Carbon Catabolite Repression and Global Control of the Carbohydrate Metabolism in *Lactococcus lactis*

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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dr. C.M. Karssen,
in het openbaar te verdedigen
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des namiddags te 13.30 uur in de Aula.

Everything should be made as simple as possible, but not simpler.

Albert Einstein.

Aan Isa

Aan mijn ouders

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Stellingen

1 Ye en co werkers gaan in hun experimenten ten onrechte uit van de afwezigheid van HPr moleculen terwijl enzyme I, dat in een grote ondermaat ten opzichte van HPr aanwezig is, verondersteld wordt in ruime hoeveelheden aanwezig te zijn.

Ye, J. J., J. Reizer, and M. H. Saier Jr. 1994. Regulation of 2-deoxyglucose phosphate accumulation in *Lactococcus lactis* vesicles by metabolite-activated, ATP-dependent phosphorylation of serine-46 in HPr of the phosphotransferase system. Microbiology **140**:3421-3429
Kohlbrecher, D., R. Eiserman, and W. Hengstenberg. 1992. Staphylococcal phosphoenolpyruvate-dependent phosphotransferase system: molecular cloning and nucleotide sequence of the *Staphylococcus carnosus ptsI* gene: expression and complementation studies of the product. J. Bacteriol. **174**:2208-2214.

2 Het observeren van cAMP-afhankelijke kataboliet repressie in Gram-positieve bacteriën berust waarschijnlijk eerder op een gedegen literatuurkennis van de wetenschapper dan op een daadwerkelijk bestaand fenomeen.

Garro, M.S., de Valdez, G.F., G. Oliver, and G.S. de Giori. 1996. Influence of carbohydrates on the α-galactosidase activity in *Lactobacillus fermentum*. Curr. Microbiol. **33**:302-305.
Poolman, B., A.J. Driessen, and W.N. Konings. 1987. Regulation of arginine-ornithine exchange and the arginine deiminase pathway in *Streptococcus lactis*. J. Bacteriol. **169**:5597-5604.
Saier, M.H. Jr., S. Chauvaux, J. Deutscher, J. Reizer, and J.J. Ye. 1995. Protein phosphorylation and regulation of carbon metabolism in Gram-negative versus Gram-positive bacteria. Trends Biochem Sci. **20**:267-271.

3 De grootte van het SacR eiwit zoals beschreven door Siegers en Entian bewijst dat de interpretatie van sequentiedata minstens zo belangrijk is als de presentatie ervan.

Siegers, K., and K. D. Entian. 1995. Genes involved in immunity to the lantibiotic nisin produced by *Lactococcus lactis*. Appl. Environ. Microbiol. **61**:1082-1089.

Dit proefschrift, Hoofdstuk 2.

4 Glucoserepressie is meer dan katabolietrepressie.

Dit proefschrift.

5 Het feit dat de metereologie zich als enige wetenschap serieus aan voorspellingen waagt kan gezien worden als bewijs voor het belang van een goede communicatie tussen wetenschap en publiek.

6 Ter bevordering van de reproduceerbaarheid is het, zeker binnen de exacte wetenschap, aan te bevelen niet met twee maten te meten.

7 De nieuwe ISO certificaten van de keizer dragen er in elk geval toe bij dat de verleners van deze certificaten er warm aangekleed bij lopen.

8 Een goede discussie, voor, tijdens en na een wetenschappelijk experiment, is het zout in de pap.

9 De efficiëntie van telewerken is omgekeerd evenredig met de afstand die overbrugd moet worden tijdens de communicatie tussen de werkgever en de werknemer.

10 Een betere harmonisatie van het geschiedenisonderwijs in de verschillende Europese landen zou een grotere bijdrage leveren aan de onderlinge integratie dan de invoering van een gemeenschappelijke munt, tegen een fractie van de kosten.

Stellingen behorende bij het proefschrift
"Carbon Catabolite Repression and Global Control
of the Carbohydrate Metabolism in
Lactococcus lactis".

Evert Luesink, Wageningen, 14 december 1998.

Voorwoord

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Isa, même si aucun mot ne suffit à décrire tout ce que tu représentes pour
moi, je tiens à te remercier pour ta patience, tes encouragements et ton amour.

Evert

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CHAPTER 1

General Introduction

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General introduction

Lactic acid bacteria.

Lactic acid bacteria represent a group of Gram-positive bacteria comprising the genera *Lactococcus*, *Lactobacillus*, *Leuconostoc* and *Streptococcus*. These bacteria are widely used in dairy fermentations where their main function is the conversion of the milk sugar lactose into lactic acid, which conserves the food-product and prevents the growth of pathogenic and spoilage bacteria (Venema *et al.*, 1996). Furthermore, these bacteria may contribute to the texture of the fermented product by the production of extracellular polysaccharides which enhance the viscosity. Finally, the hydrolysis of the milk protein casein, to peptides and free amino acids followed by their further modifications catalyzed by proteolytic enzymes and amino acid convertases of lactic acid bacteria contribute to the taste and the aroma of the final product. Since industrial dairy fermentations are used on a large scale world-wide, their economic value is considerable.

Bacteria belonging to the genus *Lactococcus lactis* are often used in starter cultures for the fermentation of different dairy products e.g. cheese, buttermilk or butter. The two most frequently used subspecies are *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* that can be distinguished by differences in specific DNA sequences including those encoding 16S rRNA (Godon *et al.*, 1992). *L. lactis* is capable of converting a limited number of mono- and disaccharides to L-lactate via a homolactic fermentation. The growth under anaerobic conditions and the absence of cytochromes reduce the possibility to generate energy by the reduction of external electron acceptors and account for the limited metabolic capacities of *L. lactis*. As a consequence, *L. lactis* can only generate energy via either substrate level phosphorylation or via a solute gradient that yields ATP through the action of a membrane associated ATPase (Konings *et al.*, 1995). The glycolysis is the main pathway by which *L. lactis* generates energy via substrate level phosphorylation, catalyzed by phosphoglycerate kinase and pyruvate kinase. Another example of the generation of ATP via substrate level phosphorylation is the arginine – deiminase pathway by which *L. lactis* subsp. *lactis* is capable of metabolizing arginine to ornithine, ammonia and carbon dioxide (Poolman *et al.*, 1987). Finally, the malolactic and citrolactic fermentation are metabolic pathways, operating in some *L. lactis* strains, where energy is generated based on a solute gradient (Renault *et al.*, 1988, Hugenholtz, 1993).

In industrial dairy fermentations the conversion of sugars to L-lactate via the glycolysis is main way for *L. lactis* to generate energy. In view of the economic importance of these fermentations the carbohydrate metabolism of *L. lactis* has been the subject of considerable research aimed at a better understanding of the processes involved (de Vos, 1996). The goal of the work described in this thesis is to gain insight in the control of the sugar metabolism in *L. lactis* in order to optimize dairy fermentations and provide tools for metabolic engineering strategies aimed at the control of metabolic fluxes.

In recent years, significant progress has been made in the characterization of sugar utilization in bacteria and an overview of relevant literature on this subject with special attention for *L. lactis* and other lactic acid bacteria will be presented below. Several strategies used to transport sugars across the cytoplasmic membrane have been identified and these will be reviewed together with some examples found in *L. lactis*. Following the uptake, the sugar substrates are usually converted into an intermediate of the central metabolic pathway by one or more sugar-specific catabolic enzymes. In addition, the catabolic pathways of some industrially important sugars will be discussed below, followed by an overview of the central carbohydrate metabolism. Specific attention will be given to the various specific and global control mechanisms involved in the regulation of the sugar utilization in *L. lactis*. First, different transcriptional control systems involved in the regulation of the carbohydrate metabolism will be discussed. Furthermore, an overview of the allosteric control of enzymatic activities by glycolytic intermediates and inducer control systems will be given. Finally, the global transcriptional control systems, i.e. carbon catabolite repression and catabolite activation will be discussed.

Sugar uptake systems.

In order to generate energy from the conversion of a sugar to L-lactate or other metabolites, the sugar has to be transported across the cytoplasmic membrane. The three principal uptake systems for sugars in bacteria are (i) group translocations like the phosphotransferase system that result in the translocation and phosphorylation of a sugar substrate at the expense of phosphoenolpyruvate (PEP) (Postma *et al.*, 1993); (ii) primary transport systems that couple ATP hydrolysis to the translocation of a sugar (Fath and Kolter, 1993); and (iii) secondary transport systems where a solute gradient drives the sugar uptake (Poolman, 1993) (Fig. 1). Phosphorylation of the sugar is required before it can be used as a substrate for enzymes of the glycolysis. This phosphorylation can occur concommitant with the translocation when the sugar is taken up via the phosphotransferase system. In case of uptake via a primary or secondary uptake system the phosphorylation succeeds the uptake and is catalyzed by a cytoplasmic sugar kinase.

The main sugar uptake system in bacteria is the PEP:dependent sugar phosphotransferase system (PTS) that catalyzes the transport and the concommittant phosphorylation of carbohydrates (Fig. 1) (Postma *et al.*, 1993). The PTS is a group-translocation process in which the transfer of the phosphate moiety of PEP to carbohydrates is catalyzed by the general non-sugar-specific proteins enzyme I (EI) and HPr in combination with sugar-specific enzyme II proteins (EII). After autophosphorylation of EI at the expense of PEP, it catalyzes the phosphorylation of HPr at residue His-15 resulting in HPr(His-P). The phosphate group from HPr(His-P) is then transferred to a sugar-specific EII protein that subsequently can take up and phosphorylate one or more specific sugar substrates.

EII proteins consist of three domains designated EIIA, EIIB, and EIIC, which can be combined in a single membrane-bound protein or can consist of two or more separate proteins. In all cases, the EIIC domain is membrane-located and most likely forms the translocation channel involved in sugar transport. The EIIA and EIIB domains are involved in the two-step phosphotransfer from HPr(His-P) to the sugar substrate and are either coupled to the membrane-linked EIIC domain or exist as separate cytoplasmic proteins (Postma *et al.*, 1993).
Preliminary data from the *L. lactis* IL1403 genome sequencing project revealed the presence of several genes which might encode proteins showing homology to EII proteins involved in the uptake of sugars like fructose and mannitol (Bolotin *et al.*, 1998).

Uptake of sugars via primary transport systems depends on the hydrolysis of ATP which provides the energy for the translocation (Fig. 1) (Fath and Kolter, 1993). An example of sugar uptake via a primary transport system is the ATP-dependent multiple sugar uptake system in *Streptococcus mutans* (Russell *et al.*, 1992). Four membrane-associated proteins are involved in the uptake of several sugars including raffinose, melibiose and sucrose. The MsmK protein is most likely involved in the hydrolysis of ATP and provides energy for the active transport of sugars across the membrane catalyzed by the MsmEFG proteins. Maltose metabolism *in L. lactis* is dependent on the presence of the *malK* gene, the deduced protein sequence of which shows homology to the *S. mutans* MsmK protein (Law *et al.*, 1995). The observed homology suggests that the *L. lactis* MalK protein is involved in the uptake of maltose via a primary transport system. Several putative genes encoding proteins showing homology to primary transport systems could be identified in the *L. lactis* IL1403 genomic DNA sequence (Bolotin *et al.*, 1998).

Secondary transport systems use the energy stored in electrochemical gradients for solute translocation (Poolman, 1993; Konings *et al.*, 1995). Three groups have been identified: (a) antiporters which transport one solute across the cytoplasmic membrane concommitant with the secretion of another; (b) symporters which import two different solutes at the same time; and (c) uniporters catalysing the uptake of a single solute (Fig. 1). Several secondary sugar transporters have been described in lactic acid bacteria and all of these proteins have been classified in the galactoside-pentose-hexuronide group of transport proteins (Poolman *et al.*, 1996). The best studied member of this group is the LacS protein of *Streptococcus thermophilus*, which is involved in the uptake of lactose. The LacS protein can function as a proton symporter taking up lactose and a proton or as a lactose / galactose antiporter depending on the concentrations of the solutes involved (Poolman *et al.*, 1989). The LacS protein contains a carboxyterminal extension that shows high homology to EIIA domains of EII proteins of the PTS (see above). The EIIA domain of the LacS protein most likely does not play a role in the uptake of lactose but is implicated in the regulation of the lactose uptake by the PTS (Poolman *et al.*, 1995, see below).

The recently identified *L. lactis* GalA protein, involved in galactose utilization, shows high sequence homology to proteins of the galactoside-pentose-hexuronide group of transport proteins, suggesting that *L. lactis* takes up galactose via a secondary transport system (Grossiord *et al.*, 1998).

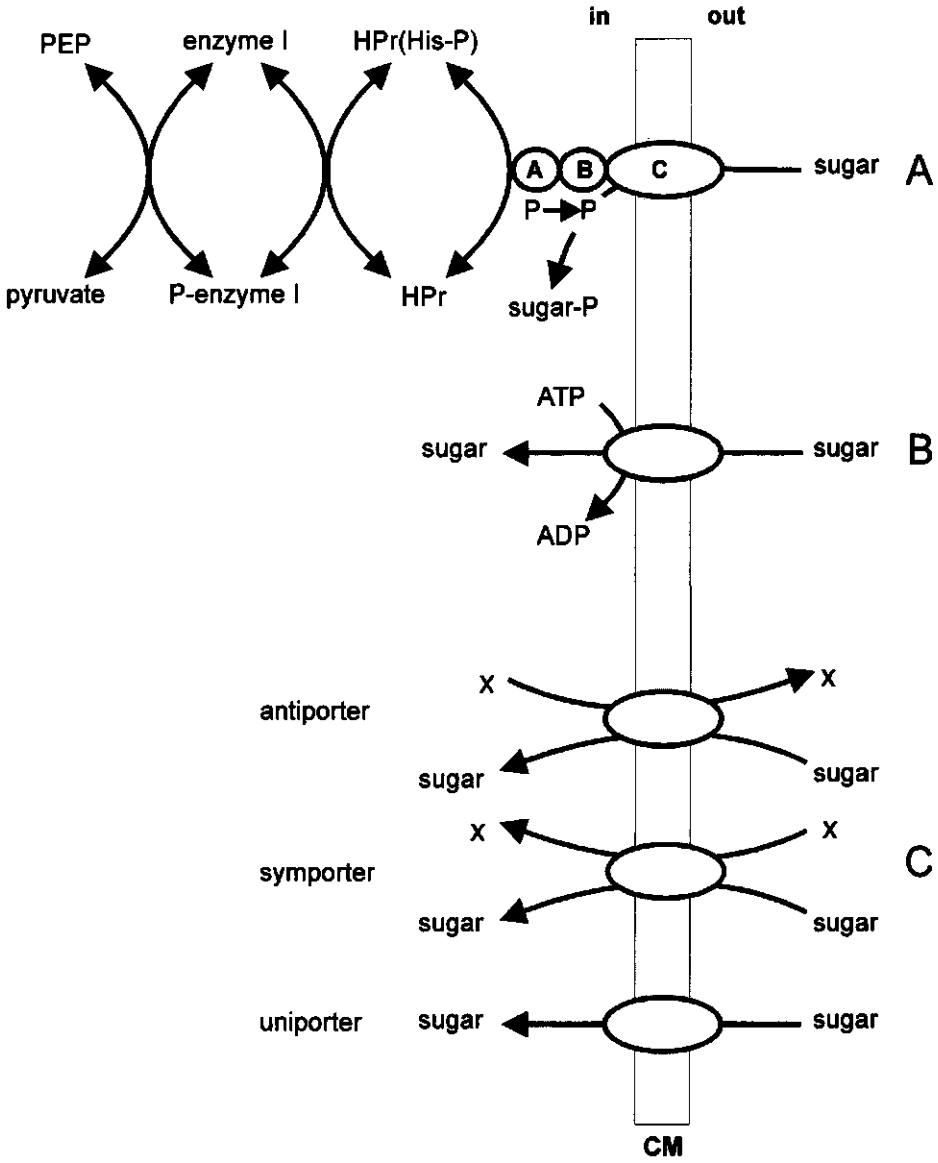


Figure 1. Schematic overview of different sugar uptake systems present in bacteria. The following systems are presented: group translocations (A), primary transport systems (B), and three types of secondary transport systems (C).

Sugar catabolic pathways in *L. lactis*.

In the following section an overview of the catabolic pathways for the utilization of the economically important sugars lactose, galactose, sucrose and glucose in *L. lactis* is presented. Some of these pathways will be analyzed in more detail in this thesis since they are subject to specific or global regulation.

L. lactis subsp. *cremoris* strain MG1820 takes up lactose via the phosphotransferase system (de Vos *et al.*, 1990). The lactose-specific EII (LacEF) takes up and phosphorylates lactose yielding lactose-6P which is then hydrolyzed by a phospho-β-galactosidase (LacG) producing galactose-6P and glucose. Glucose can enter the glycolysis, after phosphorylation by a glucokinase, while the galactose-6P moiety is metabolized via the tagatose-6P pathway (LacABCD; van Rooijen *et al.*, 1991). The galactose-6-phosphate isomerase (LacAB) catalyzes the conversion of this intermediate into tagatose-6-phosphate which is the substrate for tagatose-6-phosphate kinase (LacC). The resulting tagatose-1,6-di-phosphate is the substrate for tagatose-1,6-di-phosphate aldolase (LacD) that catalyzes the conversion to the glycolytic intermediate glyceraldehyde-3-phosphate. The genes encoding the enzymes for the lactose metabolism are plasmid-encoded and their expression increases when lactose is present in the growth medium (Fig. 2; see below).

Two different mechanisms have been reported for the uptake and catabolism of galactose in *L. lactis*. The galactose-specific PTS that has been identified in strain *L. lactis* subsp. *cremoris* E8 takes up and phosphorylates galactose yielding galactose-6-P (Thomas *et al.*, 1980). The galactose-6-P is then catabolized via the tagatose-6-P pathway that is described above. Uptake of galactose via a permease and the subsequent catabolism via the Leloir pathway has been reported for strain *L. lactis* subsp. *cremoris* ML3 (Thomas *et al.*, 1980). Galactose is the substrate for a galactokinase, which in an ATP-dependent reaction phosphorylates the galactose yielding galactose-1-P. Galactose-1-P then acquires an uridyl group from UDP-glucose in a reaction catalyzed by the galactose-1-P uridylyltransferase resulting in the production of glucose-1-P and UDP-galactose. The glucose-1-P moiety can, after isomeration to glucose-6-P by phosphoglucomutase, enter the glycolysis. The UDP-galactose is the substrate for a UDP-galactose-4-epimerase that converts UDP-galactose into UDP-glucose that serves as a substrate for the galactose-1-P uridylyltransferase. In *E. coli* the activity of a galactose mutarotase which converts β-D-galactose into α-D-galactose, the substrate for the galactokinase, results in a more efficient utilization of galactose (Bouffard *et al.*, 1994). Recently, a gene cluster was identified in *L. lactis* which encodes all four enzymes of the Leloir pathway as well as a gene encoding a permease (Fig. 2; Grossiord *et al.*, 1998). Disruption of the permease and the galactokinase gene confirmed the functionality of these genes in the utilization of galactose.

