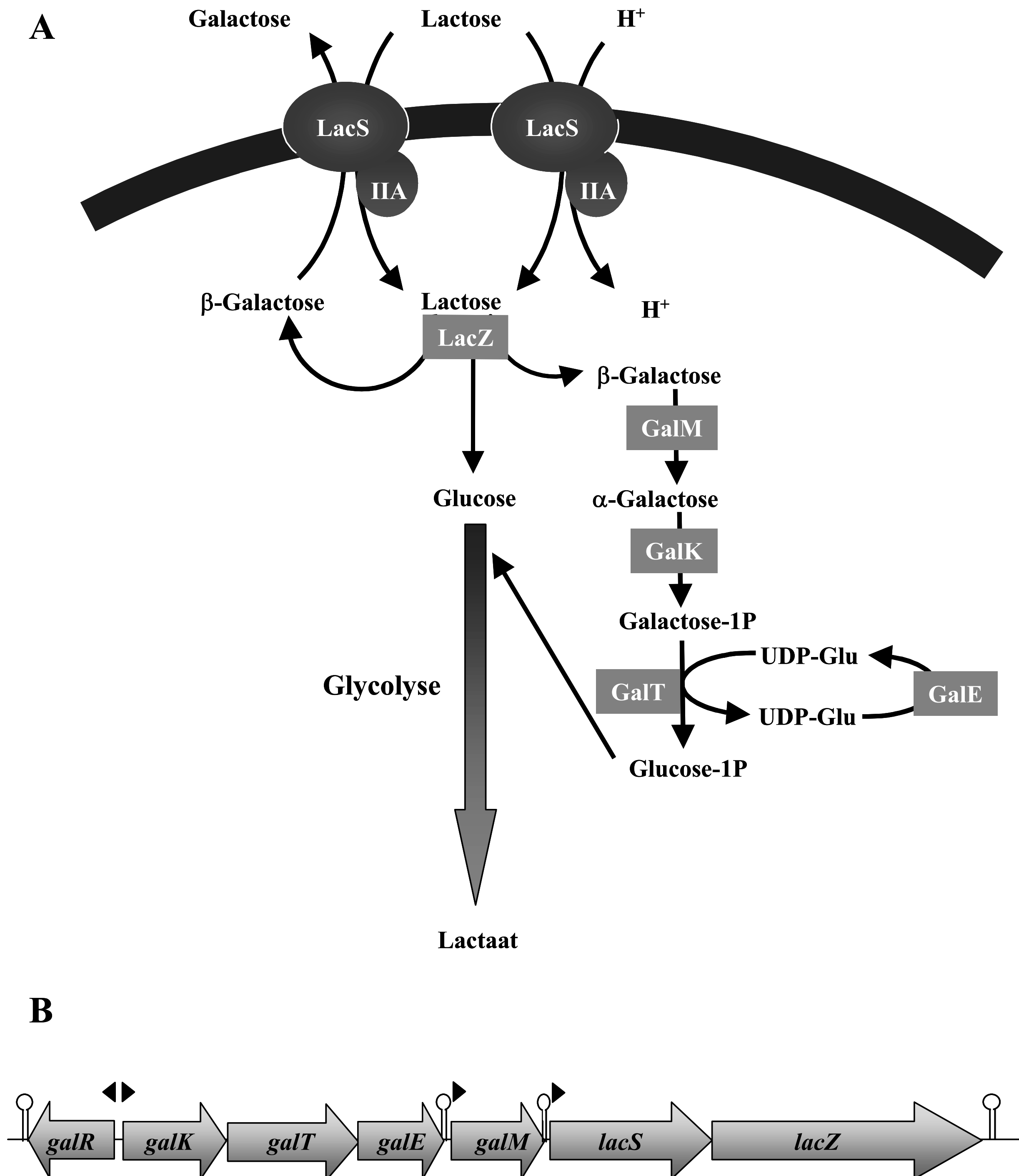
Door bestudering van het algemene suikergebruik van de melkzuurbacterie *Streptococcus thermophilus* en het lactosemetabolisme in het bijzonder, kan men de kennis vergaren om fermentatie-eigenschappen van deze bacterie gericht te veranderen. Een basis hiervoor wordt gelegd in dit proefschrift. Melkzuurbacteriën worden gebruikt voor de productie van zuivelproducten zoals kaas en yoghurt en zijn van groot industrieel belang. Lactose, ofwel "melksuiker", is de voornaamste koolstof- en energiebron in melk en wordt in deze zuivelfermentaties omgezet in lactaat, ofwel melkzuur. Deze verzuring resulteert in de remming van de groei van bederforganismen en om die reden wordt fermentatie van voedingsmiddelen al eeuwen gebruikt als methode voor conservering. Verder dragen melkzuurbacteriën bij aan de smaak, aroma en consistentie van het gefermenteerde product.

Micro-organismen prefereren over het algemeen glucose als favoriete koolstof- en energiebron, maar voor *S. thermophilus* is dit een slecht groeisubstraat. Dit organisme is gespecialiseerd in de fermentatie van lactose en groeit hierop sneller dan op andere suikers. Deze preferentie voor lactose heeft directe consequenties voor de regulatie van het totale suikermetabolisme. In dit proefschrift worden de resultaten beschreven van de studie naar specifieke en globale regulatie van lactose metabolisme in de melkzuurbacterie *S. thermophilus*.