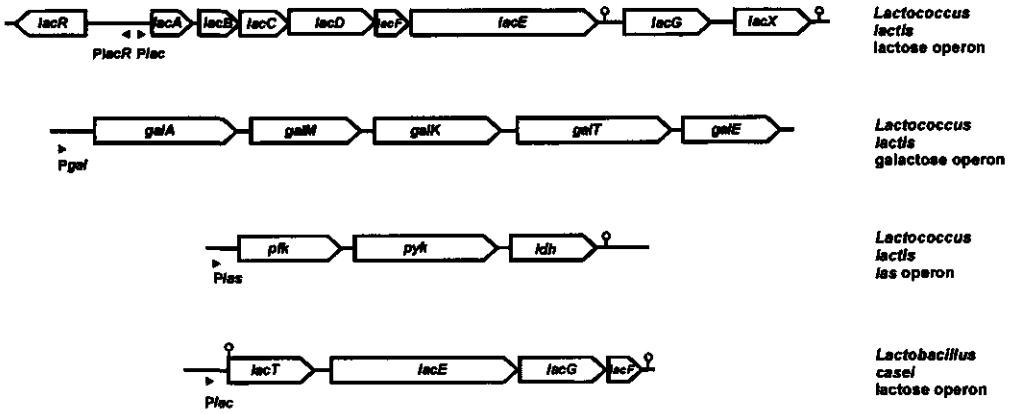


Figure 2. Overview of the *L. lactis* lactose (*lac*) operon (van Rooijen *et al.*, 1991), galactose (*gal*) operon (Grossiord *et al.*, 1998), the glycolytic *las* operon involved in lactic acid formation (Llanos *et al.*, 1992; Llanos *et al.*, 1993) and the *Lactobacillus casei* lactose operon (Alpert and Siebers, 1997). The location of the mapped promoters (triangles), concensus *cre* sites (dots) and putative terminators (open dots).

The ability of some *L. lactis* strains to utilize sucrose as a carbon source can be conjugally transferred and appears to be linked to the capacity to produce the antimicrobial peptide nisin (Rauch *et al.*, 1994). The uptake of sucrose is catalyzed by a sucrose-specific EII of the PTS and the resulting sucrose-6-P is hydrolyzed by a sucrose-6-P hydrolase. The products of this hydrolysis are glucose-6-P, that can be readily used in the glycolysis, and fructose which has to be phosphorylated by an ATP-dependent fructokinase before it enters the glycolysis (Fig. 3; Thompson *et al.*, 1991). An ATP-dependent fructokinase has been purified and characterized from *L. lactis* K1 and hybridization studies with oligonucleotides based on the NH_2-terminal amino acids linked the fructokinase gene to other genes involved in sucrose metabolism located on a transposon (Thompson *et al.*, 1991). In *L. lactis* strain R5 the sucrose-inducible *sacA* gene encoding a sucrose-6-P-hydrolase has been cloned and analysed. This gene is located on a 70-kb conjugative transposon, designated Tn*5276*, that also contains the genes involved in nisin biosynthesis (Rauch and de Vos, 1992a; Rauch and de Vos, 1992b; de Vos *et al.*, 1995).

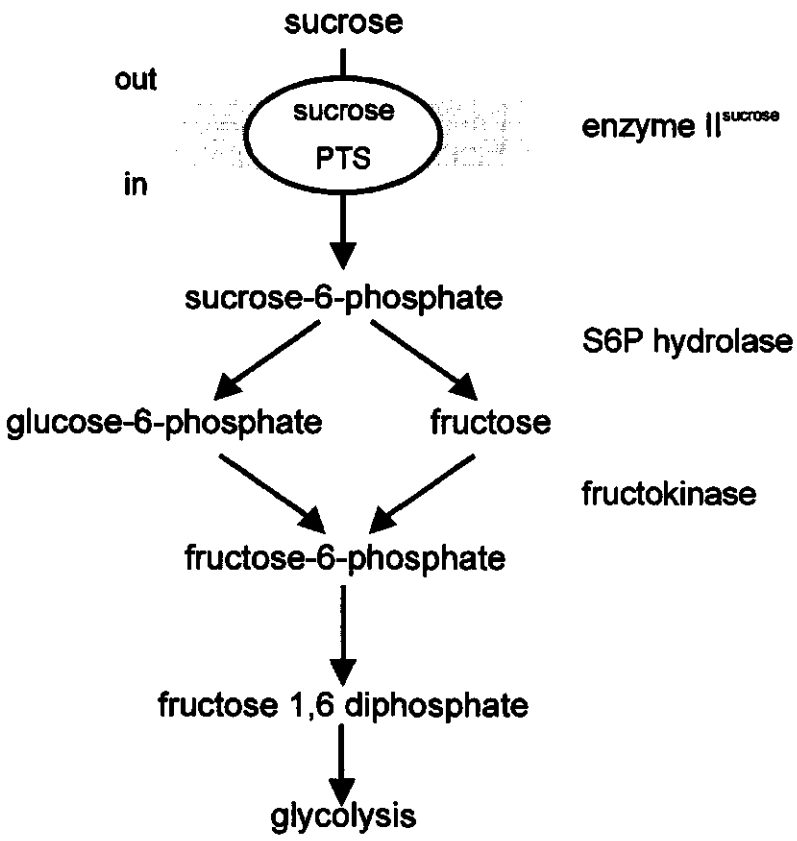


Figure 3. Schematic presentation of the catabolic pathway of sucrose that has been reported to operate in *L. lactis* (Thompson and Chassy, 1983; details see text).

By selection for resistance against the toxic glucose analog 2-deoxyglucose, Thompson and co-workers have shown that *L. lactis* 133 takes up glucose via the PTS. A mutation that is most likely located in the gene encoding the mannose-specific EII strongly reduced the growth on both mannose and glucose (Thompson and Chassy, 1982). The observation that the growth on glucose was not completely abolished by this mutation suggested that an additional glucose uptake system was functional. The isolation of a double mutant that was completely unable to utilize glucose and that lacked glucokinase activity strongly suggests that the second glucose uptake system is not a PTS and therefore most likely involves a permease (Thompson *et al.*, 1985). The presence of a mannose-specific EII protein in *L. lactis* was confirmed by the identification of a protein that cross-reacted with polyclonal antibodies raised against the *Streptococcus salivarius* mannose-specific EII protein (Pelletier *et al.*, 1995).

Central metabolic pathway in *L. lactis*.

The main pathway for energy generation in *L. lactis* is the glycolysis. Several glycolytic enzymes like pyruvate kinase (Collins and Thomas, 1974), phosphofructokinase (Fordyce *et al.*, 1982), ß-phosphoglucomutase (Qian *et al.*, 1997) and L-lactate dehydrogenase (Hardman *et al.*, 1985; Garrigues *et al.*, 1997) were found to be subject to carbon source-dependent regulation. During the growth on glucose or lactose, *L. lactis* converts about 90 % of the fermented sugar to L-lactate (Thomas *et al.*, 1980). Under homolactic circumstances *i.e.* growth on glucose or lactose in combination with a high growth rate, the endproduct of the glycolysis, pyruvate, is converted into L-lactate by L-lactate dehydrogenase. A mixed-acid fermentation, characterized by the presence of additional metabolites like acetate, ethanol or formate is observed when cells are growing slowly due to either low concentrations of sugar or growth on less-preferred carbon sources like galactose or maltose (Garrigues *et al.*, 1997; Qian *et al.*, 1997). The latter growth conditions result in lower intracellular concentrations of glycolytic intermediates including fructose-1,6-diphosphate. Fructose-1,6-diphosphate allosterically activates the glycolytic enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase and lower concentrations therefore lead to reduced enzymatic activities and an altered product formation (see below). The *L. lactis* genes encoding the key glycolytic enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase, *pfk*, *pyk* and *ldh*, respectively, have been cloned and analyzed (Fig. 2; Llanos *et al.*, 1992; Llanos *et al.*, 1993). The organization of these genes in an operon structure designated *las* (lactic acid synthesis) under the control of a single promoter allows a coordinated expression of these genes (Llanos *et al.*, 1992; Llanos *et al.*, 1993).

Transcriptional control of gene expression in lactic acid bacteria.

In order to prevent the synthesis of catabolic enzymes in the absence of their substrates, bacteria have developed various mechanisms that affect the transcription of genes encoding these enzymes. The three most commonly observed transcriptional control mechanisms in prokaryotes *i.e.* negative and positive control of transcription initiation and antitermination, are functional in the regulation of the carbohydrate metabolism of lactic acid bacteria, and will be discussed below.

The regulation of the expression of the lactose gene cluster of *L. lactis* is an example of negative control of transcription (see Fig. 2). In the absence of the inducing sugar lactose, the operon-specific regulator LacR binds to an operator site located in the promoter region of the lactose gene cluster. By binding to this operator site LacR prevents the transcription of the lactose genes. The presence of lactose in the growth medium leads to an increased intracellular concentration of tagatose-6-P, an intermediate of the tagatose-6-P pathway involved in lactose catabolism.

Tagatose-6-P can interact with the LacR protein and this most likely results in a conformational change of LacR which leads to the dissociation of LacR from the operator site and an increased transcription of the lactose genes (van Rooijen *et al.*, 1990; van Rooijen *et al.*, 1993).

Malolactic fermentation is a secondary fermentation which can be performed by several organisms when L-malate is present in the growth medium. The malolactic fermentation reduces the acidification of the medium due to the decarboxylation of L-malate to L-lactate. Under circumstances where the glycolysis is not active the malolactic fermentation can produce metabolic energy based on a membrane potential generated by the L-malate / L-lactate exchanger, MleP. The combination of the malolactic fermentation and the MleP activity results in the generation of a proton gradient which can be used to generate ATP (Bandell *et al.*, 1997). MleR most likely activates the expression of the genes involved in malolactic fermentation since a functional *mleR* gene is required for induction. The substrate induction of the *L. lactis* malolactic fermentation is most likely mediated by the predicted MleR protein which shows homology to proteins of the LysR family of bacterial activator proteins (Renault *et al.*, 1989).

Sequence analysis of the *Lactobacillus casei* lactose operon revealed the presence of the *lacT* gene the deduced protein sequence of which shows significant homology to transcriptional antiterminators (Fig. 2; Alpert and Siebers, 1997). The antitermination activity of LacT was demonstrated by use of a reporter system in *Bacillus subtilis*. Furthermore, a consensus RNA binding site of transcriptional antiterminators was identified in this *lac* operon. These findings suggest that the *Lactobacillus casei* LacT protein regulates the expression of the lactose operon via antitermination. Antiterminator proteins modulate the formation of full-length transcripts by controlling the functionality of terminator-like structures located in the transcribed RNA. This can be accomplished in two ways. First through an interaction of the antiterminator protein with the RNA polymerase, which results in reduced recognition of the terminator (Greenblatt *et al.*, 1993). Secondly, the antiterminator protein can interact with the terminator structure preventing or enhancing its formation (Rutberg, 1997). In *L. lactis* the *bglR* gene was identified which encodes a protein showing high homology to transcriptional antiterminators (Bardowski *et al.*, 1994). Disruption of this gene resulted in a reduced growth on different ß-glucoside sugars suggesting that the BglR protein is involved in the regulation of the expression of genes involved in the utilization of sugars like cellobiose in *L. lactis* through antitermination.

Allosteric control systems.

Several glycolytic enzymes of *L. lactis* are subject to allosteric control mechanisms that allow a rapid modulation of the enzymatic activity in response to the carbon source availability. The intracellular concentration of glycolytic intermediates like fructose-1,6-diP (FDP) and PEP varies in response to the carbon source provided; higher values are measured during growth on glucose (118 mM and 25 mM, respectively) compared to growth on galactose (36 mM and <0.6 mM, respectively) (Garrigues *et al.*, 1997). FDP allosterically activates the activity of pyruvate kinase and L-lactate dehydrogenase (Hardman *et al.*, 1985). The importance of the allosteric activation of these enzymes remains to be determined since the intracellular concentrations of the activator molecules are under physiological circumstances always sufficiently high to ensure full activation e.g. 50 % of the maximal activity of L-lactate dehydrogenase was measured at a FDP concentration of 2.2 μM (Hardman *et al.*, 1985).

Furthermore, a recent study showed that the activity of L-lactate dehydrogenase is controlled by the $NADH/NAD^+$ ratio (Garrigues *et al.*, 1997). Phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase are ideal candidates for regulation since they catalyze steps that are under physiological circumstances almost irreversible.

Apart from their function in the uptake of sugars, the HPr and EI proteins of the PTS also modulate the activities of several catabolic pathways. In *E. coli* and most likely other Gram-negative bacteria the cytoplasmic glucose-specific EIIA of the PTS is involved in the allosteric control of different proteins. The phosphorylation state of the glucose-specific EIIA depends on the phosphorylation by HPr(His-15) and the dephosphorylation by the glucose-specific EIICB protein. Therefore, the concentration of the unphosphorylated form increases during growth on PTS substrates compared to growth on non-PTS substrates. An interaction between the unphosphorylated form of the glucose-specific EIIA and the lactose carrier, LacY, has been shown to reduce the uptake of lactose in *E. coli* (Osumi and Saier, 1982). This phenomenon termed inducer exclusion has also been observed for several other sugar permeases (Postma *et al.*, 1993).

In Gram-positive bacteria regulatory functions for the PTS have also been described. In *Enterococcus* spp. the HPr(His-P) mediated phosphorylation of two glycerol kinases results in an increased activity of both enzymes (Charrier *et al.*, 1997). In contrast, EI/HPr(His-P) mediated phosphorylation of the lactose permease in *Streptococcus thermophilus* results in a reduced protein activity leading to a decreased uptake of lactose (Poolman *et al.*, 1995). A different type of PTS-mediated regulation was observed in *B. subtilis* where the PTS regulates the expression of the levanase operon by HPr(His-P) mediated phosphorylation of the transcriptional regulator LevR resulting in activation of transcription (Stülke *et al.*, 1995).

Inducer exclusion and inducer expulsion in Gram-positive bacteria.
Apart from the phosphorylation of HPr at residue His-15 by EI, a second phosphorylation site has been described that appears to be unique for Gram-positive bacteria, including *L. lactis*. The phosphorylation of HPr at residue Ser-46 is catalysed by a protein kinase at the expense of ATP. The HPr kinase is activated by elevated concentrations of early glycolytic intermediates such as FDP (Fig. 4) (Deutscher *et al.*, 1983). Recently, the gene encoding the *B. subtilis* HPr kinase was identified and its inactivation confirmed the role of HPr kinase in carbon catabolite repression (see below; Reizer *et al.*, 1998). HPr phosphorylated on residue Ser-46 (HPr(Ser-P)) is a poor substrate for the catalytic phosphorylation on residue His-15 by EI. Consequently, the FDP concentration modulates the activity of the PTS.

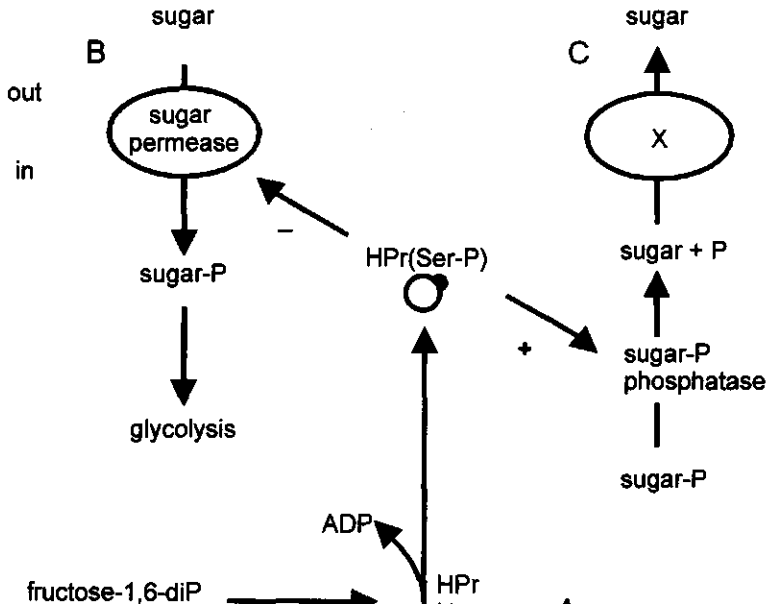


Figure 4. Overview of the formation of seryl-phosphorylated HPr after activation of HPr kinase by metabolites like fructose 1,6-di phosphate (A). The resulting HPr(Ser-P) can interact with sugar uptake proteins and reduce the sugar uptake in a process called inducer exclusion (B). The interaction of HPr(Ser-P) with a sugar-phosphate phosphatase results in the dephosphorylation and subsequent exit from the cell of sugar phosphates, in a process termed inducer expulsion (C).

HPr(Ser-P) plays a role in inducer exclusion, a mechanism by which the cell can immediately reduce the uptake of a sugar upon the addition of a rapidly metabolizable carbon source to the medium (Fig. 4). The uptake of [^{14}C]TMG, a lactose analog, via the lactose PTS in *L. lactis* was inhibited when glucose was added to the medium (Ye *et al.*, 1994). Membrane vesicles of *L. lactis* take up [^{14}C]TMG but the reduced uptake in the presence of glucose, as observed in intact cells, is not occurring. These findings indicated that cytoplasmic components play an important role in inducer exclusion. Electroporation of *Bacillus subtilis* HPr protein into the vesicles resulted in reconstitution of the reduced uptake in the presence of glucose, indicating that HPr is an essential component for inducer exclusion (Ye *et al.*, 1994). To investigate the role of the phosphorylation of HPr on residue Ser-46 in inducer exclusion, a mutant of HPr was used carrying on position 46 an alanine which can not be phosphorylated. Electroporation of Ser46Ala-HPr into vesicles did not lead to a reduced uptake of [^{14}C]TMG in the presence of glucose, indicating that phosphorylation of HPr on residue Ser-46 is a key step in the inducer exclusion mechanism (Ye *et al.*, 1994).

Inducer expulsion is a mechanism by which the cell can reduce the intracellular concentration of sugar phosphates (Fig. 4). The sugar phosphates have to be dephosporylated before leaving the cell via a sofar unidentified mechanism. A cytoplasmic hexose-6-phosphate hydrolase from *L. lactis* has been purified and characterized that catalyzes the dephosphorylation of several sugar phosphates (Thompson and Chassy, 1983). Since this hydrolase is constitutively produced and has a broad substrate range, it may play an important role in inducer expulsion. Recently, another membrane-associated hexose-6-phosphate hydrolase has been purified from *L. lactis*. The activity of this protein was activated 10-fold by Ser46Asp-HPr which conformationally resembles HPr(Ser-P) (Ye *et al.*, 1995). Although vesicle studies revealed the functionality of this hydrolase, its overall contribution to inducer expulsion in *L. lactis* remains to be established.

Carbon catabolite repression and catabolite activation.

The presence of a rapidly metabolizable carbon source in the growth medium of bacteria in many cases reduces the expression of genes involved in the utilization of other carbon sources. This phenomenon, termed carbon Carbon Catabolite Repression (CCR) is a global regulatory mechanism identified in both Gram-negative and Gram-positive bacteria. In *E. coli* and other Gram-negative bacteria CCR is mediated by the cyclic adenosine monophosphate (cAMP) receptor protein (CRP) which through an interaction with cAMP activates the expression of certain catabolic genes by binding to an operator site located in the promoter region. The intracellular concentration of cAMP is the central signal molecule in catabolite repression in *E. coli* and its concentration varies in response to the carbon source provided. The synthesis of cAMP by adenylate cyclase is controlled by the phosphorylated form of the glucose-specific EIIA of the phosphotransferase system (PTS).

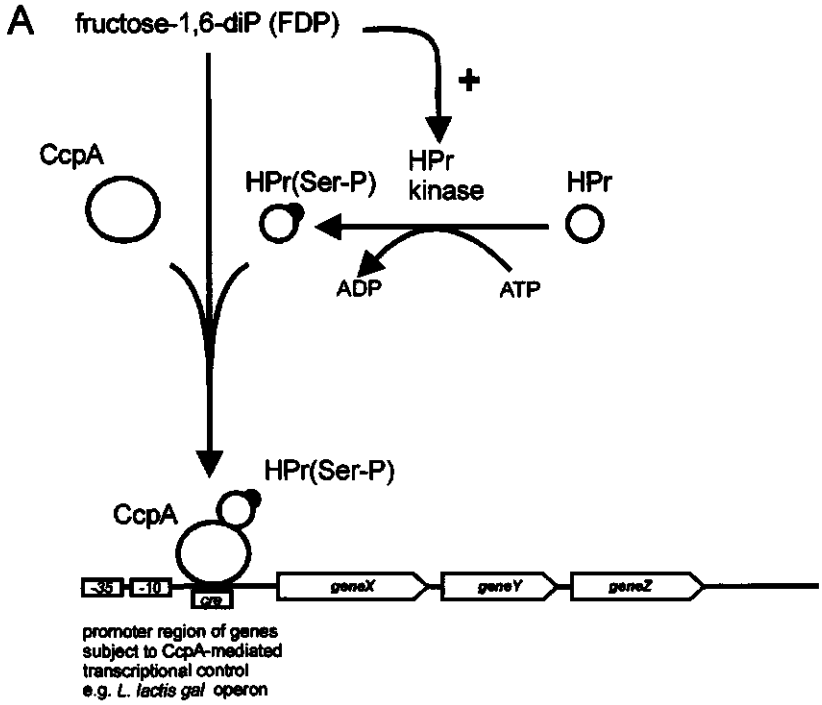
The concentration of the phosphorylated form of the glucose-specific EIIA depends on the carbon source provided; growth on PTS substrates like glucose results in lower concentrations while non-PTS sugars result in increased concentrations (Postma *et al.*, 1993).

CCR in Gram-positive bacteria is mediated via the catabolite control protein A (CcpA) and a *cis*-acting catabolite responsive element (*cre*) located near the transcription start site of genes affected by CCR (Hueck and Hillen 1995). In the studied examples, CCR was found to be a negative regulatory mechanism. The first *cre* site was identified following the observation that point mutations in the promoter region of the *B. subtilis* α-amylase gene lead to reduced CCR (Nicholson *et al.*, 1987). Using site-directed mutagenesis a consensus sequence was defined that conformed to the sequence TGWNANCGNTNWCA (Weickert and Chambliss, 1990). An extensive database search showed the presence of *cre* elements near the transcription start of several genes involved in carbohydrate metabolism in Gram-positive bacteria (Hueck *et al.*, 1994). Several *cre* elements could also be identified in genes involved in carbohydrate utilization in *L. lactis* (Fig. 5). The CcpA protein that was first identified in *B. subtilis* contains a helix-turn-helix DNA binding domain in the NH_2-terminal part of the protein (Henkin *et al.*, 1991). CcpA together with HPr(Ser-P) and possibly together with early glycolytic intermediates like FDP or glucose-6-P form a complex that is able to bind to *cre* boxes (Fujita *et al.*, 1995, Goesseringer et al., 1997).

By binding to these *cre* boxes, this complex affects the transcription of these genes. (Fig. 5) The interaction between CcpA and HPr(Ser-P) and the involvement of fructose-1,6-diphosphate and glucose-6-P in the binding to *cre* boxes links glycolytic activity to CcpA-mediated CCR (Deutscher *et al.*, 1995). It was also found that the replacement of the wild-type *ptsH* gene with a gene encoding S46A HPr, in which residue Ser46 was changed to an alanine that can not be phosphorylated, rendered the same genes insensitive to CCR as did the inactivation of the *ccpA* gene (Deutscher *et al.*, 1994). In addition, these authors showed that expression of the gene encoding S46D HPr, which has a similar structure as HPr(Ser-P), leads to CCR of the *B. subtilis* gluconate kinase gene, even in the absence of glucose. These experiments demonstrated the involvement of HPr(Ser-P) in the negative transcriptional control of several genes in *B. subtilis* through an interaction with CcpA. In addition to its contribution to the negative control, CcpA also acts as a positive regulator of the *B. subtilis alsS* and *ackA* genes encoding α-acetolactate synthase and acetate kinase, respectively (Grundy *et al.*, 1993; Renna *et al.*, 1993). Both enzymes are involved in the pyruvate metabolism and catalyse the conversion of pyruvate to compounds that can be easily removed from the cell. The transcription of the *ackA* and *alsS* genes is induced when glucose is present in the growth medium. The activation of the expression of these genes can be seen as a mechanism to prevent the accumulation of end-products of the glycolysis. These findings indicated that CcpA acts as a transcriptional regulator involved in the global metabolic control.

Recently a gene, *glkA* encoding a glucose kinase, was cloned form *Staphylococcus xylosus* that participated in CCR, in addition to the CcpA-mediated mechanism (Wagner *et al.*, 1995). The exact mechanism by which GlkA exerts CCR remains unclear but it is likely that GlkA-mediated phosphorylation of glucose results in the occurrence of a signal that plays a role in CCR.

CCR has also been reported to occur in lactic acid bacteria. The *ccpA* genes from *Lactobacillus casei* and *Lactobacillus pentosus* have been (partly) cloned and disruption of these genes reduced CCR (Monedero *et al.*, 1997: Lokman *et al.*, 1997). Futhermore, the expression of the *Lactobacillus plantarum bglH* gene, encoding a phospho-β-glucosidase, was found to be subject to CCR (Marasco *et al.* 1998). In *L. lactis* the expression of genes involved in galactose catabolism appears to be subject to CCR, since growth in a medium containing both galactose and glucose resulted in the inhibition of the galactose uptake (Thompson *et al.*, 1977). Furthermore, the addition of glucose to cells growing on galactose resulted in an immediate reduction of the galactose uptake. These findings suggest that in addition to CCR also more rapid control mechanisms, like inducer exclusion or inducer expulsion, affect the galactose catabolism. A better understanding of the mechanisms involved in CCR and catabolite activation in *L. lactis* will yield new insights leading to a more efficient utilization of carbon sources. Furthermore, this information will provide useful tools for metabolic engeneering strategies.



B

TGWNANCGNTNWCA consensus *cre* (Weickert and Chambliss 1990)

TGATATCGCTTCCA *L. lactis ccpA* gene (Chapter 4)
TGAAAACGTTATCT *L. lactis lac* operon *cre* (van Rooyen *et al.*, 1991)
TGTAAGCGAAATCA *L. lactis sac* operon *cre* (Chapter 2; Rauch and de Vos 1992b))
TGTGAGCGATTTCA *L. lactis gal* operon *cre* (Chapter 4; Grossiord *et al.*, 1998)
TGAAAACGTTT-CA *L. lactis las* operon *cre* (Chapter 4; Llanos *et al.*, 1993,1993)

Figure 5. Proposed mechanisms for the CcpA-mediated transcriptional control in Gram-positive bacteria (Saier *et al.*, 1995). After phosphorylation of HPr on residue Ser-46 by a metabolite activated kinase, the resulting HPr(Ser-P) can interact with the transcriptional regulator CcpA. The complex between HP(Ser-P), CcpA and possibly fructose 1,6-diphosphate (FDP) can bind to *cre* elements located in the promoter regions of genes subject to CcpA-mediated control (A). Alignment of *cre* elements identified in genes involved in the carbohydrate metabolism of *L. lactis*. Residues which perfectly match the proposed consensus are indicated in bold (B).

Outline of this Thesis

Given the industrial importance of *L. lactis* for the production of fermented dairy products, this organism was chosen as a model system to study the regulatory mechanisms underlying metabolic control in Gram-positive bacteria. The aim of the work described in this thesis was to investigate different specific and global control systems that modulate the carbohydrate catabolism of *L. lactis*. Specific attention was given to carbon catabolite repression since this process has been shown to affect the expression of genes involved in sugar uptake, the central metabolism, and the export of metabolites.

In **Chapter 2** the cloning and the analysis of the genes involved in sucrose catabolism are reported. The transcriptional analysis of the *sacKBAR* genes involved in the utilization of sucrose is described. Furthermore, the role of SacR in the transcriptional control of the sucrose gene cluster is discussed.

Chapter 3 deals with the identification of proteins which cross-react with polyclonal antibodies raised against *Bacillus megaterium* CcpA in different Gram-positive bacteria. The implications of the possible functionality of CcpA-mediated carbon catabolite repression in Gram-positive bacteria is discussed.

In **Chapter 4** the identification and characterization of the *L. lactis ccpA* gene is reported. The role of CcpA in the metabolic control of *L. lactis* is described. Both the negative and the positive regulation mediated by CcpA are discussed using two different model systems.

The identification of the *L. lactis ptsHI* genes encoding the general PTS proteins HPr and EI, respectively, is reported in **Chapter 5.** The transcriptional and functional analysis of the *ptsHI* genes is discussed. Furthermore, the regulatory role of the seryl-phosphorylated form of HPr in the inducer control mechanisms and in the CcpA-mediated transcriptional control is analyzed.

A summary of the work presented in this thesis and concluding remarks concerning the impact of the obtained results are presented in **Chapter 6.**

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CHAPTER 2

Characterization of the divergent *sacBK* and *sacAR* operons involved in sucrose utilization by *Lactococcus lactis*

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Abstract

The structural, transcriptional and functional analysis is reported of the divergently transcribed *sacBK* and *sacAR* operons, which are involved in sucrose utilization of *Lactococcus lactis* NZ9800. The deduced amino acid sequence of the *sacB* gene shows homology to sucrose-specific enzyme II proteins of the phosphotransferase system and the *sacK* gene encodes an ATP-dependent fructokinase. The *sacB* and *sacK* genes were disrupted by using replacement recombination and subsequent growth experiments indicated that the utilization of sucrose is dependent on an intact *sacB* gene but not on an intact *sacK* gene. Hybridization studies with *sacK* suggest that a second chromosomally located *sacK*-like gene is present in *L. lactis*, which could explain the normal growth on sucrose upon disruption of the *sacK* gene. Downstream of the previously described *sacA* gene encoding a sucrose-6-phosphate hydrolase, another gene, *sacR*, was identified that encodes a protein belonging to the LacI/GalR family of bacterial regulator proteins. Northern analysis of RNA isolated from cells grown on different carbon sources indicated three sucrose-inducible transcripts: one of 3.2 kb containing *sacB* and *sacK*, a second of 3.4 kb containing *sacA* and *sacR*, and a third one of 1.8 kb containing only *sacR*. The inactivation of the *sacR* gene by replacement recombination resulted in the constitutive transcription of the *sacBK* and *sacAR* operons on different carbon sources, indicating that SacR acts as a repressor of transcription. Carbon catabolite repression of the *sac* genes is dependent on SacR and no evidence for the involvement of additional regulatory mechanisms was found.

Introduction

Sucrose can be utilized as a sole carbon source by many bacteria and it is known to be transported into the cell via primary (32) or secondary transport systems (1). However, the vast majority of bacteria take up this disaccharide via the sucrose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) (18). In Gram-negative bacteria the PTS-dependent sucrose transport most likely requires the additional activity of a sucrose-specific porin responsible for the transport across the outer membrane (29, 35). In Gram-positive bacteria no additional components are required for sucrose transport and several sucrose-specific PTS systems have been identified and genetically characterized (7, 26, 4, 39). The PTS catalyses the transport of sucrose across the cytoplasmic membrane concomitant with its phosphorylation by a sucrose-specific enzyme II. The product of this translocation, sucrose-6-phosphate, is then hydrolyzed to glucose-6-phosphate and fructose by a sucrose-6-phosphate hydrolase (EC 3.2.1.26). The glucose-6-phosphate can readily be used, while the fructose has to be phosphorylated by an ATP-dependent fructokinase (EC 2.7.1.4), before it can be metabolized via the glycolytic pathway. These two cytoplasmic enzymes involved in the generation of fructose and its phosphorylated derivative have been characterized in detail in the Gram-positive lactic acid bacterium *Lactococcus lactis* (33, 34).

The expression of sucrose catabolic genes is in most cases regulated at the transcriptional level. The transcription of the *Bacillus subtilis sacPA* operon is inducible by sucrose and this regulation is mediated by the SacT protein that shares homology with transcriptional terminators (5). The sucrose genes located on plasmid pUR400 of *Escherichia coli* K12 are regulated by the repressor ScrR, and are inducible by sucrose, fructose and fructose-containing oligosaccharides (28). The *scrB* gene of *Staphylococcus xylosus* encoding a sucrose-6-phosphate hydrolase is negatively regulated by the regulator ScrR and is induced by the presence of sucrose in the growth medium. In addition to the ScrR specific regulation, the *scrB* gene from *Staphylococcus xylosus* also appears to be controlled by catabolite repression (9).

Genes involved in the utilization of sucrose can be located either on the chromosome like in *B. subtilis* (7) and *Staphylococcus xylosus* (3) or on plasmids, such as plasmid pUR400 found in enteric bacteria (28). In contrast, the ability to ferment sucrose by *L. lactis* strains was found to be linked to the presence of conjugative transposons, like Tn*5276*, that also contain the genes for the biosynthesis of the antimicrobial peptide nisin (19, 20). Previously, the molecular cloning and sequence analysis of the Tn*5276*-located sucrose-6-phosphate hydrolase gene, designated *sacA* was reported (21). Here, we present the sequence, transcriptional and functional analysis of three other Tn*5276*-located genes involved in sucrose metabolism in *L. lactis*. These genes form the divergent operons *sacBK* and *sacAR*, and include *sacB* encoding the sucrose-specific enzyme II of the PTS, *sacK* encoding a fructokinase, and *sacR* that encodes a regulatory protein. The results show that the transcription of the sucrose genes can be induced by sucrose, that SacR acts as a repressor of transcription and that *sacK* is dispensible for sucrose utilization in *L. lactis*.

Materials and Methods

Bacterial strains, media, and transformation. The *L. lactis* strains used in this study (Table 1) were cultivated without aeration at 30°C in M17 broth complemented with 1 % (w/v) sugar. *L. lactis* was transformed by electroporation as described previously (10). The *E. coli* strain MC1061 was used as a host for cloning experiments and grown in L-broth based medium with aeration at 37°C. Antibiotics were used in the following concentrations: ampicillin 50 μg/ml, chloramphenicol 5 μg/ml and erythromycin 2.5 μg/ml.

DNA techniques and sequence analysis. All manipulations with recombinant DNA were carried out according to standard procedures (24) and according to the specifications of the enzyme manufacturers (Gibco/BRL Life technologies or United States Biochemicals). Plasmid and chromosomal DNA of *L. lactis* was isolated as described previously (38). The DNA sequence of the sucrose gene cluster was determined on double stranded plasmid DNA by the chain termination method (25).