Het lactose/galactose metabolisme in *S. thermophilus*

S. thermophilus wordt voornamelijk gebruikt als starterculture voor de producties van yoghurt en verschillende kaassoorten, waarbij een relatief hoge bereidingstemperatuur gebruikt wordt zoals Mozzarella, Gruyère and Emmental. De voornaamste taak van deze melkzuurbacterie is het snel omzetten van lactose in lactaat. Lactose bestaat uit de twee aan elkaar gekoppelde suikers glucose en galactose, en al decennia lang is bekend dat *S. thermophilus* alleen de glucose gebruikt en niet de galactose. Lactose wordt door *S. thermophilus* in de cel opgenomen door een transport eiwit genaamd de lactose permease LacS. Deze LacS transporter is een speciaal geval. Het herkent en transporteert specifiek galactose moleculen, zowel de suiker alleen maar ook wanneer het gekoppeld is aan andere soorten suikermoleculen als lactose (glucose+galactose) en raffinose (galactose+glucose+fructose). Wanneer de galactose concentraties in de cel hoog genoeg zijn kan LacS kan het galactose ook vanuit de cel het medium in transporteren. Verder heeft het LacS nog een extra gedeelte dat sterk lijkt op een eiwit van een andere soort suikeropname complex dat vooral snel afbreekbare suikers opneemt (zier ook hieronder). Wanneer lactose eenmaal opgenomen is in de cel wordt het in de twee suikers glucose en galactose gesplitst door het β-galactosidase enzym LacZ (Fig. 1A). Glucose kan door de centrale afbraakroute van de cel, glycolyse genaamd, worden omgezet in lactaat waardoor de cel energie genereert voor groei en andere cellulaire processen. Galactose kan niet direct dezelfde afbraakroute volgen als glucose. Galactose moet eerst volgens een andere weg specifiek voor deze suiker, die Leloir-route wordt genoemd, worden omgezet in een product alvorens het verder door de glycolyse kan worden afgebroken. In *S. thermophilus* wordt het galactose echter niet gebruikt maar uitgescheiden in het groeimedium, door dezelfde transporter LacS die de lactose opneemt. Recentelijk zijn in *S. thermophilus* alle genen van de Leloir-route gevonden die normaal gesproken nodig zijn om bacteriën in staat stellen galactose te gebruiken als groeisubstraat. Deze genen zijn gelegen in een cluster (zie hoofdstuk 2). Dit gencluster bevat



Figuur 1. Schematische voorstelling van het lactosemetabolisme in *Streptococcus thermophilus* en de genen die daarbij betrokken zijn. A: de twee transportvormen van de lactosepermease LacS voor lactose en de verdere afbraak van dit suiker. Eenmaal in de cel wordt lactose gesplitst in galactose en glucose door de β-galactosidase LacZ. Glucose wordt via de glycolyse omgezet tot lactaat waar de cel energie uit wint. Galactose kan verder worden afgebroken door de enzymen van de Leloir-route tot een tussenproduct van de glycolyse. De leloir-route voor galactose afbraak bestaat uit de enzymen galactokinase (GalK), galactose-1-phosphate uridylyltransferase (GalT), and UDPglucose 4-epimerase (GalE), die worden gecodeerd door het *gal-lac* gencluster (B). Galactose wordt echter in de meeste *S. thermophilus* stammen naar buiten getransporteerd in een uitwisselingsreactie met lactose.

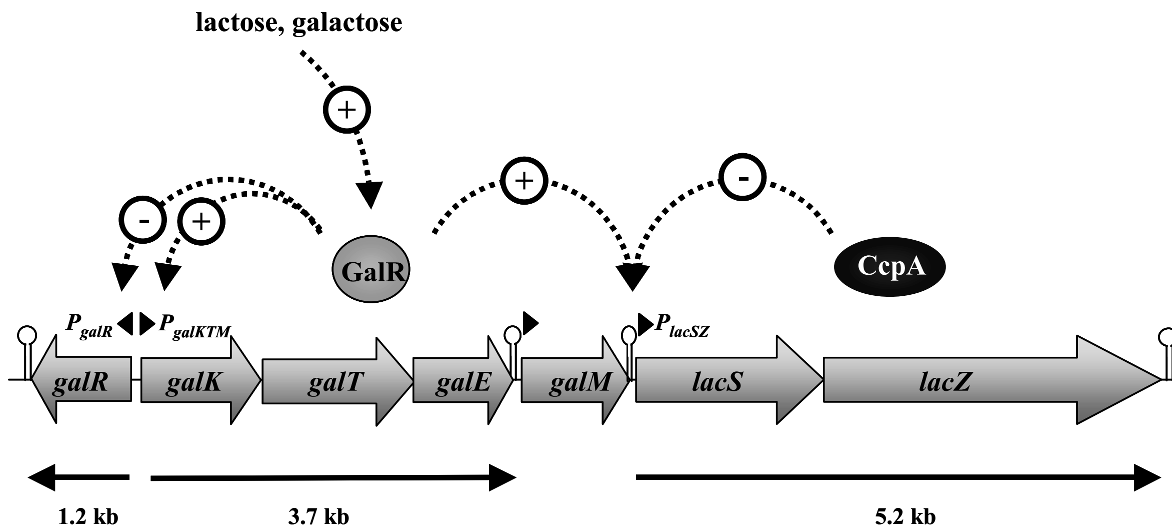
naast alle galactose-genen (*gal*-genen) ook de genen die nodig zijn voor de opname en splitsing van lactose en wordt het *gal-lac* gencluster genoemd (Fig. 1B).

Het *S. thermophilus gal-lac* gencluster

De *gal*-genen van *S. thermophilus* liggen achtereen in de volgorde *galKTE*. Dit is ook de volgorde waarin de enzymproducten van deze genen (GalK, GalT en GalE) hun functie uitoefenen in de Leloir-route voor galactoseafbraak. Het aflezen, (transcriptie), van deze genen gebeurt alleen wanneer galactose of lactose in het groeimedium aanwezig is. Hiervoor is dus een vorm van regulatie nodig waarbij de aanwezigheid van lactose of galactose wordt herkend waarna een signaal voor het activeren van de *gal*-genen wordt gegeven. In *S. thermophilus* en ook in sommige andere streptococcen gebeurt dit via de specifieke galactose-regulatoreiwit GalR (zie hoofdstuk 2)(Fig. 2). Deze activeert een genetische schakelaar, (promoter), waardoor transcriptie van de *gal*-genen plaatsvindt en de cel het vermogen krijgt galactose te fermenteren. Dezelfde GalR activeert ook de promoter van de *lac*-genen (lacSZ). In *S. thermophilus* CNRZ302 zijn deze *gal*-genen dus wel gevonden, en gebleken is dat ze inderdaad ook worden aangezet door GalR wanneer galactose of lactose in het groeimedium aanwezig is. Maar de transcriptie-efficiëntie, ofwel de promoteractiviteit, is heel erg laag waardoor er niet genoeg enzymen van de Leloir-route geproduceerd worden. Dit is waarschijnlijk te wijten is aan een inefficiënte *galKTE* promoter. In een mutant van deze stam die wel galactose kan fermenteren werd in deze promoter een mutatie gevonden waardoor de efficiëntie van deze promoter duidelijk toeneemt. Bij onderzoek in meerdere stammen van *S. thermophilus* die uit verschillende fermentatieproducten werden geïsoleerd werd gevonden dat in al deze stammen het *gal-lac* gencluster op dezelfde manier georganiseerd is en ook een zelfde inefficiënte *galK* promoter bevatten (zie hoofdstuk 3). Verder blijken de *gal* en *lac* genen in al de stammen nagenoeg identiek te zijn en ook de omliggende gebieden in het chromosoom van *S. thermophilus* zijn gelijk. Doordat *S. thermophilus* nagenoeg alleen maar in melkfermentaties gebruikt en gevonden wordt heeft het blijkbaar het vermogen om galactose te fermenteren verloren, hetgeen overigens met een enkele mutatie weer herkregen kan worden (zie hoofdstuk 2).

Globale suikerregulatie.

Naast suiker specifieke regulatie, zoals voor het gebruik van galactose, hebben micro-organismen ook een vorm van globale regulatie. Overleving en uitgroei van de soort wordt bij deze organismen hoofdzakelijk bepaald door de relatieve groeisnelheid van die populatie. Welke soort het snelst kan delen zal de concurrerende organismen overwoekeren en zo een dominante positie verkrijgen in de betreffende ecologische niche. Hiervoor is het belangrijk dat de cel de beschikbare energie zo goed mogelijk besteedt, en dat betekent dat de cel er voor zal kiezen om eerst de suikers uit het medium op te nemen die zo snel mogelijk energie kunnen genereren voor groei. Hiervoor is een complex regulatienetwerk aanwezig waardoor een “snel” metaboliseerbaar suiker in het groeimedium het gebruik van een “langzamer” metaboliseerbaar suiker blokkeert. Dit fenomeen heet catabolietrepressie (CR) en verschillende groepen van bacteriën hebben hier verschillende regulatiemechanismen voor ontwikkeld. De belangrijkste regulatiecomponenten in melkzuurbacteriën zijn de eiwitten CcpA en HPr (Fig. 3). Hoofdstuk 4 behandelt de invloed van CcpA in het lactose metabolisme van *S. thermophilus*. CcpA is de centrale transcriptionele regulator in CR en binding aan een “catabolite responsive element” (*cre*) in promotoren zorgt voor repressie dan