Table 1. *L. lactis* strains and plasmids

Strain or plasmid	Relevant properties	Reference
Strains		
MG1614		(8)
NZ9700	MG1614 derivative containing Tn*5276*	(13)
NZ9800	NZ9700 derivative; Δ*nisA*	(13)
NZ9840	NZ9800 derivative; Δ*sacB*	This work
NZ9841	NZ9800 derivative; Δ*sacK*	This work
NZ9860	NZ9800 derivative; Δ*sacR*	This work
Plasmids		
PNZ755	pACYC184 derivative containing a 6.6 kb *Bam*HI fragment with the *sacR* and *sacA* genes	(21)
PUC19E	pUC19 derivative containing an erythromycin resistance cassette	(14)
PNZ9250	pUC19 derivative containing a 4.5 kb *Kpn*I-*Xba*I fragment with the *sacB* and *sacK* genes	This work
PNZ9251	pUC19E derivative containing an internal *Pst*I-*Bam*HI fragment of the *sacB* gene	This work
PNZ9252	pUC19E derivative containing an internal *Mun*I-*Bgl*II fragment of *sacK*	This work
PNZ9253	pUC19E derivative containing a 5.6-kb *Pst*I-*Bam*HI fragment from pNZ755	This work
PNZ9254	pNZ9253 derivative containing a frame shift in the *sacR* gene.	This work
PNZ8020	lactococcal cloning and inducible expression vector, with the *nisA* promoter	(22)
PNZ9255	pNZ8020 derivative containing the entire *sacR* gene	This work

Construction of plasmids. Previously, the cloning of a 6.6-kb *Bam*HI fragment of Tn*5276* resulting in pNZ755 has been described (Fig. 1) (21). The *sacB* and *sacK* genes were cloned by using a 1.0-kb *Pst*I-*Bam*HI fragment from the plasmid pNZ755 as a probe to clone a 4-kb *Xba*I-*Kpn*I fragment from the chromosomal DNA of *L. lactis* NZ9800 into *Xba*I-*Kpn*I digested pUC19 (43), resulting in pNZ9250. The same 1.0-kb *Pst*I-*Bam*HI fragment from plasmid pNZ755, located in the *sacB* open reading frame, was cloned into *Pst*I-*Bam*HI digested pUC19E (14), resulting in plasmid pNZ9251, which was used to disrupt the *sacB* gene. The *sacK* gene was disrupted using plasmid pNZ9252 which was constructed by cloning a 0.7-kb *Mun*I-*Bgl*II fragment from plasmid pNZ9250, after treatment with Klenow DNA polymerase, into *Sma*I digested pUC19E. For the overexpression of the *sacR* gene a 2.5-kb *Nco*I-*Hae*III fragment containing the *sacR* gene was, after Klenow DNA polymerase treatment, cloned into *Sma*I digested plasmid pNZ8020 (22) resulting in plasmid pNZ9255. For the disruption of the *sacR* gene via double-crossover recombination, a 5.6-kb *Pst*I-*Bam*HI fragment from plasmid pNZ755, containing the *sacA* and *sacR* genes was cloned into *Pst*I-*Bam*HI digested pUC19E, resulting in pNZ9253. Plasmid pNZ9253 was digested with *Mun*I, treated with Klenow DNA polymerase and ligated, resulting in pNZ9254.

Disruption of the *sacB* and *sacK* genes. Plasmids pNZ9251 and pNZ9252 were transformed in strain NZ9800 in order to disrupt the chromosomal copies of the *sacB* and *sacK* genes, respectively. Erythromycin-resistant (Ery^R) colonies were obtained and were analyzed by Southern hybridization. Strains that contained a single copy of the plasmids pNZ9251 and pNZ9252 were designated NZ9840 and NZ9841, respectively.

Inactivation of chromosomal *sacR* by gene replacement. Plasmid pNZ9254 was transformed to *L. lactis* NZ9800 and Ery^R colonies were obtained which contained a single chromosomal copy of pNZ9254. One transformant was selected and grown for 150 generations in glucose M17 without selection on erythromycin. Cells were plated and analyzed for the occurrence of the second recombination event, i.e. the removal of the plasmid sequences including the erythromycin-resistance gene from the chromosome. Erythromycin sensitive (Ery^S) colonies were further screened by Southern analysis, and one, designated NZ9860, was selected that contained a disrupted copy of the *sacR* gene.

RNA isolation and analysis. RNA was isolated from *L. lactis* cultures as described previously (13). RNA was denatured and size fractionated on a 1 % agarose gel containing formaldehyde according to standard procedures (24). The RNA was stained by adding ethidium bromide to the sample buffer. As molecular weight markers the 0.24-9.5 kb RNA ladder from BRL was used. The gel was blotted to a nylon membrane (Gene Screen, New England Nuclear) as recommended by the manufacturer.

Primer-extension analysis. The oligonucleotide used for priming cDNA synthesis was SACRPE (5'-GTCACTCAAATATCCTTTACG-3') complementary to nucleotides 1197 to 1217 in the coding strand of the *sacR* gene in the sequence data. Primer extension reactions were performed by annealing 2 ng of oligonucleotide to 100 μg of total RNA as described (13).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide sequence databases under the accession number Z97015

Results

Isolation and characterization of genes involved in sucrose utilization. Sequence analysis of plasmids containing DNA fragments from Tn*5276* isolated from *L. lactis* NZ9800 revealed three new genes, i.e. *sacR*, *sacB* and *sacK* (Fig. 1; see below), in addition to the previously described *sacA* gene encoding a sucrose-6-phosphate hydrolase (21). The GC content of the *sac* genes (33 %) and the nisin genes (32 %) located on Tn*5276* or its homologs is significantly lower than that described for the *L. lactis* genome (38 %) (12). The *sacA* stopcodon partly overlaps with the putative SacR start codon (GTG). Based on this start codon, the *sacR* gene encodes a 318-residue protein containing a helix-turn-helix DNA binding motif in its NH_2-terminal sequence (2).

The deduced amino acid sequence of the *sacR* gene shows significant sequence similarity to proteins of the LacI/GalR family of bacterial regulator proteins (28 % identical residues compared to *E. coli* RbsR) (17, 40). Further analysis of Tn*5276*-derived DNA fragments revealed an IS*981*-like element, located downstream of *sacR*. Downstream of this iso-IS*981* sequence the last genes of the Tn*5276*-located nisin gene cluster were identified, i.e. the *nisFEG* genes involved in immunity to the antimicrobial peptide nisin (Fig. 1). These data are in analogy with the finding that in *L. lactis* strain 6F3 the *nisFEG* genes are followed by an iso-IS*981* sequence and, in opposite orientation, a gene encoding a SacR-like protein (30). The *sacB* gene, which is located in the opposite orientation to the *sacA* gene, encodes a protein that contains the EIIABC domains expected for an enzyme II protein of the PTS (23). The highest sequence homology was found with the sucrose-specific EII, ScrA, from *S. mutans* (54% identical residues) (26) that also contains the EIIABC domains. The *sacB* gene is immediately followed by another gene, *sacK*, which encodes a 290-residue protein.

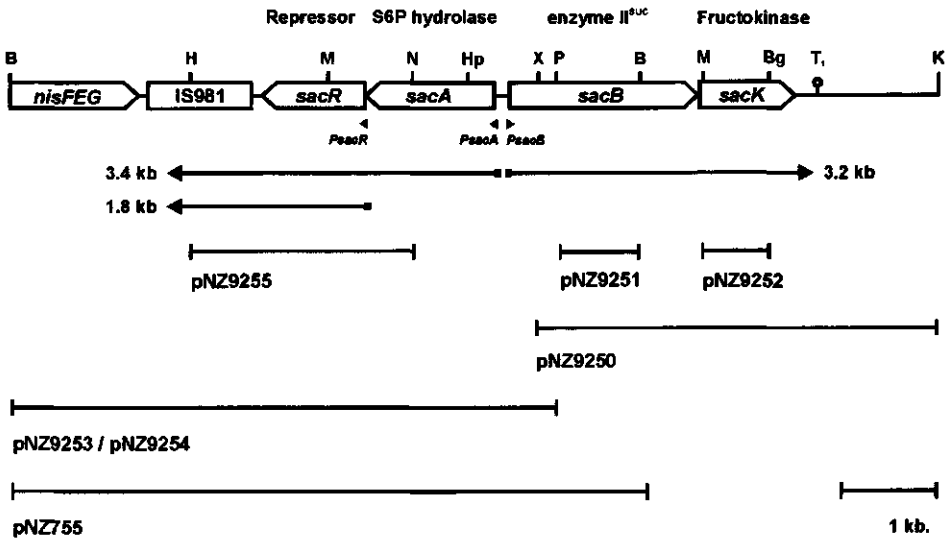

Figure 1. Genetic and transcriptional organization of the Tn*5276*-located sucrose gene cluster of *L. lactis* NZ9800. The genes (open arrows) are shown with their products and mapped promoters as well as the mapped transcripts (arrows). The relevant cloned chromosomal DNA fragments are shown and the names of the derived plasmids. The putative terminator downstream of *sacK* is indicated (T_1). Relevant restriction sites are B, *Bam*HI; Bg, *Bgl*II; H, *Hae*III; Hp, *Hpa*II; K, *Kpn*I; M, *Mun*I; N, *Nco*I; P, *Pst*I; X, *Xba*I.

The NH_2-terminal amino acids 2 – 26 of the deduced SacK sequence are identical to those determined of the purified fructokinase I from *L. lactis* KI (34) (Fig. 2). Moreover, the total amino acid composition and the calculated molecular mass of the deduced protein (31,626 Da) are highly similar to that of the purified lactococcal fructokinase (34). The amino acid sequence of SacK shows high similarity to several bacterial fructokinases (56.9 % identical residues compared to *S. mutans* ScrK (27)) (Fig. 2) and a consensus pattern was identified that is usually found in proteins belonging to a family of sugar kinases and regulatory proteins (36) (Fig. 2).

***sacB* but not *sacK* is essential for the utilization of sucrose.** A disruption of the *sacB* gene was constructed by transforming strain NZ9800 with the non-replicating plasmid pNZ9251, which contains an internal fragment of the *sacB* gene and an Ery^R cassette. Growth experiments indicated that the resulting *sacB* mutant strain, NZ9840, was no longer able to grow on sucrose, while the growth rate on glucose and fructose was not affected. Since the integration of pNZ9251 into the *sacB* gene could have polar effects on the expression of the *sacK* gene, a *sacK* disruption mutant was constructed by integration of plasmid pNZ9252 into the *sacK* gene of *L. lactis* NZ9800 resulting in two incomplete copies of the *sacK* gene. The *sacK* mutant strain NZ9841 showed the same rapid growth as the wild-type strain on glucose, sucrose and fructose. Furthermore, growth experiments in broth containing limiting concentrations of sucrose indicated that the same amount of biomass was produced by NZ9841 as compared to the wild-type strain, suggesting that both the glucose and the fructose moiety of sucrose were metabolized (data not shown). A second protein with fructokinase activity has been shown to be present in strain *L. lactis* K1 (34) and this might be able to complement the *sacK* deficiency in strain NZ9841. To verify the presence of a second *sacK*-like gene in *L. lactis* NZ9800, an internal fragment of the *sacK* gene was used as a probe against chromosomal DNA of strain NZ9800 digested with *Hin*dIII. In addition to the Tn*5276* derived hybridizing band of 7 kb, another hybridizing fragment of 5 kb was identified. This DNA fragment was of chromosomal origin, but not located on Tn*5276*, since it was also detected in chromosomal DNA isolated from the isogenic, Tn*5276*-free strain MG1614 (data not shown).