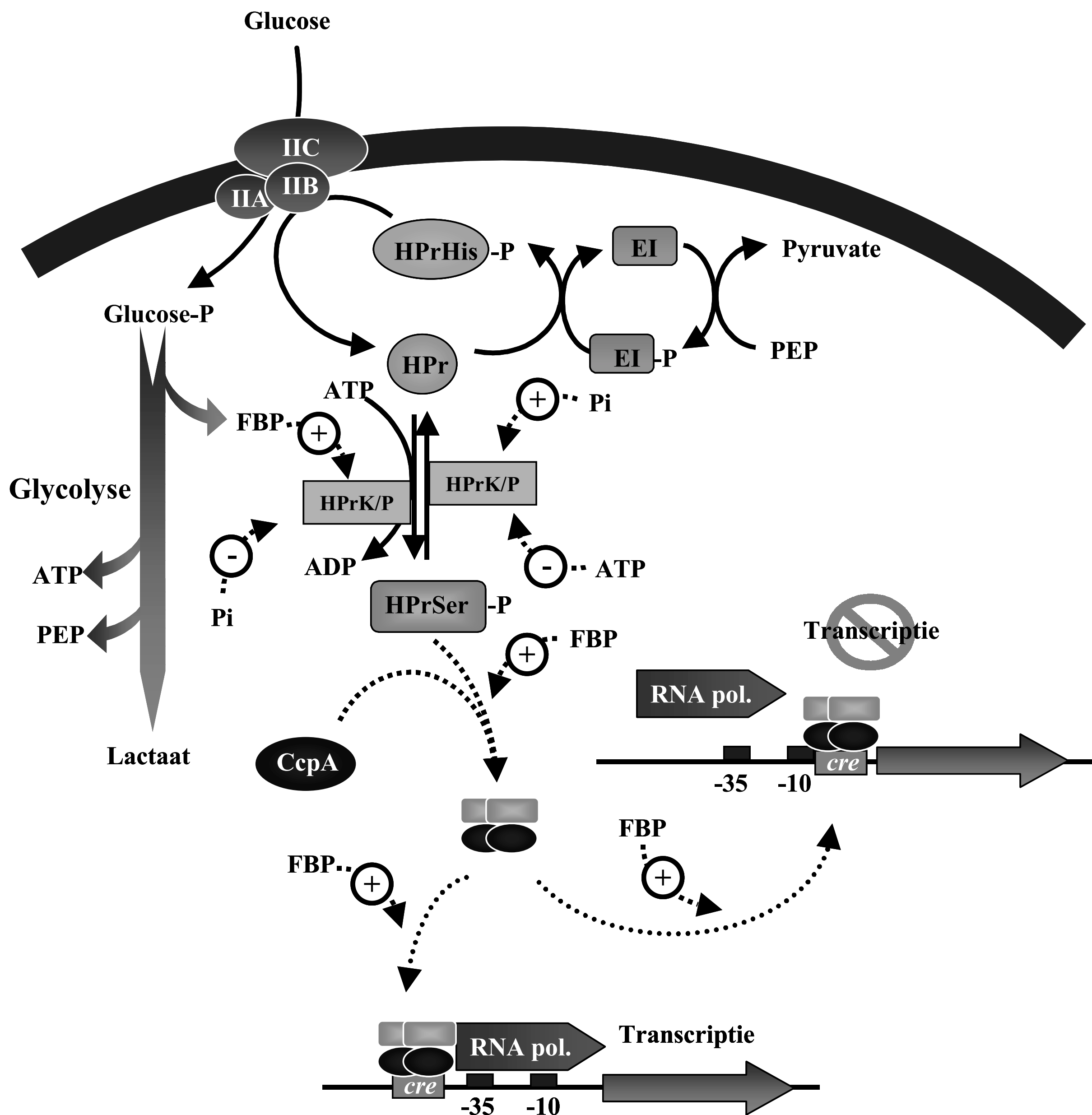
Figuur 2. Regulatie van het *gal-lac* gencluster in *Streptococcus thermophilus*. De aanwezigheid van lactose of galactose in het groeimedium geeft een signaal in de cel waardoor regulator GalR de transcriptie van de *galKTE* en *lacSZ* genen activeert, en tevens de transcriptie van het eigen *galR* gen represeert. De gevonden transcripten zijn aangevenen met pijlen onder de genen. De transcriptie van *lacSZ* promoter wordt verder nog door CcpA gereguleerd (lees verder in de tekst).

wel activatie van transcriptie van bepaalde genen, in respons op de aanwezigheid van een bepaald suiker in het groeimedium. In de *lacS* promoter van *S. thermophilus* bevind zich zo'n *cre*-sequentie en CcpA bleek tijdens groei op lactose hieraan te binden en voor repressie van de *lac*-genen te zorgen. Deze repressie werd niet waargenomen tijdens groei op glucose of galactose. In een mutant van stam CNRZ302 die geen CcpA meer kan vormen was deze repressie op de *lacSZ* transcriptie niet meer waarneembaar. Dit resulteerde in sterk verhoogde opname en splitsing van lactose, terwijl de groei op lactose sterk vertraagd was. Verder werd gevonden dat deze mutant veel minder lactaat produceerde dan de ouder stam en een grote hoeveelheid glucose in het medium uitscheidde. Bovendien bleek dat de promoter van het gen dat verantwoordelijk is voor de lactaat productie (*ldh*) ook een *cre*-sequentie bevatte. Transcriptie studies wezen uit dat CcpA de eigenschap om lactaat te produceren ook reguleert, maar in dit geval wordt deze activiteit niet gerepresseerd maar geactiveerd. Blijkbaar is CcpA nodig in *S. thermophilus* om de opname van lactose af te stemmen op de productie van lactaat, om daardoor zo efficiënt mogelijk op deze suiker te kunnen groeien (zie hoofdstuk 4).

Transport en glycolyse

Lactose is dus de geprefereerde koolstof- en energiebron voor *S. thermophilus* maar wanneer opname niet wordt afgestemd op de capaciteit van de glycolyse dan raakt het metabolisme van de cel ontregelt. Er moet dus een vorm van communicatie zijn tussen de capaciteit van de glycolyse en het opnamesysteem van een bepaalde suiker. In melkzuurbacteriën speelt het eiwit HPr hierin een belangrijke rol. Voor de meeste bacteriën, buiten *S. thermophilus*, is glucose de geprefereerde koolstof- en energiebron. Deze suiker wordt veelal opgenomen door een zeer efficiënt suiker opnamesysteem, genaamd fosfo-enolpyruvaat: fosfotransferase systeem (PTS) (zie Fig. 3). De zo opgenomen suiker wordt na transport door hetzelfde systeem geactiveerd voor afbraak in de glycolyse door fosforylering,

waarbij de fosfaatgroep via een keten van andere eiwitcomponenten, waaronder HPr, wordt gedoneerd. Deze fosfaatgroep komt oorspronkelijk van fosfoenolpyruvaat, een tussenproduct van de glycolyse, vanwaar het wordt doorgegeven aan het PTS via enzym I (EI) en HPr. Het HPr wordt tijdens dit proces op een specifieke plaats namelijk residu histidine 15 (P-His-HPr). Een ander belangrijk tussenproduct van de glycolyse, fructosebisfosfaat (FBP) geeft aan wat het energie niveau in de cel is. De FBP concentratie in de cel is afhankelijk van de snelheid waarmee een bepaald suiker gemetaboliseerd kan worden en is daarmee een primair



Figuur 3. Schematische weergave van P-Ser-HPr/CcpA-gemedieerde catabolietrepressie in Gram-positive bacteriën. Snel metaboliseerbare suikers (zoals glucose) worden veelal opgenomen via een PTS waardoor het glycolytische intermediair fructose-1,6-bisfosfaat zich ophoopt wat resulteert in de activatie van HPrK en vervolgens vorming van P-Ser-HPr. Het P-Ser-HPr/CcpA complex reguleert transcriptie van verschillende genen door binding aan *cre*-sequentie in de promoters van deze genen. Afhankelijk van de positie van de *cre*-sequentie in de promoters van deze genen leidt binding tot transcriptionele repressie dan wel activatie.

signaal voor CR. Bij groei op "snelle" suikers hoopt FBP zich in de cel op waardoor een eiwit wordt geactiveerd, het HPr-kinase, dat ook fosforylering van HPr verzorgt, maar nu specifiek op het residu serine 46. Deze vorm van HPr, P-Ser-HPr complexeert met CcpA waardoor deze globale transcriptionele regulator actief wordt en zo expressie van genen voor afbraak van "langzame" suikers blokkeert en specifieke genen die coderen voor glycolytische enzymen activeert. Het is duidelijk dat de tussenproducten van de glycolyse (glycolytische intermediairen) belangrijke indicatoren zijn voor de energiestatus van de cel en als signaalmoleculen kunnen fungeren in regulerende processen.

In hoofdstuk 5 wordt een methode beschreven om de glycolytische intermediairen te kunnen meten. Allereerst moeten deze intermediairen uit de cel geïsoleerd worden en omdat ze een zeer korte levensduur hebben, moet dit erg snel gebeuren. Door cellen in kokend water op te nemen worden de enzymen van de glycolyse momentaan geïnactiveerd waardoor conversies van de meeste intermediairen worden stopgezet. Vervolgens kan de concentratie van die intermediairen worden gemeten in specialistische apparatuur. Zo blijkt dat de concentraties van de meeste glycolytische intermediairen in de cel variëren met de soort suiker waarop *S. thermophilus* groeit. Zo zijn de concentraties van de vroege intermediairen glucose-6-fosfaat (G6P), fructose-6-fosfaat (F6P) en FBP lager wanneer op langzame suikers worden gegroeid dan wanneer op snelle suikers worden gegroeid. Voor G6P en F6P is dit onafhankelijk van de aanwezigheid van een functioneel CcpA eiwit terwijl de concentratie van FBP hier wel afhankelijk van blijkt te zijn. ATP is geen intermediair van de glycolyse maar de algemene energiedrager in de cel, en de intracellulaire concentratie varieert ook met het groeisubstraat op een CcpA afhankelijke manier, vergelijkbaar met FBP. In een soort gelijke serie experimenten werd gekeken hoe snel de glycolytische intermediairen gevormd konden worden wanneer de cellen in hoge en lage lactoseconcentraties gegroeid werden (zie hoofdstuk 6). Duidelijk werd dat de concentraties van de glycolytische intermediairen veel lager waren in cellen die in lage lactose concentraties groeiden dan in cellen die in hoge lactose concentraties groeiden. De vormingssnelheid van de intermediairen gaf aan dat de capaciteit van het bovenste deel van de glycolyse niet verschilde maar dat vanaf de FBP vormende stap door het enzym fosfofructokinase (PFK) de glycolysecapaciteit hoger was in de cellen die in hoge lactose concentraties gegroeid waren en dat dit afhankelijk was van CcpA. Het niveau van transcriptie werd vervolgens geanalyseerd van alle genen die coderen voor de glycolytische enzymen coderen en hierbij bleek dat naast het eerder gevonden *ldh*-gen, ook het *pfk*- en het *pyk*-gen geactiveerd werden door CcpA (Fig. 4). Deze activatie bleek ook afhankelijk van de soort en concentratie suiker in het groeimedium. Uit deze experimenten kon worden geconcludeerd dat naast de opname en hydrolyse van lactose ook de glycolyse op verschillende snelheid bepalende stappen gereguleerd wordt door de concentratie lactose in het groeimedium en dat dit afhankelijk is van CcpA.

De functie van HPr in het lactosemetabolisme van *S. thermophilus*.