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                                                                                                    100
L. l.  MS---VYTGSIEAGGTKFVLAIADEKFNIIKKFKFATTTPQETISKTIKYFK------ENRVSAIGLGSFGPIDLNLSSKTYGYITSTPKVGWKNINLVGQI
S. m.  MS---KLYGSIEAGGTKFVCAVGDENFQILEKVQFPTTTPYETIEKTVAFFKK----FEADLASVAIGSFGPIDIDQNSDTYGYITSTPKPNWANVDFVGLI
Z. m.  MKNDKKIYGCIEGGGTKFMLALIDSDRKMLAVERVPTTTPEETLGKSVEFFKKALPQYADSFASFGIASFGPLCLDRKSPKWGYITNTPKPFWPNTDVVTPF
       *     ** ** *****  * *          **** ** *   **              ****      *  **** *** * *  *
                                                                                                    200
L. l.  KEALDIPIYFTTDVNASAYGE-MKNT--GIKNLVYLTIGTGIGGGAIQNGYFIGGIGHSEMGHQRINRHRDV--NTFEGICPFHGDCLEGVAAGPSLEARTG
S. m.  SKDFKIPFYFTTDVNSSAYGETIARS--NVKSLVYYTIGTGIGAGAIQNGEFIGGMGHTEAGHVYMAPHPNDVHHGFVGTCPFHKGCLEGLAAGPSLEARTG
Z. m.  KEAFGCPVEIDTDVNGAALAENFWGASKGTNTSVIYVTVGTGFGGGVLIDGKPIHGLAMPEMGHGIPIRHPD--DRDFEGCCPYHGGCYEGLASGTAIRKRWG
         *   ****  * *          ** * *** * *    * * * * * **     *      * * ** *  * ** * *    * *
                                                                                                    300
L. l.  ILGEKISSDD--PIWDILSYYIAQAAINATLTLAPECIILGGGVMEKPNMISLIQKQFISMLNNYIDLPCSVEKYIRLPTVKENGSATLGNFYLAYSLFTKE
S. m.  IRGELIEQNS--EVWDIQAYYIAQAAIQATVLYRPQVIVFGGGVMAQEHMLNRVREKFTSLLNDYLPVP-DVKDYIVTPAVAENGSATLGNLALAKKIAAR-
Z. m.  KALNEMEPAEFEKAREIIAFYLAHFNVTLQAFISPERIVPGGGVMHVDGMLASVRRQTAEIANSYFEGA-DFEKIIVLPG-LGDQAGMMGAFALALAAENK-
                  *  * *          * *  *****    **          * *          * *          *  **
```

Figure 2. Alignment of the fructokinase members of the ROK family. Conserved aminoacids are indicated with asterisks, the region containing the consensus pattern for the ROK (NAGC/XYLR) family of proteins ([LIVM]-x-(2)-G-[LIVMFCT]-G-x-[GA]-[LIVMFA]-x(8)-G-x(3,5)-[GATP]-x(2)-G-[RKH]) is underlined. The fructokinase protein sequences of *L. lactis* (*L. l.*), *Streptococcus mutans* (*S. m.*) (27) and *Zymomonas mobilis* (*Z. m.*) (46) were analysed using the Clustal alignment program from the PC/GENE package (Intelligenetics Inc.). The NH_2 terminal amino acids in the *L. lactis* sequence that are identical to the previously purified fructokinase are indicated in bold.

Transcriptional analysis of the *sac* genes. Northern analyses were performed to determine the transcriptional organization of the *sacBK* and *sacAR* genes. The RNA from cells of strain NZ9800 grown on glucose and sucrose was probed with internal DNA fragments of all *sac* genes (Fig. 3). After hybridization with both a *sacB* and a *sacK*-specific probe a transcript of approximately 3.2 kb was observed in RNA isolated from sucrose grown cells but not in RNA isolated from glucose grown cells, indicating that the *sacBK* genes are located on a single sucrose-inducible transcript. The size of this transcript suggests that the transcription terminates at a putative *rho*-independent terminator structure that was identified immediately downstream the *sacK* gene (Fig. 1). Another sucrose-inducible transcript of approximately 3.4 kb was observed when RNA from sucrose-grown cells was hybridized with a *sacA* or a *sacR* probe. This transcript was absent in RNA isolated from glucose-grown cells. When *L. lactis* NZ9800 was grown on a mixture of sucrose and glucose a severe reduction of the *sacBK* and *sacAR* transcription could be observed (Fig. 3). A third sucrose-inducible transcript of 1.8 kb was shown to hybridize with a *sacR*-specific probe, suggesting a second regulated promoter driving transcription of the *sacR* gene. This transcript is likely to end at the same transcriptional terminator as the 3.4-kb transcript that initiates from the *sacA* promoter. The sucrose-specificity of the *sac*-transcripts was further established by the finding that no transcription of any of the *sac* genes could be detected when the cells were grown on fructose, maltose or galactose (not shown).

Analysis and comparison of the *sac* promoters. Primer extension analysis was performed using total RNA isolated from strain NZ9800 grown on sucrose and glucose. A transcription initiation start site was mapped upstream of the *sacR* gene, which is preceded by a sequence corresponding to consensus *L. lactis* promoters (37) (Fig. 4). A very low level of the *sacR* primer extension product was detected in RNA isolated from cells grown on glucose compared to that obtained with RNA isolated from sucrose-grown cells (data not shown). Because the previously mapped *sacB* and *sacA* promoters (21) and the *sacR* promoter appeared to be regulated in a similar way, a comparison was made between the promoter regions.

Strikingly, the *sacB, sacA* and *sacR* promoters contain a similar imperfect inverted repeat located upstream of the transcription start sites (Fig. 4), which could represent the binding site of a factor involved in sucrose specific regulation.

***sacR* encodes a repressor of the *sac* operons.** To investigate the role of *sacR* in gene regulation, this gene was overexpressed in the wild-type strain NZ9800 by introducing plasmid pNZ9255. When protein extracts of strain NZ9800 with and without plasmid pNZ9255 were separated by SDS-PAGE an additional protein band was visible in the former extract with a size of approximately 37 kDa (data not shown). We conclude that this is the SacR protein since its calculated molecular mass is 37,856 Da.

The maximal specific growth rate of strain NZ9800 harboring plasmid pNZ9255 grown on glucose was not affected by the overexpression of *sacR*. The growth rate of this strain on sucrose, however, was reduced to less than 0.2 h^{-1} compared to 1.22 h^{-1} of the wild-type strain, indicating that SacR acts as a repressor of one or more of the *sac* genes. To further substantiate this finding, the *sacR* gene was disrupted by using replacement recombination. No differences in growth rate could be observed between strain NZ9860 (Δ*sacR*) and the wild-type strain NZ9800 when the cells were grown on either glucose or sucrose.

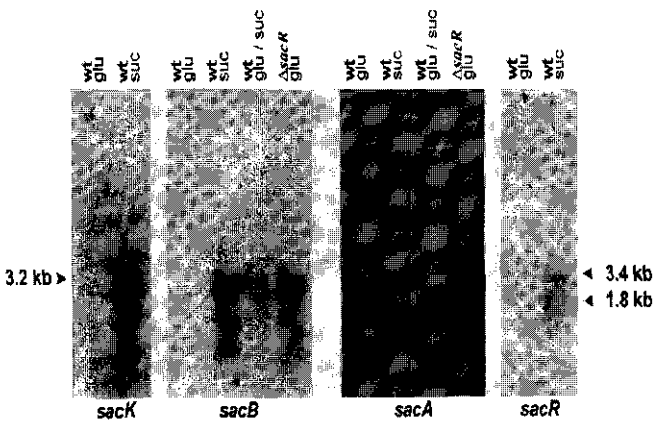

Figure 3. Northern analysis of *sacK*, *sacB*, *sacA* and *sacR* gene expression in *L. lactis* strains NZ9800 and NZ9860. Cells were grown to mid logarithmic phase on glucose (glu), sucrose (suc) and a mixture of glucose and sucrose (glu / suc) and RNA was isolated, separated and blotted. Blots were hybridized with DNA probes specific to the indicated *sac* genes. The size of the transcripts was estimated by comparing their migration distance to that of RNA markers run in parallel.

Northern analysis of RNA isolated from strain NZ9860 (Δ*sacR*) grown on glucose showed that the level of *sacBK* and *sacAR* transcription had become constitutive at a level slightly increased compared to that of the wild-type strain grown on sucrose (Fig. 3). In addition, the transcription level of *sacBK* and *sacAR* in strain NZ9860 did not vary when cells were grown on glucose or sucrose (data not shown). These results demonstrate that SacR acts as a repressor of both *sacBK* and *sacAR* transcription when the cells are grown on glucose. Furthermore, they suggest that SacR-mediated repression prevents maximal transcription of the *sacBK* and *sacAR* genes in sucrose-grown cells.

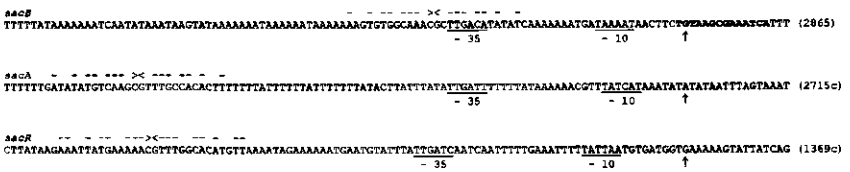

Figure 4. Alignment of the *sacB, sacA,* and *sacR* promoter regions. The –35 and –10 boxes are underlined. Further relevant features are indicated and include the mapped transcription start sites (arrows), a putative *cre* box (bold), and the imperfect inverted repeats (facing arrows). The numbers in brackets correspond to the positions in the deposited sequence data.

Discussion

In this study we describe the transcriptional and functional analyses of the sucrose gene cluster of *L. lactis* located on the conjugative transposon Tn*5276*. The *L. lactis* gene cluster consists of two divergent operons *sacBK* and *sacAR* and its comparison with other sucrose gene clusters shows it to be the only one so far known containing all three structural genes necessary for the complete catabolism of sucrose as well as a specific regulatory gene (Fig. 5). A common feature is that the *S. mutans, Staphylococcus xylosus* and *B. subtilis* gene clusters contain genes homologous to the *L. lactis sacB* and *sacA* genes, encoding sucrose-specific enzyme II proteins and sucrose-6-phosphate hydrolases involved in sucrose transport and hydrolysis of the intracellular sucrose-6-P, respectively (Fig. 5). While the sucrose-6-P hydrolases are similar in size and highly conserved, the sucrose transporters show several striking differences in organization and predicted structure. The *L. lactis* SacB protein is predicted to contain enzyme IIA, B and C domains necessary for the phosphorylation and translocation of a sugar across the cytoplasmic membrane (23). The disruption of the *L. lactis sacB* gene indicated that it is essential for the utilization of sucrose in *L. lactis* because strain NZ9840 was no longer able to grow on sucrose. So far, only in *S. mutans* a sucrose-specific enzyme II protein has been reported that also contains all these three domains (26). In contrast, the sucrose-specific enzyme II proteins from *B. subtilis* (7) and *Staphylococcus xylosus* (39) lack the EIIA domain explaining the smaller size of the *sacP* and *scrA* genes, respectively (Fig. 5).

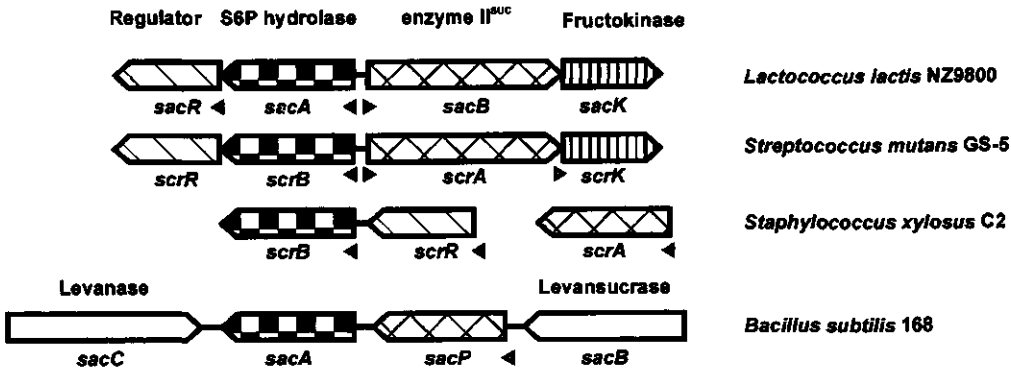

Figure 5. Comparison of the sucrose gene clusters from the Gram-positive bacteria *L. lactis*, *S. mutans* (26, 27), *B. subtilis* (7) and *Staphylococcus xylosus* (3, 7, 39). The function of the proteins encoded by the *L. lactis* genes are indicated. Homologous genes in other gene clusters have identical shading. Mapped (closed triangles) and proposed (open triangles) promoters are indicated. The ORF encoding a putative regulator protein in the *S. mutans* gene cluster is indicated as an open box. The *Staphylococcus xylosus scrA* gene is arbitrary placed in the same orientation as the *scrR* and *scrB* genes which are located at a different locus in the chromosome.

In *B. subtilis* the phosphate group necessary to energize the translocation of sucrose across the membrane by the sucrose-specific EIIBC protein is provided by the membrane-located glucose-specific enzyme IIABC protein (31). In *Staphylococcus xylosus* it has not been clarified which PTS component phosphorylates the sucrose-specific enzyme II (39).Immediately downstream of the *L. lactis sacB* gene, the *sacK* gene is located, coding for a fructokinase, since its deduced protein sequence has 25 identical NH_2-terminal amino acids, the same molecular weight, and a similar amino acid composition as the fructokinase that was previously purified from *L. lactis* subsp. *lactis* K1 (34). Since SacK showed high sequence homology to fructokinases classified in the ROK family (36) and based on the fact that SacK contains the consensus pattern found in proteins belonging to this family, we propose to classify the SacK protein within the ROK family (Fig . 2).

The ROK family of proteins groups together sugar kinases and regulator proteins of sugar catabolic operons, based on the presence of conserved residues that are suggested to be involved in sugar binding (36). The putative fructokinase members of the ROK family from *Z. mobilis* and the Gram-positive bacteria *S. mutans* and *L. lactis* were compared. These fructokinases show significant sequence identities (39.3% – 56.9 %) among each other but the sequence homology to other fructokinases is lower indicating a separate evolutionary development of these enzymes (Fig. 2). The observation that the *sacK* mutant strain NZ9841 appeared to be able to utilize both the glucose and the fructose moiety of sucrose indicated that *sacK* is not essential for the utilization of sucrose in *L. lactis*.

Furthermore, it suggested that the fructose moiety of sucrose can be metabolized via a *sacK*-independent pathway. This can be explained by the activity of a second fructokinase, which has been described in *L. lactis*, in addition to the Tn*5276*-located *sacK* gene described here (34). A second DNA band that hybridizes with a *sacK*-specific probe suggests the presence of a chromosomally located *sacK*-like gene, which may encode a protein that complements the *sacK* deficiency. An alternative strategy for the utilization of the fructose moiety might be its export and subsequent uptake and phosphorylation via the fructose-specific PTS system, which was recently shown to be the sole fructose-uptake system in *L. lactis* (16). Similar observations were made in the sucrose metabolism in *Corynebacterium glutamicum* (6).

The deduced amino acid sequence of the protein encoded by the *sacR* gene, which is located downstream of the *sacA* gene, shows sequence homology to proteins of the LacI/GalR family of transcriptional regulators (40). So far, genes encoding regulatory proteins belonging to the LacI/GalR family of transcriptional regulators have only been reported for the *L. lactis* and *Staphylococcus xylosus* sucrose gene clusters (Fig. 5). Interestingly, careful analysis of the *S. mutans* sucrose gene cluster (accession number M36849) revealed the presence of the 5' end of a *sacR*-like gene located downstream of the *scrB* gene, suggesting a similar organization as the *L. lactis* gene cluster (Fig. 5).

Northern analysis of cells grown on different carbon sources indicated that the sucrose gene cluster gives rise to three transcripts. These results extend previous transcription studies (21) and show that a 3.2-kb sucrose-inducible transcript contains both the *sacB* and *sacK* genes. This transcript initiates from the *sacB* promoter and is likely to terminate at the inverted repeat located downstream of the *sacK* gene. A transcript of 3.4 kb, only observed when the cells were grown on sucrose, was shown to contain the *sacA* and *sacR* genes and initiates from the *sacA* promoter. Since its size exceeds that of the combined sizes of the *sacA* and *sacR* genes, this transcript most likely terminates approximately 0.8 kb downstream of the *sacR* gene.

We also report the presence of a third sucrose-inducible transcript of 1.8-kb, which contains the *sacR* gene and is likely to terminate at the same transcriptional terminator as the 3.4-kb *sacAR* transcript that initiates from the *sacA* promoter. The *sacR* transcript starts at a promoter which was mapped upstream of the *sacR* gene and was found to be activated when sucrose is present in the growth medium. Primer extension studies also showed a low level of transcription from this promoter when the cells were grown on glucose. The *sacR* promoter shows striking homology to the *sacB* and *sacA* promoters since all three promoters contain a highly conserved inverted repeat located at different positions upstream of the transcription start sites (Fig. 4). It is feasible that this structure acts as an operator site since the *sacB, sacA* and *sacR* promoters are regulated in a similar way.

Under non-induced circumstances a regulator protein (probably SacR; see below) might bind to these structures and thus repress the transcription of the *sacBK, sacAR* operons, and the *sacR* gene. In the presence of an inducer molecule, probably sucrose-6-P (see below), the repressor is likely to dissociate from the operator and the transcription of the sucrose genes initiates. In contrast to the sucrose gene cluster from *E. coli* (28), the transcription of the *L. lactis sac* genes is not induced by the addition of fructose to the growth medium. The uptake of fructose in *L. lactis* is catalyzed by the PTS (16) and yields fructose-6-P, which is also the product of the fructokinase. Since growth on fructose does not induce the transcription of the *sac* genes, induction by fructose-6-P can be ruled out and therefore sucrose-6-P remains the most probable inducer molecule.

Inactivation of *sacR* indicated that SacR acts as a repressor of transcription because the transcription of the *sacB* and *sacA* promoters became constitutive in strain NZ9860 (Δ*sacR*). Moreover, the observation that the level of *sacBK* and *sacAR* transcription in strain NZ9860 is slightly elevated compared to that of the wild-type when the cells were grown on sucrose could suggest that SacR-mediated repression prevents full induction of the *sac* genes, even under induced conditions. Overproduction of SacR confirmed its repressor function since growth on sucrose was almost completely abolished probably due to complete repression of the *sacBK* and *sacAR* expression.

A striking difference with the regulation of the *Staphylococcus xylosus scrB* gene is the fact that the regulatory *scrR* gene appears to be constitutively transcribed while the transcription of the *L. lactis sacR* gene is sucrose-inducible (9). Similar negative autoregulation of a repressor gene by its own geneproduct has also been reported for the GalS protein involved in the regulation of the galactose genes in *E. coli* (41). Similar to the GalS system a higher level of SacR under induced circumstances would allow faster repression of the *sac* genes when the concentration of the inducer molecule decreases. A low concentration of SacR protein under non-induced circumstances would allow an immediate derepression of *sacBK* and *sacAR* transcription, assuming that low concentrations of the inducer molecule result in the dissociation of SacR from the operator site.

The observed substrate induction and negative autoregulation of *sacR* by its gene-product results in a very efficient regulation of the transcription of the *sac* genes in response to variations in extracellular sucrose concentrations.

The transcription of *sacBK* and *sacAR* was strongly reduced when the wild-type strain NZ9800 was grown on a mixture of sucrose and glucose as compared to cells grown on sucrose alone, indicating a form of glucose repression. An explanation for the observed glucose repression could be catabolite repression which in Gram-positive bacteria is mediated by the *trans*-acting transcriptional regulator CcpA (Catabolite control protein A) in combination with the *cis*-acting *cre* (catabolite responsive element) (11).

Covering the transcription start site of the *sacB* gene a putative *cre* site was identified (Fig. 4) with only one mismatch compared to the consensus *cre* (42), which may indicate the involvement of CcpA in the glucose repression of the *sac* genes. This situation would resemble that of the *Staphylococcus xylosus sacB* gene where a limited CcpA-dependent catabolite repression was observed (9). However, our results show that glucose repression of the *L. lactis sacB* gene is strictly dependent on the presence of SacR. In addition, the inactivation of the *L. lactis ccpA* gene does not affect the transcription of the *sac* genes (15). This indicates that, under these conditions, the CcpA-mediated control does not play a significant role in the glucose repression of the *sac* genes in *L. lactis* NZ9800. The level of *sacBK* and *sacAR* transcription in strain NZ9860 (Δ*sacR*) grown on glucose or sucrose appeared to be similar, indicating that SacR is not only involved in the substrate induction but also in mediating the glucose repression.

The glucose repression found in the wild-type strain NZ9800 can most likely be explained by the inducer exclusion and expulsion mechanisms that are active in *Lactococcus lactis* (44, 45). Both of these mechanisms might lead to a reduction of the putative inducer (sucrose-6-P) concentration explaining the requirement of SacR for the observed glucose repression. The tight regulation of the expression of the *sac* genes by the operon-specific regulator SacR and the apparent independence of the chromosomally encoded CcpA-mediated catabolite repression, may be a consequence of their location on a conjugative transposon probably of non-lactococcal origin that may be transferable to a variety of hosts.

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CHAPTER 3

Immunological crossreactivity to the catabolite control protein CcpA from *Bacillus megaterium* is found in many Gram-positive bacteria

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Immunological crossreactivity to the catabolite control protein CcpA from *Bacillus megaterium* is found in many Gram-positive bacteria

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Abstract

The catabolite control protein CcpA from *Bacillus megaterium* was overproduced as a fusion protein to a 6xhis affinity tag and purified to homogeneity. Polyclonal antibodies of high affinity and specificity were raised against the purified protein. The serum did not crossreact with purified Lac repressor despite the fact that CcpA and LacI belong to the same protein family. Using this antiserum we identified proteins that share antigenic determinants with CcpA in many Gram-positive bacteria, including bacilli, staphylococci, streptococci, lactic acid bacteria, and some actinomycetes.

Keywords: LacI/GalR family; Carbon catabolite repression; Immunoblot assay; *Bacillus megaterium*; Gram-positive bacteria

1. Introduction

Carbon catabolite repression (CCR) in *Bacillus subtilis* and *B. megaterium* is distinct from the well known CRP and cyclic AMP mediated regulatory network in *Escherichia coli* [20,3,23]. It involves the catabolite control protein CcpA [9,12], which can act as a repressor or an activator of transcription (reviewed in [10]), and a catabolite responsive element (*cre*) [26] acting in *cis* and occurring in many genes [11]. CcpA can interact specifically with P-ser46-HPr [6], a component of the phospho*enol*pyruvate:sugar phosphotransferase system (PTS) [20], whose presence in the seryl-phosphorylated form is a consequence of the availability of high amounts of PTS substrates [5,22]. It was also shown that CcpA can bind to *cre* both in the absence [14] and in the presence of P-ser46-HPr [8]. Thus, the available evidence indicates clearly that a completely different molecular signalling chain is employed in CCR in *Bacillus* as compared to *E. coli* [23].

CcpA belongs to the LacI/GalR family of bacterial regulatory proteins [25]. It shows 28% (*B. megaterium*) and 25% (*B. subtilis*) identical residues with LacI. We describe here the use of polyclonal antibodies raised against *B. megaterium* CcpA to screen for immunological crossreactivity in a large variety of Gram-positive bacteria. The results suggest that this new type of CCR mechanism may be widely distributed among the Gram-positive bacteria.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli RB791/pWH1564, carrying the *ccpA(his)* gene, was grown in Luria-Bertani medium at 37°C. Overexpression was induced by addition of 0.5 mM IPTG as the culture reached an OD_{600} of 0.7. Growth was allowed to proceed for another 3 h, then the cells were harvested. After centrifugation, the cell paste was stored at −20°C until use.

Bacterial strains used for the immunoblot assays are listed in Table 1. They were grown in PA (Oxoid), JP [19], MRS (Difco), glucose M17 (GM17, Merck) or brain heart infusion (BHI, Difco) medium at the temperatures indicated in Table 1 and with the supplements given therein. 10 ml aliquots were harvested after overnight incubation or when the culture reached visible turbidity (1–2 days). Cell pellets were either used immediately or stored at −20°C.

2.2. Determination of protein concentration

To measure protein concentration, the Bio-Rad dye binding assay was used. Bovine serum albumin served as the protein standard.

Table 1
Bacterial strains used in this study

Organism	Strain/relevant genotype	Source or reference	Growth medium (temperature, °C)
Actinoplanes brasiliensis		*	JP (18)
Arthrobacter crystallopoites	DSM 20117	DSM	PA (28)
Bacillus cereus	DSM 626	DSM	PA (28)
Bacillus cereus var. mycoides	DSM 299	DSM	PA (28)
Bacillus circulans	DSM 11	DSM	PA (28)
Bacillus licheniformis	BGSC 5A24	BGSC	PA (37)
Bacillus megaterium	WH 320	[13]	LB or PA (37)
Bacillus megaterium	WH 367/Δ*ccpA*	[13]	LB or PA (37)
Bacillus stearothermophilus	Merck 11499	Merck, Darmstadt, Germany	PA (60)
Bacillus subtilis 168	BGSC 1A1	BGSC	LB or PA (37)
Bacillus thuringiensis	DSM 350	DSM	PA (28)
Enterococcus faecalis	ATCC 11700	ATCC	GM17 (37)
Escherichia coli	RB791	[2]	LB (37)
Lactobacillus casei	ATCC 393	ATCC	MRS (30)
Lactobacillus plantarium	DSM 20174	DSM	PA + 1% glucose (28)
Lactococcus lactis	NZ9700	[17]	GM17 (30)
Micrococcus luteus	DSM 20030	DSM	PA (28)
Mycobacterium phlei	DSM 43214	DSM	PA + 1% glycerol (37)
Nocardia rubra		*	JP (28)
Sporosarcina ureae	DSM 317	DSM	PA + 2% urea (28)
Staphylococcus carnosus SK311	DSM 20501	DSM	PA (37)
Staphylococcus xylosus C2A	DSM 20267	DSM	PA (37)
Staphylococcus xylosus TX154	TX154/*ccpA::ermB*	[7]	PA + erm 2.5 mg/l (37)
Streptococcus salivarius	DSM 20067	DSM	PA (37)
Streptococcus sanguis	SM 101	[1]	BHI (30)
Streptococcus thermophilus	CNRZ 302	INRA	GM17 (37)
Streptomyces coelicolor	DSM 40233	DSM	JP (28)
Streptomyces lividans	1B26	*	JP (28)
Streptomyces reticuli WT	Tü45	*	JP (28)

* Strains were obtained from H. Schrempf, Osnabrück, Germany.
BGSC: Bacillus Genetic Stock Center, Ohio State University, Columbus, OH, USA.
DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.
ATCC: American Type Culture Collection, Rockville, MA, USA.
INRA: Institut National de la Recherche Agronomique, France.

2.3. Purification of CcpA(his)

Frozen cell pellets of *E. coli* RB791/pWH1564 grown as stated above were thawed on ice and resuspended in SBT buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5 mM 2-mercaptoethanol, 0.1 mM PMSF). All subsequent steps were performed at 4°C. Cells were disrupted by sonication applying 5 bursts of 50 W, 30 s in duration with 30 s intervals. The lysate was centrifuged at 25 000 × *g* for 30 min. The supernatant was immediately used for chromatography.

The crude cell extract was passed over a Ni^{2+}-NTA-agarose column (Qiagen, Hilden, Germany) mounted on a Pharmacia FPLC apparatus and CcpA(his) was eluted using a linear 30–150 mM gradient of imidazole. Fractions were analyzed by SDS-PAGE for the presence of protein of the expected molecular mass.

2.4. Western blotting and immunodetection

Cell pellets of different Gram-positive bacteria were resuspended in 200 μl of SBT. An equal amount of SBT containing 5 mg/ml lysozyme was added (for *Staphylococcus* sp.: +70 μg/ml lysostaphin) followed by a 30 min incubation at 37°C. The cells were returned on ice and sonicated until the lysate cleared (up to 4 20 s bursts of 50 W). Alternatively, resuspended cell pellets were subject to mechanical disruption in the presence of Zirconium glass beads as previously described [17]. Lysates were not centrifuged prior to the addition of sample loading buffer for denaturing gel electrophoresis. They were heated in boiling water for 10 min and samples of 1–15 μl were loaded on a 10% SDS-PAGE gel. Permanently dyed proteins (Amersham Rainbow Marker) were used as molecular mass indicators. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Fluorotrans, Pall) by means of an electroblotting device (V8-10, BRL). CcpA was detected with rabbit polyclonal serum produced in response to repetitive immunization with purified CcpA(his) and Freund's adjuvant. Antibodies were in turn detected using alkaline phosphatase-conjugated protein A (Sigma) or goat anti-rabbit peroxidase conjugate (Gibco/BRL) as described by the manufacturer. Western blots of each sample were repeated at least twice.

3. Results

3.1. Cloning, overproduction and purification of CcpA(his)

pWH1564 is a derivative of pQE9 (Qiagen) into which *ccpA* from *B. megaterium* was translationally fused to a region encoding a 6xhis affinity tag and an enterokinase cleavage site [5]. The encoded protein has a 17 residue N-terminal extension including the affinity tag (underlined) and the protease recognition site (italics): Met-Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser-*Asp-Asp-Asp-Asp-Lys*-**Met**... *E. coli* RB791 transformed with pWH1564 yielded an intense band of overproduced protein migrating at the expected deduced mass of about 38 kDa in SDS-PAGE.

CcpA(his) was purified by passing crude cell extract of overproducing *E. coli* RB791/pWH1564 over a Ni^{2+}-NTA-agarose column followed by elution with an imidazole gradient. The fusion protein typically eluted at about 100 mM imidazole. A 500 ml culture yielded about 25 mg of pure protein.

3.2. Specificity and sensitivity of anti-CcpA antibodies

Rabbits were injected with complete Freund's adjuvant and purified CcpA(his) to raise polyclonal anti-CcpA antibodies. Serum from these rabbits was used in immunoblot experiments. The reactivity of the antiserum was confirmed by dot blot experiments using different amounts of purified CcpA(his) and bovine serum albumin as a control. The serum detected down to 10 ng of purified protein (data not shown). No crossreactivity with an unrelated protein containing the same 17 residue N-terminal extension was observed, indicating that the serum detects only CcpA specific epitopes. CcpA belongs to the LacI/GalR family of bacterial regulatory proteins [25]. We have therefore tested the cross-reactivity of anti-CcpA with LacI (see Fig. 1). A Western blot containing 2 μg of purified LacI (kindly provided by Dr. B. Müller-Hill, Cologne, Germany) did not show any signal with anti-CcpA.

Wild-type *B. megaterium* WH320 and WH367 *ΔccpA* containing an in-frame deletion in the chromosomal *ccpA* gene [13] were propagated in LB and

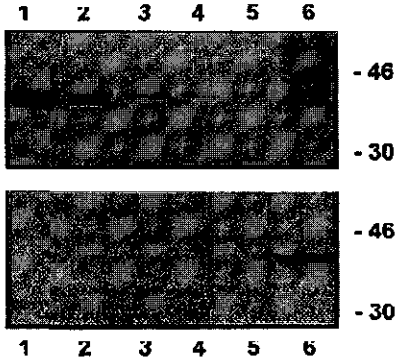

Fig. 1. Specificity of anti-CcpA antibodies. Two SDS-PAGE gels were loaded with identical samples of purified LacI and *B. megaterium* WH320 protein extract. One was Coomassie-stained (top panel), the other one subjected to Western blot with anti-CcpA (bottom panel). Positions of molecular mass markers of 46 and 30 kDa are given on the right. Lanes 1–5 contain: 2 μg, 1 μg, 500 ng, 250 ng, and 100 ng LacI, respectively, and lanes 6 were loaded with 1 μg of *B. megaterium* WH320 crude protein extract. Exposure time of the blot was 5 times longer than that shown in Fig. 2A.

harvested at an OD_{600} of 0.6. Total cell protein equalling 0.01 OD_{600} was subjected to Western blotting with anti-CcpA (see Fig. 2a). A crossreacting protein with a size of approximately 38 kDa was detected in the extract from wild-type cells, while proteins from the *ΔccpA* strain did not yield any background signal under these conditions. Furthermore, *E. coli* RB791, the expression host for CcpA(his), did not yield any specific band in the Western blot (see Fig. 2a, lane 10). We conclude from this result that the anti-CcpA serum is highly specific for CcpA.

3.3. Immunological screening for CcpA-like proteins in different Gram-positive bacteria

To screen Gram-positive bacteria for immunological crossreactivity to anti-CcpA, strains of different species were propagated in complex liquid media supplemented with additives (see Table 1). Crude cell extracts were produced by incubation with lysozyme (and lysostaphin for *Staphylococcus* sp.) followed by sonication or by mechanical disruption.

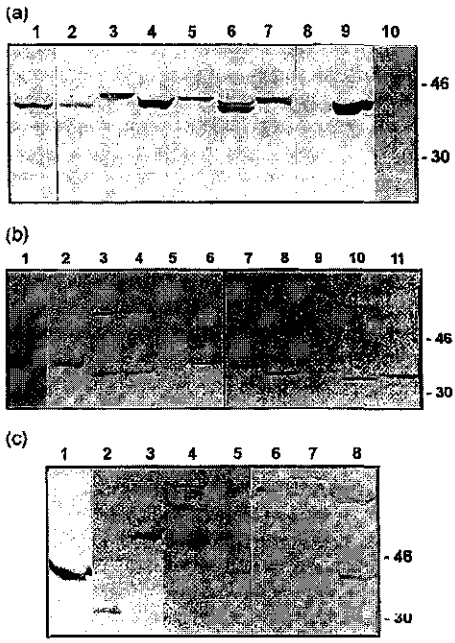

Fig. 2. Western blot analysis of cell extracts of different groups of Gram-positive bacteria. Lanes in panel A were loaded with 4 μg of total cell protein (0.005 OD_{600} equivalents), lanes in panels B and C and lane 10 of panel A contain 10–20 times more protein as measured by BioRad dye binding assay. Sizes of molecular mass markers of 46 and 30 kDa (Amersham Rainbow Marker) are indicated on the right. Extracts of the following species were applied in the lanes indicated in brackets. a: *Bacillus* species and *Escherichia coli*, *Bacillus cereus* (1), *Bacillus cereus var. mycoides* (2), *Bacillus circulans* (3), *Bacillus licheniformis* (4), *Bacillus stearothermophilus* (5), *Bacillus subtilis* (6), *Bacillus thuringiensis* (7), *Bacillus megaterium* WH367 *ΔccpA* (8), *Bacillus megaterium* WH320 (9), *Escherichia coli* RB791 (10). b: Non-*Bacillus* Gram-positive bateria of low GC content. *Lactobacillus plantarum* (1), *Sporosarcina ureae* (2), *Staphylococcus carnosus* (3), *Staphylococcus xylosus* (4), *Staphylococcus xylosus ccpA::ermB* (5), *Streptococcus salivarius* (6), *Lactococcus lactis* (7), *Streptococcus thermophilus* (8), *Streptococcus sanguis* (9), *Enterococcus faecalis* (10), *Lactobacillus casei* (11). c: Gram-positive bacteria of high GC content. *Arthrobacter crystallopoites* (1), *Actinoplanes brasiliensis* (2), *Micrococcus luteus* (3), *Mycobacterium phlei* (4), *Nocardia rubra* (5), *Streptomyces coelicolor* (6), *Streptomyces lividans* (7), *Streptomyces reticuli* (8).

Complete cell proteins were then used for Western blot analysis.

All the *Bacillus* species showed a signal at about the same molecular mass as *B. megaterium* CcpA (see Fig. 2a). Cell extracts used for this analysis were derived from equal amounts of cells as determined by optical densities of the original cultures and by quantifying the protein contents of the extracts. We have repeatedly observed a double band in the blot of proteins from *B. subtilis*.

To obtain signals in Western blots of non-*Bacillus* Gram-positives, about 10–20 times as much protein extract compared to the *B. megaterium* blots was applied to the gels (Fig. 2b,c). All of the Gram-positive bacteria with low GC content showed one or two distinct bands in the immunoblot (Fig. 2b). Since the apparent crossreactivity is much weaker in this group of bacteria we aimed to provide additional evidence for the specificity of this result. We have achieved this using extracts from *Staphylococcus xylosus* TX154 (kindly provided by R. Brückner, Tübingen, Germany) which is isogenic to *S. xylosus* C2A except for a *ccpA* inactivation constructed by insertion of an antibiotic resistance cassette. The bands seen clearly in the extract of *S. xylosus* C2A are not present in the one from that mutant (see Fig. 2b, lanes 4 and 5). This result leads to the important conclusion that the signals are indeed caused by the protein expressed from the *ccpA* gene and that both bands originate from the CcpA protein. In addition, extracts of several other species belonging to the low GC content group of Gram-positive bacteria showed a signal with anti-CcpA of approximately 38 kDa (*Leuconostoc*, *Listeria*, and *Pediococcus*, data not shown).

Gram-positive bacteria of high GC content were also analyzed for crossreactivity to anti-CcpA antibody. The results are shown in Fig. 2c. Some of these bacteria were only poorly lysed or (e.g. *Mycobacterium phlei*) showed several bands in the Western blot. The second observation can be explained by the fact that complete Freund's adjuvant was used for immunization. It contains *M. tuberculosis* cells and, thus, induces antibody production against mycobacterial proteins. A number of species showed signals similar in intensity and molecular mass to those obtained from Gram-positive bacteria with low GC content.

4. Discussion

The polyclonal antiserum used in this study is exceptionally specific for CcpA. This is highlighted by the fact that *B. megaterium ccpA* deletion mutant WH367 did not show any background signals in the Western blot. Furthermore, LacI of *E. coli* shares 28% identical amino acid residues with CcpA of *B. megaterium* and yet, 2 μg of purified LacI does not show any crossreactivity. The greatest sequence similarity of CcpA with LacI occurs in the helix-turn-helix motifs of their DNA-binding regions, where 15 out of 21 residues are identical (71%), and in the entire DNA-binding regions with 29 out of 59 (49%) identical residues. Thus, the lack of crossreactivity even at a prolonged incubation time is remarkable. It is not surprising in the light of that result that *E. coli* RB791 carrying the *lacI*^{q} allele overproducing LacI [16] shows no crossreactivity, either. These results clearly show that crossreacting proteins either share a greater overall identity with CcpA than LacI, or contain more highly conserved sequence motifs.

Genetic evidence for the occurrence of a *ccpA* gene has been obtained for *B. megaterium* [12], *B. subtilis* [9], *Clostridium acetobutylicum* [4], *Staphylococcus xylosus* [7], *Lactobacillus pentosus* [21], *Leuconostoc lactis* [15], and *Lactobacillus casei* [18]. All of those species belong to the low GC content group. Western blot analysis of these and other species from this group clearly revealed the existence of anti-CcpA crossreacting proteins in all of the strains studied here. Immunological reactivity was substantially higher with extracts from the *Bacillus* species compared to the non-*Bacillus* bacteria. This indicates a higher degree of sequence conservation for CcpA in that genus. The molecular masses of the crossreacting proteins as estimated by migration in SDS-PAGE range around 40 kDa, which is in the range of the masses proposed for the Bacillus CcpA proteins.