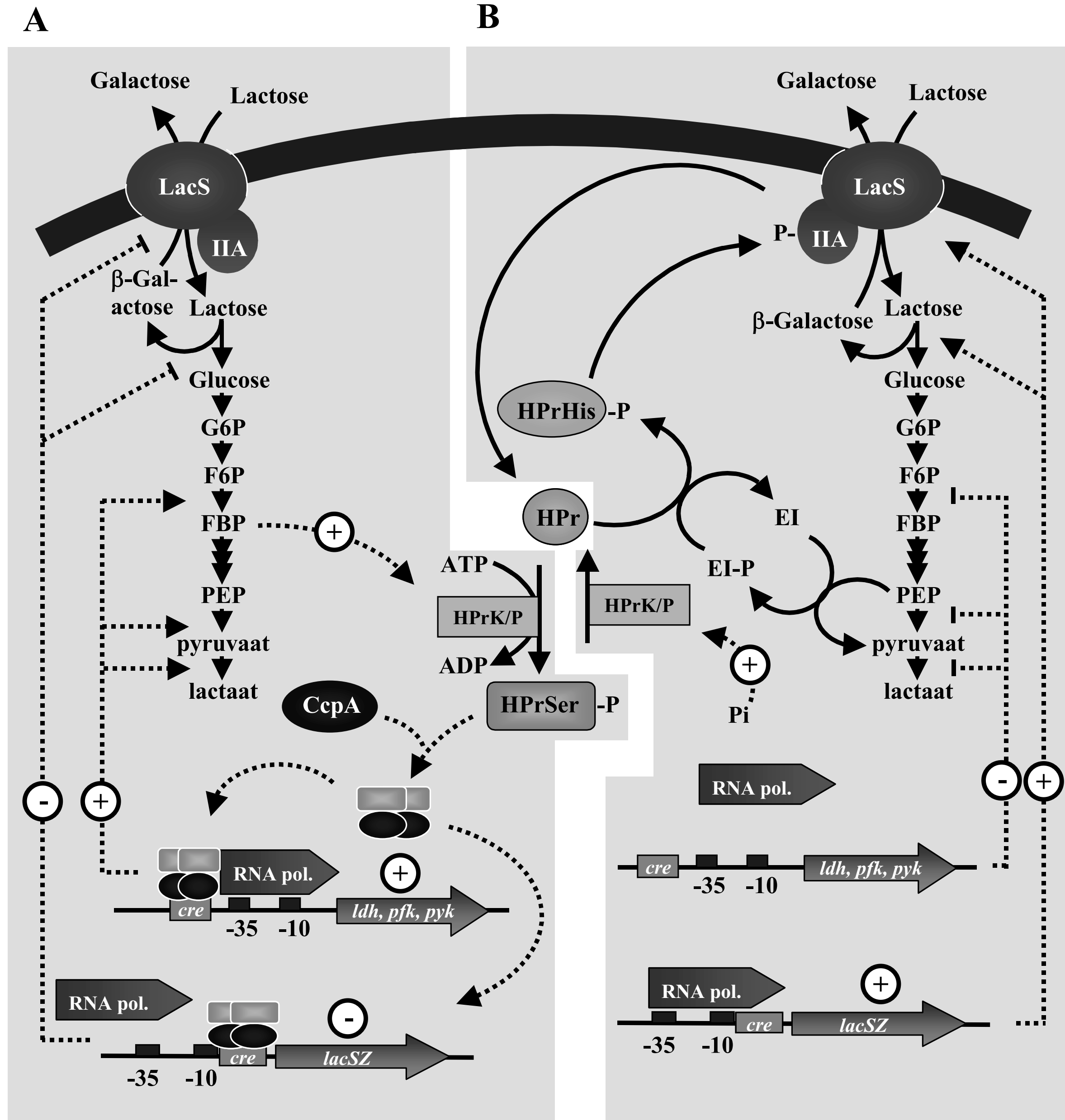
In samenwerking met CcpA is HPr de belangrijkste globale regulator van het koolstofmetabolisme in melkzuurbacteriën. Het levert niet alleen de energie voor PTS transport (in de vorm van een hoog energetische fosfaatbinding; P-His-HPr), maar ook activeert het CcpA voor transcriptionele regulatie (P-Ser-HPr), afhankelijk van welk residu van HPr gefosforyleerd is. Hier ligt al een subtiele vorm van regulatie daar beide gefosforyleerde HPr's voortkomen uit dezelfde HPr-populatie. Nu zijn voor beide gefosforyleerde vormen nog meer functies beschreven. Zo wordt gespeculeerd dat P-Ser-HPr een rol speelt bij de modulatie van transportactiviteiten van zowel PTS systemen als niet-PTS

systemen (permeases). Ook zou P-Ser-HPr bepaalde enzymen activeren die suikerfosfaten (onder andere glycolytische intermediairen) defosforyleren waarna de vrije suiker door een nog onbekende transporter de cel uit wordt gepompt. Via deze mechanismen kan de cel een overschot aan glycolytische intermediairen (hetgeen toxisch is voor de cel) voorkomen dan wel wegwerken. Echter harde bewijzen voor deze rol van P-Ser-HPr moeten nog geleverd worden. Wel duidelijk is dat P-His-HPr in *S. thermophilus* nog een tweede rol heeft naast de rol als fosfo-donor in PTS transport. De lactose permease LacS van *S. thermophilus* bevat een extra domein dat niet voorkomt bij andere vergelijkbare permeases. Dit extra domein bevindt zich aan de binnenkant van de cel en lijkt sterk op het (IIA) domein van het PTS systeem dat in suikertransport gefosforyleerd wordt door P-His-HPr. Ook het IIA domein van LacS kan door P-His-HPr gefosforyleerd worden, alleen leidt dit nu niet tot fosforylering van het getransporteerde lactose molecuul, maar resulteert in de modulatie van de transportactiviteit van LacS. Zoals boven beschreven transporteert LacS lactose de cel in en transporteert gelijktijdig galactose de cel uit in een zogenaamde uitwisselingsreactie. Door fosforylering van het IIA domein van LacS wordt deze uitwisselingsreactie gestimuleerd. Nu is recentelijk bekend geworden dat de gefosforyleerde HPr-populatie voornamelijk in de serinegefosforyleerde vorm is wanneer *S. thermophilus* in hoge lactose concentraties groeit (Fig. 4). Onder deze condities wordt de import van lactose nauwkeurig afgestemd op de capaciteit van de glycolyse waarbij de concentratie van het glycolytische intermediair FBP (via P-Ser-HPr en CcpA) als primaire indicator dient voor de mate van transportremming en glycolyse versnelling met als doel zo snel mogelijk te kunnen groeien. Wanneer de lactose concentratie in het medium heel laag wordt, neemt de concentratie FBP in de cel ook af en wordt de hoeveelheid P-Ser-HPr sterk verminderd. Hierdoor worden de *lac*-genen niet meer gerepresserd en de glycolytische genen niet meer geactiveerd. Onder deze omstandigheden is P-His-HPr de dominante vorm en wordt ook de uitwisselingsreactie van LacS gestimuleerd. Zo wordt de transportcapaciteit van *S. thermophilus* voor lactose sterk verhoogd om de kleine hoeveelheid beschikbare lactose in het medium toch nog op te kunnen nemen.