The CcpA protein from *S. xylosus* exhibits 56% identity of amino acids with the one from *B. megaterium* [7]. Despite the high sequence conservation the signal with anti-CcpA is weak. The significance of the weaker signals obtained from the non-*Bacillus* bacteria compared to the bacilli is underlined by the lack of crossreactivity in the *S. xylosus ccpA* disruption mutant TX154. The fact that two bands are

present in *S. xylosus* could be due to incomplete denaturation of oligomeric CcpA, since CcpA is thought to occur as a dimer [14]. However, other non-trivial reasons are also possible. Nevertheless, both signals have disappeared in the *ccpA* mutant.

Crossreacting proteins were also detected in Gram-positive bacteria of high GC content. The signals are of about the same intensity as the one in *S. xylosus*, for which specificity for CcpA was demonstrated. They are of a wider range of molecular masses. The double bands observed in some lanes may also result from incomplete denaturation. Another explanation, especially for the faster migrating protein seen in extract from *Actinoplanes brasiliensis*, might be partial proteolysis.

Recently, components of the PTS were found in *Streptomyces* sp., however, ATP-dependent phosphorylation of HPr at ser46 could not be demonstrated [24]. It is, therefore, possible that a CcpA-like protein in these bacteria may respond to different signals.

The immunological results presented here provide evidence for the existence of CcpA-like proteins in a large number of Gram-positive bacteria. This, in turn, indicates that CCR in these bacteria may be exerted by mechanisms similar to the one found in *B. subtilis* and *B. megaterium* [8,13,14].

Acknowledgements

We thank R. Brückner for making *S. xylosus* TX154 available to us, B. Müller-Hill for providing purified LacI, and H. Schrempf for providing us with a number of Actinomycetes. This work was supported by the European Community Biotech Programme (Contract No. BIO2-CT92-0137), the Fonds der Chemischen Industrie, and the Deutsche Forschungsgemeinschaft. E.K. received a personal grant from the Friedrich-Ebert-Stiftung.

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Results

Cloning and characterization of the lactococcal ccpA *gene*

Using polyclonal antibodies raised against the purified CcpA protein from *B. megaterium* a protein band of approximately 37 kDa was identified on a Western blot of a lactococcal extract (Küster *et al.*, 1996). Therefore, a lambda-based genomic library of *L. lactis* NZ9800 was screened with the polyclonal antibodies. A recombinant phage was isolated, which upon infection of *E. coli* XL1 resulted in the production of a 37 kDa protein cross-reacting with the anti-CcpA antibodies. Partial sequence analysis of *L. lactis* DNA present in this phage revealed the presence of a *ccpA*-like gene. Overlapping fragments were cloned, combined, and sequenced, resulting in plasmid pNZ9243 carrying an intact gene, which could encode a protein of 333 amino acids with a calculated molecular mass of 36,684 Da. Since the deduced protein sequence showed 48 % identical residues compared to *B. subtilis* CcpA (Henkin *et al.*, 1991) this gene was designated *ccpA* (see below; Fig. 1).

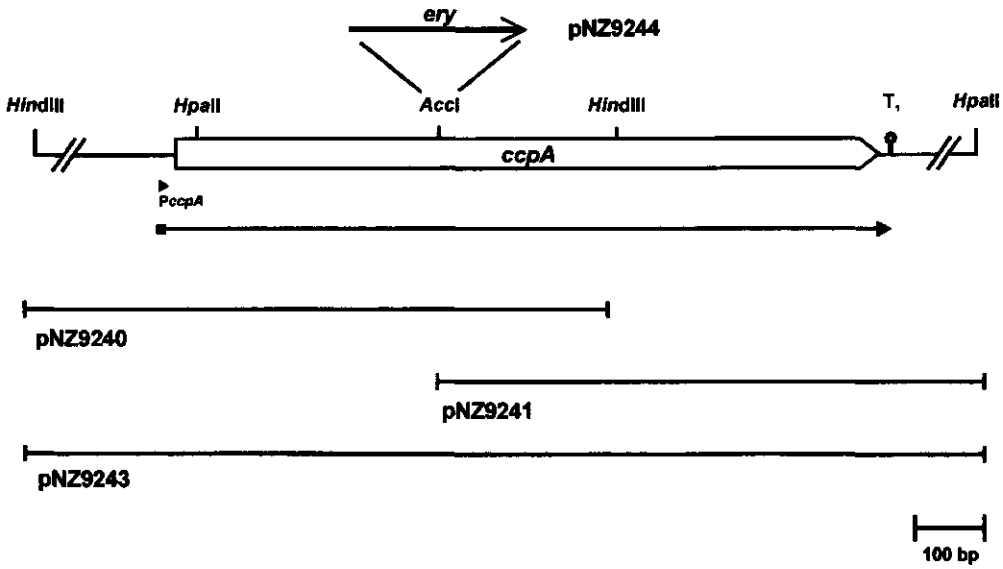

Figure 1. Transcriptional organization of the *L. lactis ccpA* gene. The *ccpA* gene is shown with its gene product and the mapped promoter. The arrows denote the promoter and transcripts mapped with primer extension and Northern analysis, respectively. Relevant restriction sites are indicated.

Disruption of the ccpA *gene and its effects on growth*

An erythromycin resistance (Ery^R) gene was introduced into a unique *Acc*I site located in the *ccpA* gene resulting in strain NZ9870. Protein extracts of strain NZ9870 no longer contained a protein of 37 kDa that cross-reacted with the antiserum raised against the *B. megaterium* CcpA (data not shown). To analyze the effect of CcpA on the carbohydrate metabolism the growth rates of strain NZ9870 on different carbon sources were compared to those of the wild-type strain NZ9800 (Table 1). Both strains were grown in M17 medium supplemented with the different carbon sources to a concentration of 1 % (w/v). A significant reduction in growth rate (ranging from 20 to 60 %) was observed on several carbon sources but the utilization of galactose was particularly affected by the disruption of the *ccpA* gene. Complementation of the *ccpA* mutation with plasmid pNZ9245 carrying the *L. lactis ccpA* gene under the control of the inducible *nisA* promoter (de Ruyter *et al.*, 1996) restored the growth defect after addition of inducing concentrations of nisin A (Table 1). Similar results were obtained with plasmid pNZ9246 that contains the *B. subtilis ccpA* gene under control of the *nisA* promoter (data not shown).

Table 1. Growth rates of strains used in this study. Average values of at least two independent determinations including the error are given.

Strains	μ_{max} (h^{-1})				
	glucose	Sucrose	fructose	galactose	maltose
NZ9800 (wild-type)	1.42 ±0.05	1.22 ±0.04	0.92 ±0.05	0.63 ±0.04	0.51 ±0.04
NZ9870 (Δ*ccpA*)	0.71 ±0.09	0.68 ±0.08	0.63 ±0.06	<0.1 ±0.10	0.30 ±0.02
NZ9870 + pNZ9245 (*L. lactis ccpA*)	1.36 ±0.11	1.20 ±0.07	0.88 ±0.03	0.60 ±0.05	0.51 ±0.09

Transcriptional analysis of the ccpA *gene*

Primer extension experiments were performed using total RNA isolated from *L. lactis* strain NZ9800 grown on glucose. Two adjacent transcriptional start sites were identified (Fig. 2) which were preceded by a sequence corresponding to consensus *L. lactis* promoters (de Vos and Simons, 1994). The same RNA was analyzed by Northern blot analysis. A band of approximately 1.2-kb hybridizing with a *ccpA*-specific probe could be identified (Fig. 3). This suggests that transcription terminates at a rho-independent terminator structure, with a ΔG value of –12.6 kcal/mol that was identified downstream of the *ccpA* open reading frame. Although the promoter region of the *L. lactis ccpA* gene does not contain a consensus *cre* site, the presence of a putative *cre* site in the *ccpA* gene at positions 436 – 449 suggested possible autoregulation of the *ccpA* expression.

Therefore, further Northern analyses were performed with RNA isolated from cells grown on different carbon sources. However, the transcription level of the *L. lactis ccpA* gene did not vary significantly in response to the carbon source, indicating that the *ccpA* gene is constitutively transcribed. This observation is in agreement with immunological data which showed that the production level of CcpA is independent of the carbon source (data not shown).

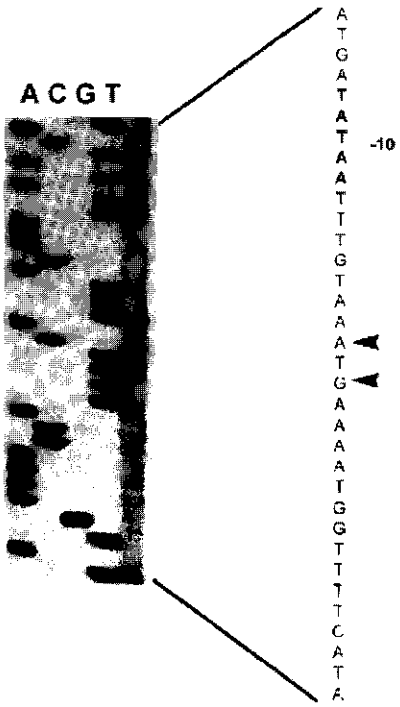

Figure 2. Primer extension analysis of the *ccpA* promoter. The transcription start sites are indicated with arrows. The putative – 10 region in the complementary strand is presented in bold. RNA was isolated from *L. lactis* strain NZ9800 grown on glucose.

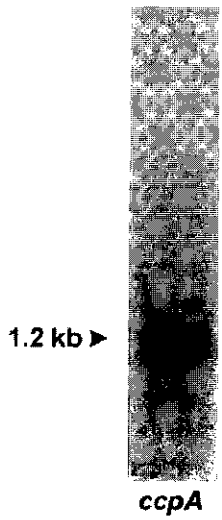

Figure 3. Northern analysis of the *L. lactis ccpA* gene. After electrophoresis the RNA was transferred to nylon membranes and probed with a *ccpA* specific probe. The size of the *ccpA* transcript is indicated with an arrow. RNA was isolated from *L. lactis* strain NZ9800 grown on glucose.

Analysis of the effect of CcpA on the transcription of the gal *operon*

The presence of a putative *cre* site in the promoter region of the recently identified *L. lactis gal* genes (Grossiord *et al.*, 1998) suggested a possible involvement of CcpA in the regulation of the expression of these genes (Fig. 4). The *gal* operon consists of five genes with the order *galAMKTE* and encodes the proteins necessary for the uptake and conversion of galactose to glucose-1-P via the Leloir pathway. The *gal* genes are located on one 7.5 kb transcript that initiates from a promoter mapped upstream of the *galA* gene (Fig. 4).

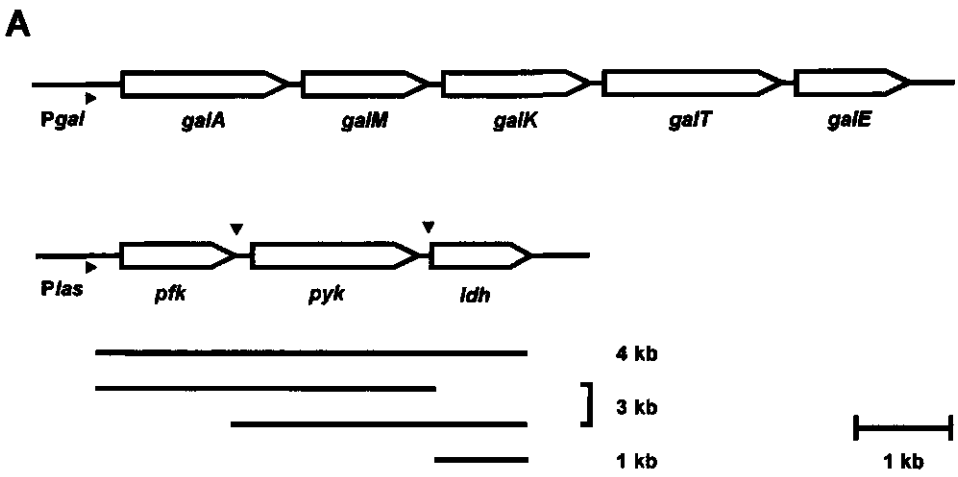

Figure 4. A. Schematic representation of the *L. lactis gal* and *las* operons. The mapped promoters are indicated with arrowheads. The proposed processing sites of the 4-kb transcript of the *las* operon are indicated with arrows. These structures with a free energy of –4.6 kcal/mol and –3.0 kcal/mol are centered around bases 1201 and 2872, respectively in the reported sequence data (Llanos *et al.*, 1993) . The putative transcripts derived from the *las* operon, observed in the Northern analyses are presented. B. Alignment of the promoter regions of the *L. lactis* NZ9800 *gal* and *las* operons. The –35 and –10 boxes are underlined and the mapped transcription start sites are indicated with arrows. The putative *cre* sites are aligned with the consensus sequence.

The *galK* gene was selected to analyze the role of CcpA in the regulation of the expression of the *gal* genes since encodes a galactokinase which is a key enzyme in the Leloir pathway. 20 μg total RNA isolated from strains grown under different circumstances was immobilized and the resulting slot blots were hybridized with a *galK*-specific probe (Fig. 5). No *gal* transcription could be detected in the wild-type strain grown on glucose but when the cells were grown on galactose the transcription increased indicating induction by a compound which is probably formed from galactose. On a mixture of 1 % glucose and 1 % galactose no transcription of the *gal* genes was detected, indicating strong glucose repression. Analysis of RNA isolated from the *ccpA* mutant strain NZ9870 indicated that no *gal* transcription could be detected on glucose but an increased transcription could be observed on the mixed substrate compared to the wild-type indicating that the *gal* gene expression was partially relieved of glucose repression. The level of *gal* transcription on the mixed substrate reached approximately 50 % of the level measured in the wild-type strain grown on galactose, indicating that the transcription initating from the *galA* promoter was not completely derepressed (Fig. 5). Similar results were obtained with different RNA concentrations (data not shown). Since the growth rate of strain NZ9870 on galactose was severely reduced, sufficient RNA from cells grown on this carbon source could not be obtained for the experiment.

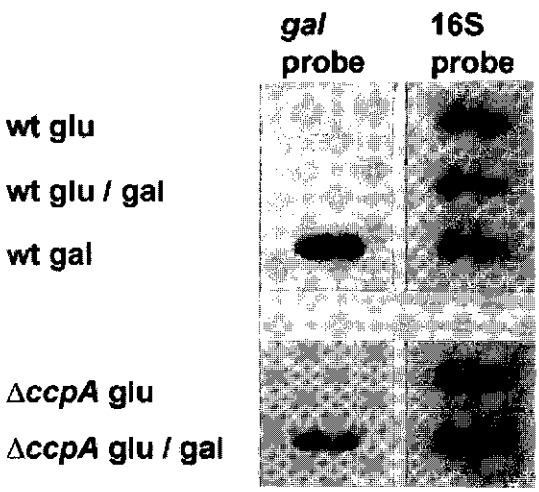

Figure 5. Slot blot analysis of 20 μg total RNA isolated from strains NZ9800 (wild-type) and NZ9870 (Δ*ccpA*) grown under different circumstances after hybridization with a *galK*-specific probe.

Analysis of the pyruvate kinase and L-lactate dehydrogenase activities in wild-type and ccpA *knock-out strains*

Careful analysis of the nucleotide sequence of the *L. lactis* LM0230 *las* operon encoding phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase (Llanos *et al.*, 1993) revealed the presence of a *cre* site located upstream of the mapped promoter, suggesting a possible involvement of CcpA in the regulation of this operon (Fig. 4). The promoter region of the *L. lactis* NZ9800 *las* operon was amplified using PCR and its nucleotide sequence was found to be identical to the published sequence (Fig. 4). In the wild-type strain the pyruvate kinase and L-lactate dehydrogenase expression levels appeared to be regulated since the enzyme actitivies in galactose grown cells were reduced to 50 % and 65 % respectively, compared to glucose grown cells (Table 2). Disruption of the *ccpA* gene resulted in a two- to fourfold reduction of both the pyruvate kinase and L-lactate dehydrogenase activities in cells grown on glucose (Table 2). Introduction of plasmid pNZ9245 into strain NZ9870 (*ΔccpA*) and induction of *ccpA* transcription by the addition of inducing concentrations of nisin A almost completely restored the activity of pyruvate kinase and L-lactate dehydrogenase indicating that CcpA plays a key role in the activation of expression of the *las* operon. The *ccpA* gene from *B. subtilis* under control of the *nisA* promoter was also able to restore the pyruvate kinase and L-lactate dehydrogenase activities in strain NZ9870 (Table 2). Remarkably, the activities of pyruvate kinase and L-lactate dehydrogenase in strain NZ9870 containing pNZ9245 grown on galactose were higher than in the wild-type strain NZ9800 grown on galactose. To analyze the effect of overproduction of CcpA on the activities of pyruvate kinase and L-lactate dehydrogenase plasmid pNZ9245 was introduced into the wild-type strain NZ9800. Induction of the transcription of the *nisA* promoter by the addition of nisin A to cells grown on glucose resulted in pyruvate kinase and L-lactate dehydrogenase activities similar to those of the wild-type strain grown on glucose. However, the values found when the cells were grown on galactose were also elevated to the level measured in glucose-grown wild-type cells suggesting that overproduction of CcpA leads to an activation of the expression of the genes of the *las* operon.

Table 2. Lactate dehydrogenase and pyruvate kinase activities of strains used in this study. Average values of at least two independent determinations as well as the error are given.

Strain	Carbon source	Pyruvate kinase µmol NADH/mg /min	Lactate dehydrogenase µmol NADH/mg /min
NZ9800 (wild-type)	glucose	3.20 ± 0.19	14.2 ± 0.41
	galactose	1.67 ± 0.13	9.05 ± 0.72
NZ9800 + pNZ9245 (*L. lactis ccpA*)	glucose	2.91 ± 0.42	13.55 ± 0.52
	galactose	2.81 ± 0.21	13.31 ± 0.71
NZ9870 (Δ*ccpA*)	glucose	0.79 ± 0.08	6.32 ± 0.55
NZ9870 + pNZ9245 (*L. lactis ccpA*)	glucose	2.72 ± 0.13	11.48 ± 1.02
	galactose	2.58 ± 0.48	11.57 ± 0.66
NZ9870 + pNZ9246 (*B. subtilis ccpA*)	glucose	2.65 ± 0.13	10.74 ± 0.34

Transcriptional analysis of the L. lactis las *operon*

The presence of a *cre* site in the promoter region of the *las* operon (Fig. 4) and the observation that the activities of pyruvate kinase and L-lactate dehydrogenase were reduced in the *ccpA* mutant NZ9870 indicated that CcpA might be involved in the transcriptional regulation of the *las* operon. Therefore, the transcription of the *las* operon was analyzed in more detail. Northern analyses were performed and the resulting blots were hybridized with *pfk*, *pyk* and *ldh* probes. Analysis of RNA isolated from the wild-type strain NZ9800 grown on glucose revealed the presence of several transcripts (Fig. 6). After probing with a *pfk*-specific probe two bands could be identified; a large transcript of 4-kb and a smaller transcript of 3-kb. Probing with a *pyk*-specific probe lead to the identification of two bands of the same sizes as those observed when probing with the *pfk* specific probe. When a *ldh*-specific probe was used three bands could be identified: in addition to the bands of 4-kb and 3-kb a small band of 1-kb was found (Fig. 6). Analysis of RNA isolated from the *ccpA* mutant strain NZ9870 grown on glucose indicated that all transcripts identified in the wild-type strain were also present in the mutant strain but at a reduced level. To correct for the amount of RNA used the same RNA was also probed with a probe specific for variable regions of the 16S rRNA. All the bands were cut from the blots and the total radioactivity of each band was determined using a liquid scintilation counter. Based on the ratio between the gene-specific and the 16S derived signals the relative mRNA levels were calculated. The reduction of the transcription levels in strain NZ9870 (Δ*ccpA*) compared to NZ9800 (wild-type) were calculated to be 3.8, 4.3 and 4.1 for the *pfk*-, *pyk*- and *ldh*-specific signals, respectively, demonstrating that CcpA acts as a transcriptional activator.

Effects of CcpA on product formation

The disruption of the *ccpA* gene did not affect the rate of glucose consumption in *L. lactis* since both the wild-type and the *ccpA* mutant strain consumed approximately the same amount of glucose (Table 3). However, the analysis of the end products formed by the wild-type and the *ccpA* mutant strain showed that a significant reduction in the L-lactate production from 50 mM in the wild-type strain to 37 mM in the *ccpA* mutant had occurred, whereas the acetate production increased from 2.4 mM to 4.9 mM. The wild-type strain did not produce any ethanol but in the medium of the *ccpA* mutant 3.2 mM ethanol was measured, characteristic of a mixed acid fermentation.

Table 3. Product formation of *L. lactis* strains used in this study. Average values two independent determinations as well as the error are given. ND not detectable.

Strain	Glucose consumption (mM)	Concentration of end products (mM)		
		L-lactate	Acetate	ethanol
NZ9800 (wild type)	33.1 ±0.9	50.1 ±2.5	2.4 ±0.1	ND
NZ9870 (Δ*ccpA*)	33.2 ±0.2	37.3 ±0.3	4.9 ±0.1	3.2 ±0.3

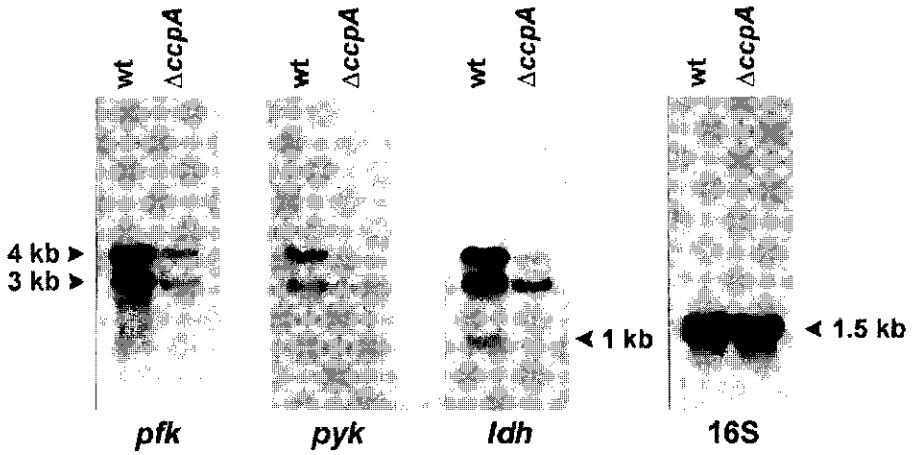

Figure 6. Northern analysis of the expression of the *pfk*, *pyk* and *ldh* genes in the *L. lactis* strains NZ9800 and NZ9870 grown on glucose. The sizes of the different transcripts are indicated with arrows.

Discussion

The *L. lactis ccpA* gene was cloned and its role in the negative regulation of the *gal* operon and the positive regulation of the *las* operon was analyzed. Although an internal *cre* site might suggest an involvement of CcpA with its own expression, as observed in *S. xylosus* and *Lb. casei*, the transcription of the *L. lactis ccpA* gene was found to be constitutive (Egeter and Brückner, 1996; Monedero *et al.*, 1997).

Disruption of the *L. lactis ccpA* gene resulted in a reduction of the growth rate on both PTS and non-PTS sugars as was also observed in other Gram-positive bacteria (Hueck *et al.*, 1995; Egeter and Brückner, 1996; Monedero *et al.*, 1997). The growth rate of the *ccpA* mutant strain on galactose was affected more severely than that on any other carbon source tested. Disruption of the *ccpA* gene might result in an altered expression of genes directly or indirectly involved in the galactose catabolism leading to a reduced growth rate. Introduction of the *L. lactis* or the *B. subtilis ccpA* gene under control of the inducible *nisA* promoter leads, after addition of inducing concentrations of nisin A, to an almost complete complementation of the observed growth defect in the *ccpA* mutant strain NZ9870, indicating that the observed effects were caused by the disruption of the *ccpA* gene.