De rol van HPr in globale suikerregulatie

Om rol van HPr in de globale regulatie van het suikermetabolisme in *S.thermophilus* te kunnen onderzoeken werd het gen geïsoleerd dat voor HPr codeert. Direct achter dit *ptsH* gen ligt het gen dat codeert voor EI, het *ptsI* gen, en beide genen staan onder de controle van de *ptsH* promoter. Deze organisatie van het zogenoemde *ptsHI* operon is al in vele verschillende soorten bacteriën gevonden en is dus niet uniek voor *S. thermophilus*. Ook de transcriptieactiviteit van de *ptsH* promoter en de soorten transcripten die daarvan geproduceerd worden verschilt niet veel. Analoog aan wat is gevonden in andere bacteriën als *Escherichia coli* en *Streptococcus salivarius* wordt de *ptsH* promoter van *S. thermophilus* niet sterk gereguleerd door het soort suiker in het groeimedium. Er zijn aanwijzingen dat condities als groeisnelheid, osmolariteit en pH van het medium een indirect effect hebben op de activiteit van de *ptsH* promoter. Hoewel er geen *cre* in de *ptsH* promoter werd gevonden was de promoteractiviteit in de $CcpA^-$ stam toch wat lager dan in de oudertam. Omdat de $CcpA^-$ stammen sterk vertraagt zijn in groei zou dit een indirect effect kunnen hebben op de *ptsH* promoteractiviteit wat een verklaring kan zijn voor de gevonden CcpA afhankelijkheid. Om dieper op de functionele rol van HPr in te kunnen gaan werd getracht om van *S. thermophilus* een stam te maken die geen HPr meer produceert. Verder werden mutanten van HPr gemaakt met specifieke aminozuur veranderingen. Door nu deze mutanten te produceren in de HPr^- stam kan bestudeert worden wat de functie van HPr en van de specifieke

aminozuurresiduen is in *S. thermophilus*. Om het *ptsH* in *S. thermophilus* gen functioneel uit te schakelen werd een methode gebruikt die eerder succesvol werd toegepast in *Lactococcus lactis*. Na veelvuldige pogingen onder verschillende condities (waaronder verschillende soorten suikers als groeisubstraat) werd geen *ptsH* knock-out gevonden.



Figuur 4. Schematisch overzicht van de voorgestelde regulatie van het lactosemetabolisme in *Streptococcus thermophilus*. A Wanneer lactose in overvloed is wordt de import van lactose nauwkeurig afgestemd op de capaciteit van de glycolyse waarbij de concentratie van het glycolytische intermediair FBP (via P-Ser-HPr en CcpA) als primaire indicator dient voor de mate van transportremming en glycolyse versnelling met als doel zo snel mogelijk te kunnen groeien. B: Wanneer de lactose concentratie in het medium heel laag wordt, neemt de concentratie FBP in de cel ook af en wordt de hoeveelheid P-Ser-HPr sterk verminderd. Hierdoor worden de *lac*-genen niet meer gerepresserd en de glycolytische genen niet meer geactiveerd. Onder deze omstandigheden is P-His-HPr de dominante vorm en wordt ook de uitwisselingsreactie van LacS gestimuleerd. Zo wordt de transportcapaciteit van *S. thermophilus* voor lactose sterk verhoogd om de kleine hoeveelheid beschikbare lactose in het medium toch nog op te kunnen nemen.

Er waren aanwijzingen dat er geen genetische beperkingen waren voor het verkrijgen van de *ptsH* knock-out, en dat een verklaring dus in de fysiologische rol gezocht moest worden. Al eerder werd genoemd dat *S. thermophilus* maar op weinig verschillende suikers kan groeien. Om de HPr$^-$ stam te selecteren kan natuurlijk niet op een PTS suiker gegroeid worden omdat P-His-HPr in deze stam niet meer gevormd kan worden. Er blijven dus maar twee suikers over om de *ptsH* mutant wel op te selecteren, nl. lactose en glucose. HPr speelt een belangrijke regulerende rol in het lactosemetabolisme, zowel op transcriptieniveau via CcpA als op transportniveau via LacS. Het uitschakelen van HPr zou dus een sterk nadeel op kunnen leveren voor groei op lactose, waardoor de *ptsH* knock-out stam niet verkregen kon worden. Hoe glucose in *S. thermophilus* over het celmembraan getransporteerd wordt is nog onduidelijk, behalve de constatering dat het geen PTS systeem betreft. Verder is bekend dat er een relatie is tussen glucose metabolisme en CR via CcpA, en dus waarschijnlijk ook via HPr. Hoewel van vele bacteriën een HPr$^-$ stam verkregen is, blijft het onduidelijk of dergelijke stam van *S. thermophilus* wel levensvatbaar zou zijn. In *S. thermophilus* heeft CcpA een aangepaste rol gekregen, welke is toegespitst op groei op lactose als primaire koolstof- en energiebron en HPr zou ook een aangepaste functie gekregen kunnen hebben in *S. thermophilus* waardoor de afwezigheid van dit eiwit lethaal zou kunnen zijn.

Om toch nog de centrale rol van HPr te kunnen bestuderen werden *S. thermophilus* HPr mutanten tot expressie gebracht in een HPr$^-$ stam van *L. lactis*. Eerst werd bekeken of het HPr van *S. thermophilus* wel in staat was om de HPr van *L. lactis* te vervangen. Dit bleek zo te zijn daar deze stam weer gewoon op PTS suikers kon groeien. De veranderde aminozuren in de *S. thermophilus* HPr mutanten waren verkozen omdat ze specifiek een rol zouden spelen in PTS transport (residu His-15, Arg-17 en Ile-47) of in CR (Ser-46). Zo bleek een HPr mutant waarvan Ser-46 veranderd was in een residu dat biochemisch gezien op een gefosforyleerde serine lijkt sterk gereduceerd te zijn in groei op alles geteste suikers. Dit wordt waarschijnlijk veroorzaakt doordat de cel maximale CR induceert vele systemen voor suikergebruik worden geremd. De residuen His-15, Arg-17 en Ile-47 zijn om verschillende reden cruciaal voor PTS transport. His–15 omdat dit residu de fosfaatgroep overdraagt aan het PTS, Arg-17 omdat dit residu de overgangsvorm van P~HPr naar P~PTS stabiliseert en Ile-47 omdat deze betrokken is bij de binding en herkenning van HPr en de PTS transporter. Een verassende observatie was dat deze residuen ook belangrijk waren voor groei op galactose, een niet-PTS suiker. Wel was uit eerdere studies bekend dat de *gal*-genen in *L. lactis* gerepresseerd werden door CcpA wanneer een “snel” metaboliseerbaar suiker (zoals glucose) in het groeimedium aanwezig was. Om te onderscheiden of de effecten van de HPr mutanten op het gebruik van galactose op transport- of transcriptieniveau plaatsvonden werd van deze mutanten zowel de GalA transportactiviteit als de transcriptieactiviteit van de *galA*-promoter gemeten. Hieruit bleek dat de HPr mutanten hun effect hadden op de transport activiteit van GalA en niet op de regulatie van transcriptie van het *galA* gen via CcpA. HPr blijkt een activator te zijn voor galactose transport maar hoe dit precies werkt is nog niet duidelijk. De galactose permease GalA behoort tot dezelfde transporterfamilie als LacS uit *S. thermophilus,* maar bevat in tegenstelling tot LacS geen IIA domein voor de interactie met HPr. Verder onderzoek moet uitwijzen wat het mechanisme is dat de rol van HPr in de galactose metabolisme van *L. lactis* beschrijft. Uit onderzoek komt duidelijk naar voren dat HPr zeer algemeen voorkomt in de bacteriewereld en dat het veelal dezelfde functies vervult. Uitzonderingen hierop zijn de specifieke functies van HPr in het lactosemetabolisme van *S. thermophilus* en het galactosemetabolisme van *L. lactis*.

Het onderzoek beschreven in dit proefschrift heeft geleid tot de identificatie en karakterisatie van de hoofdrolspelers in de globale suikerregulatie en de specifieke regulatie van het lactosemetabolisme in *S. thermophilus*. Het *gal-lac* gencluster is zeer geconserveerd in *S. thermophilus* en in verreweg de meeste stammen worden de *gal*-genen niet meer afgelezen. Omdat lactose in melk, het natuurlijke goeimedium van *S. thermophilus*, altijd in overvloed aanwezig is, is de *gal*-promoter waarschijnlijk door mutaties minder actief geworden zodat alleen nog maar de glucose deel van de lactose verbruikt wordt. Het levert de cel een hogere groeisnelheid wanneer de galactose, door de relatief langzame Leloir-route, niet meer verbruikt wordt, maar in de LacS gemedieerde uitwisselingsreactie wordt gebruikt voor de opname van een nieuw lactosemolecuul. Dus niet alleen de transcriptie van de *gal*-genen maar ook de activiteit van de LacS transporter heeft zich hierop aangepast. De preferentie voor lactose als koolstof- en energiebron wordt teruggevonden in de intracellulaire concentraties van specifieke glycolytische intermediairen en gekenmerkt door de inzet van de globale suikerregulatie componenten CcpA en HPr voor de regulatie van het lactosemetabolisme. Door de optimale afstemming van lactose opname en de verwerking tot lactaat wordt maximale groei op deze suiker gehandhaafd, ook wanneer de lactose concentratie in het medium daalt. Naast hun wetenschappelijke waarde leveren deze bevindingen de basis om gericht te kunnen ingrijpen in het suikermetabolisme van *S. thermophilus* en zo de fermentatie-eigenschappen van dit organisme gericht te veranderen.

Curriculum Vitae

Patrick van den Bogaard werd geboren op 11 november 1970 in Mook. De middelbare schoolopleiding begon hij aan het Stedelijke Scholengemeenschap Nijmegen waar hij in 1989 het HAVO-diploma behaalde. In hetzelfde jaar startte hij met de studie Biotechnologie aan het Hoger Laboratorium onderwijs (HLO) in Nijmegen. De afstudeerstage werd volbracht bij bij de toenmalige werkgroep Moleculaire Genetica, afdeling Biofysische Chemie van het NIZO food research te Ede (prof. dr. O.P. Kuipers). In juni 1993 werd het HLO-diploma behaald.
In 1993 begon hij de studie Biologie aan de Katholieke Universiteit Nijmegen met als oriëtatie de fysiologische/biochemische richting. Tijdens de doctoraalfase verrichtte hij onderzoek bij de afdeling Biochemie, faculteit der medische wetenschappen, KUN (prof. dr. J.J.H.H.M. de Pont, drs. M. de Moel) en bij de afdeling Moleculaire biologie vakgroep Moleculaire biologie en celbiologie, faculteit der natuurwetenschappen, KUN (prof. dr. R.N.H. Konings, drs. K. Dechering). In oktober 1996 werd het doctoraaldiploma behaald.
In januari 1997 startte hij zijn promotie-onderzoek bij NIZO food research te Ede binnen de sectie Microbial ingredients (prof. dr. W.M. de Vos, prof. dr. O.P. Kuipers, dr. M. Kleerebezem). In 1998 werd dit project opgenomen binnen het Wageningen Centre for Food Sciences (WCFS). Vanaf 15 maart 2002 is hij werkzaam als postdoctoraal onderzoeker aan de Rijksuniversiteit Groningen binnen de afdeling Eukaryote Microbiologie.

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