The *L. lactis gal* genes are subject to catabolite repression and the presence of a *cre* site in the *galA* promoter region hinted at the involvement of CcpA in this repression. This suggestion was confirmed by the disruption of the *ccpA* gene since this resulted in a higher transcription of the *gal* genes when the cells were grown on a mixture of glucose and galactose. The disruption of the *ccpA* gene did not result in a complete derepression of the *galA* transcription since the transcription level in the *ccpA* mutant strain grown on a mixed substrate of glucose and galactose did not reach the level observed for the wild-type strain grown on galactose. This suggests that either the induction of the *gal* transcription is reduced by the disruption of the *ccpA* gene or that an additional system of glucose repression might be active.

The observed residual glucose repression in the *ccpA* mutant could be mediated by inducer exclusion and inducer expulsion that have been described in *L. lactis* and which have been proposed to play an important role in the regulation of the transcription of the lactose gene cluster by regulating the level of inducer (Ye *et al.*, 1994a; Ye *et al.*, 1994b). These mechanisms in combination with the operon-specific regulator could lead to a lower level of transcription of the *gal* operon in cells grown in a medium containing galactose and glucose.

In the wild-type strain the expression of the genes encoding pyruvate kinase and L-lactate dehydrogenase is subject to glucose activation since increased activities were measured in glucose-grown cells compared to galactose-grown cells. The reduced pyruvate kinase and L-lactate dehydrogenase activities measured in the *ccpA* mutant strain suggested that CcpA acts as a positive regulator of the *las* operon, although alternative explanantions like indirect effects on the transcription or changes in the RNA stability can not be excluded.

Because the intracellular concentration of early glycolytic intermediates like glucose-6-P and fructose-1,6-diP varies in response to the carbon source provided (Garrigues *et al.*, 1997) and these factors enhance the binding of CcpA to *cre* sites (Fujita *et al.*, 1995; Gösseringer *et al.*, 1997), the level of CcpA-mediated activation of the *las* operon most likely depends on the concentration of these metabolites.

Northern analysis of RNA isolated from the wild-type and the *ccpA* mutant strain grown on glucose showed that the observed regulation occurred at the transcriptional level since a four-fold reduction in the transcription of all three genes of the *las* operon was observed. The presence of additional bands can be explained by RNA processing, as has been proposed previously (Llanos *et al.*, 1992). Alternatively, more promoters may be present but this is unlikely since no promoter-like sequences were found in the entire *las* operon and previous experiments indicated that the upstream region of the *ldh* gene did not show any promoter activity (Llanos *et al.*, 1992; Llanos *et al.*, 1993). The fact that CcpA dependent regulation appears to be identical for all *las* operon derived mRNA products, combined with the observation that the only *cre* site identified in the *las* operon is present in its promoter region, argues for the presence of a single promoter. Possible sites for processing are two stem-loop structures located in the intergenic region upstream of the *pyk* and the *ldh* genes (details in Fig. 4). The fact that not all bands that could be expected after processing at the proposed sites were identified can be explained by assuming differences in the stability of the transcripts.

In addition to the transcriptional control, the enzymes encoded by the genes of the *las* operon are also subject to allosteric control by metabolites. Fructose-1,6-diP allosterically activates the activity of phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase and in addition PEP activates pyruvate kinase (Fordyce *et al.*, 1982; Hardman *et al.*, 1985). Furthermore, a recent study showed that the $NADH/NAD^+$ ratio plays an important role in the allosteric control of L-lactate dehydrogenase (Garrigues *et al.*, 1997). Phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase are ideal candidates for regulation since they catalyse steps that are almost irreversible under physiological circumstances. The control mechanisms mentioned above result in regulation of the metabolism at two levels: the allosteric control results in a rapid fine tuning of the enzymatic reactions and the transcriptional control provides the cell with an additional but slower process. Both mechanisms allow the cell to adjust the metabolic activity in response to the carbon source availability.

The lower production of L-lactate and the increased concentrations of acetate and ethanol, as observed in strain NZ9870, indicate that more pyruvate is converted into acetyl-CoA via the activity of pyruvate formate lyase or the pyruvate dehydrogenase complex which convert pyruvate into acetyl-CoA and formate or into acetyl-CoA and CO_2, respectively. Further analysis is required to analyze whether the concentrations of other possible end-products like α-acetolactate, diacetyl or acetoin are also affected by the disruption of the *ccpA* gene. Our results show that in addition to allosteric factors affecting the enzymatic activity the regulation of the expression of the *las* operon genes is an important factor in the shift from homolactic to mixed-acid fermentation in *L. lactis*. Moreover, they confirm the important role of early glycolytic intermediates as signals reflecting the energy state of the cell. Apart from their role in the previously reported allosteric control these molecules are most likely also involved as signal molecules mediating catabolite repression and catabolite activation in *L. lactis*.

So far, CcpA mediated catabolite activation has only been reported for the *B. subtilis ackA* and *alsS* genes encoding acetate kinase and α-acetolactate synthase respectively, enzymes involved in carbon secretion (Grundy *et al.*, 1993; Renna *et al.*, 1993). The role of both enzymes is similar to the role of L-lactate dehydrogenase in *L. lactis* since they are part of the pyruvate metabolism and catalyse the conversion of pyruvate to compounds that can be easily removed from the cell. Activation of the expression of these genes can be regarded as a mechanism to prevent the possible toxic accumulation of end products of the glycolysis.

Several authors have suggested that the overall reduction of the growth rate observed in *ccpA* knock out strains might be caused by the interference of CcpA with central metabolic pathways like the glycolysis (Hueck *et al.*, 1995; Monedero *et al.*, 1997). Here, we provide for the first time direct evidence that in *L. lactis* the transcriptional regulator CcpA not only mediates catabolite repression of the catabolic *gal* operon but also activates the transcription of the *las* operon encoding the glycolytic enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase.

Experimental procedures

Media and bacterial strains

The *Escherichia coli* strains MC1061 (Casadaban and Cohen, 1980) and XL1 (Stratagene) were used for cloning experiments. *E. coli* was grown in L-broth based medium with aeration at 37°C. The *Lactococcus lactis* stains used in this study are the wild-type strain NZ9800 (Kuipers *et al.*, 1993) and NZ9870 which was obtained by transforming strain NZ9800 with plasmid pNZ9244 and selecting for a double cross-over integration resulting in a disrupted *ccpA* gene (This work). *L. lactis* strains were cultivated without aeration at 30°C in M17 broth supplemented with different carbon sources. *L. lactis* was transformed by electroporation as described by Holo and Nes (1989). Antibiotics were used in the following concentrations: ampicillin 50 mg/l, chloramphenicol 5 mg/l and erythromycin 2.5 mg/l.

DNA techniques and sequence analysis

All manipulations with recombinant DNA were carried out following standard procedures (Sambrook *et al.*, 1989) and according to the specifications of the enzyme manufacturers (Gibco/BRL Life technologies or United States Biochemicals). Plasmid and chromosomal DNA of *L. lactis* was isolated as described previously (Vos *et al.*, 1989). The DNA sequence of the *ccpA* gene was determined on both strands using an ALF DNA sequencer (Pharmacia Biotech). PCR was performed with a total volume of 50 µl containing 10mM Tris/HCl (pH8.8), 50 mM NaCl, 2 mM $MgCl_2$, 10 µg gelatin, 200 µM of each deoxynucleoside triphosphate, 1 U Taq polymerase (GIBCO-BRL Gaithersburg, Md.), 10 pmol of each primer and 10 to 100 ng of template DNA. A small volume of mineral oil was added to prevent evaporation. PCR amplifications were performed in 25 cycles, each cycle consisting of a denaturation step at 95°C for 1 min, a primer annealing step at the appropriate temperature for 1 min, and a primer extension step at 72°C for 2.5 min, with a DNA Thermocycler (Perkin Elmer).

Construction of plasmids

Plasmid pNZ9240 was constructed by cloning a 1.7-kb *Hin*dIII DNA fragment from a phage containing a gene encoding a protein that cross-reacted with the CcpA antibodies into *Hin*dIII digested pUC19 (Yanisch-Perron *et al.*, 1985). A 0.5-kb *Hpa*II – *Hin*dIII fragment from plasmid pNZ9240 was used as a probe to clone a 1.7-kb *Hpa*II fragment from the chromosomal DNA from strain NZ9800 into *Acc*I digested pUC19 yielding pNZ9242. After the orientation of both inserts was determined a 1.4-kb *Acc*I – *Kpn*I fragment from pNZ9242 was cloned into *Acc*I – *Kpn*I digested pNZ9240. The resulting plasmid was designated pNZ9243 and contains the intact *ccpA* gene.

A 0.9-kb *Acc*I fragment from pUC19E containing an Ery^R gene was cloned into pNZ9243 digested with *Acc*I resulting in plasmid pNZ9244 carrying an interrupted *ccpA* gene. A *Nco*I site was introduced at the ATG start codon of the *L. lactis ccpA* gene. PCR was performed using primers CCPANCO (5'-GATAGCCAACCATGGTAGAATC-3') containing the *Nco*I site (underlined) and the antiparallel primer CCPAR5 (5'-cggttgattaacagaagtt-3') using chromosomal DNA from strain NZ9800 as template. The obtained PCR product containing the 5' end of the *ccpA* gene was digested with *Nco*I and *Hin*dIII and cloned in *Nco*I - *Hin*dIII digested pNZ8030 (de Ruyter *et al.*, 1996). The resulting plasmid was digested with *Xho*I and made blunt using Klenow DNA Polymerase. Afterwards the plasmid was digested with *Hpa*II and a 1.7-kb *Hpa*II - *Sma*I fragment from plasmid pNZ9243 containing the 3' end of the *ccpA* gene was cloned in these sites. The resulting plasmid pNZ9245 carried the entire *ccpA* gene translationally fused to the *nisA* promoter.

The nucleotide sequence of the DNA obtained by PCR was analyzed and found to contain no deviations. Oligonucleotides BSCCPA5 (5'-CAGTGGATCCAGTAAAAGGAGTGG-3') and BSCCPA3 (5'-CGCAGAATTCACCATAAAGGTGAAGC-3') based on the sequence data published under accession number M85182 were used to amplify the *B. subtilis* IG33 *ccpA* gene.

The oligonucleotides were based on bases 306 to 322 and the complementary strand of bases 1352 to 1373, respectively, in order to amplify the *B. subtilis ccpA* gene without its promoter but with its ribosome binding site. The obtained PCR product was cloned in *Bam*HI and *Eco*RI digested pNZ8020 (de Ruyter *et al.*, 1996) resulting in plasmid pNZ9246. Its nucleotide sequence was determined and found to be identical to the published sequence.

Western blot analysis

Cells were grown to an OD_{600} of 1 and concentrated by centrifugation. Cell pellets were resuspended in 1 ml of a Sodium phosphate buffer 0.1 M pH 7. The resulting suspension was subjected to mechanical disruption in the presence of Zirkonium as described previously (van der Meer *et al.*, 1993). Proteins were separated by SDS-PAGE and transferred to Gene Screen-plus membranes (Dupont) using electroblot equipment (LKB, 2051 Midget Multiblot). CcpA proteins were detected using polyclonal anti-CcpA antibodies as described previously (Küster *et al.*, 1996). These antibodies were detected using goat anti-rabbit peroxidase conjugate (Gibco/BRL) as described by the manufacturer.

Enzyme assays

Pyruvate kinase and L-lactate dehydrogenase activities were determined according to standard methods (Collins and Thomas, 1974; Hillier and Jago, 1982). Protein was quantified using bovine serum albumin as standard (Bradford, 1976).

RNA analysis

RNA was isolated from *L. lactis* cultures as described previously (Kuipers *et al.*, 1993). Northern analysis was performed with 20 μg RNA which was denatured and size fractionated on a 1-% agarose gel containing formaldehyde according to standard procedures (Sambrook *et al.*, 1989). The RNA was stained by adding ethidium bromide to the sample buffer. As molecular weight markers the 0.24-9.5-kb RNA ladder from BRL was used. The gel was blotted to a nylon membrane (Gene Screen, New England Nuclear) as recommended by the manufacturer. Slot blot analyses were performed using several dilutions steps resulting in different RNA concentrations. Blots were probed with the following oligonucleotides: PECCPA (5'-GTGCCACATCATAAATTGTTGTTGTTG-3'; *ccpA*), GALR1 (5'-ACCGACAACTTCTTCGTA-3';*galK*), LAS2 (5'-CTGCACGAATAGCCGCATTC-3'; *pfk*), LAS3 (5'-CATCATTGGGATAACACCC-3'; *pyk*), LAS4 (5'-GCATCAGAGTAGTCTGCAGAG-3'; *ldh*) and 3.2 (5'-ATCTACGCATTTCACCGCTAC-3'; 16S rRNA; Klijn *et al.*, 1991).

Following autoradiography bands were cut out and total radioactivity was determined using a liquid scintilation counter (Beckmann LKS 7500). RNA amounts were corrected by probing with probe 3.2 specific for variable regions of the *L. lactis* 16S rRNA.

Primer-extension analysis

The oligonucleotide used for priming cDNA synthesis was PECCPA (5'-GTGCCACATCATAAATTGTTGTTGTTG-3') complementary to nucleotides 189 to 215 in the coding strand of the *ccpA* gene in the sequence data. Primer extension reactions were performed by annealing 2 ng of oligonucleotide to 100 μg of total RNA as described previously (Kuipers *et al.*, 1993).

End-product determination

Cells were grown to an OD_{600} of 1, concentrated by centrifugation and resuspended to a final OD_{600} of 10 in 100 mM of sodium phosphate buffer pH 7.0 containing 50 mM of glucose. After 1 h. incubation at 30°C under continuous aeration the cells were pelleted by centrifugation and the L-lactate, acetate and ethanol concentrations in the supernatant were determined by HPLC as described previously (Starrenburg and Hugenholtz, 1991).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide sequence databases under the accession number Z97202

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CHAPTER 5

Molecular characterization of the *Lactococcus lactis ptsHI* operon and analysis of the regulatory role of HPr

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Abstract

The *Lactococcus lactis ptsH* and *ptsI* genes encoding the general proteins of the phosphoenolpyruvate-dependent phosphotransferase system HPr and Enzyme I, respectively, were cloned and the regulatory role of HPr was studied by mutational analysis of its gene. A promoter sequence was identified upstream of the *ptsHI* operon and the transcription start site was mapped by primer extension. Northern analysis showed the presence of two glucose-inducible transcripts: one of 0.3 kb containing *ptsH* and a second of 2.0 kb containing both *ptsH* and *ptsI*. Disruption of the *ptsH* and *ptsI* genes in strain NZ9800 resulted in reduced growth rates on glucose, sucrose and fructose, confirming the dominant role of the phosphotransferase system in the uptake of these sugars in *L. lactis*. Complementation of the *ptsH* and *ptsI* mutants with the intact genes under control of the inducible *nisA* promoter resulted in the restoration of the wild-type phenotype. The role of HPr(Ser-P) in the recently established CcpA mediated control of the galactose metabolism as well as the glycolysis was analyzed by producing a HPr mutant carrying an aspartic acid on residue 46 which mimicks a phosphorylated serine. These experiments demonstrated the role of HPr(Ser-P) as co-repressor in the catabolite repression of the *gal* operon. Furthermore, we show that HPr(Ser-P) functions as a co-activator in the CcpA-mediated catabolite activation of the pyruvate kinase and L-lactate dehydrogenase genes.

Introduction

The main sugar uptake system in many bacteria is the phosphoenolpyruvate: sugar phosphotransferase system (PEP-PTS) that mediates the uptake and the phosphorylation of carbohydrates (27). The PTS is a group translocation process in which the transfer of the phosphate moiety of PEP to carbohydrates is catalyzed by the general non-sugar-specific proteins enzyme I and HPr in combination with sugar-specific enzyme II proteins (EII). Following autophosphorylation of enzyme I at the expense of PEP, enzyme I catalyzes the phosphorylation of HPr at residue histidine 15 resulting in HPr(His-P). The phosphate group from HPr(His-P) is then transferred to the sugar substrate via a two-step phosphorylation reaction mediated by a dedicated EII protein. Enzymes II can consist of one or more proteins and are composed of three domains, i.e. the enzyme IIA and IIB domains that are involved in the phosphotransfer, and the membrane located enzyme IIC domain that is most likely involved in the translocation of the sugar substrate (27). The genes encoding HPr and enzyme I, i.e. *ptsH* and *ptsI*, respectively, have been cloned from several bacteria and are often organized in an operon structure with the gene order *ptsHI*. The expression of the *Escherichia coli* and the *Bacillus subtilis ptsHI* operons appears to be regulated at the transcriptional level since higher mRNA levels were detected in glucose-grown cells compared to cells grown on non-PTS sugars (29, 35).

Apart from its function in the uptake of sugars, the PTS also plays a regulatory role. In *E. coli* and other Gram-negative bacteria the PTS regulates the concentration of cAMP via activation of adenylate cyclase by the phosphorylated form of the glucose-specific EIIA, the concentration of which increases in the absence of PTS substrates (27). Elevated concentrations of cAMP lead to transcriptional activation of several genes via the binding of the cyclic AMP receptor protein (CRP) complexed with cAMP to operator sites located in the promoter regions of affected genes. Furthermore, the unphosphorylated form of the glucose-specific EIIA reduces the uptake of several non-PTS sugars via an interaction with the uptake protein (27).

In Gram-positive bacteria regulatory functions for the PTS have also been described. In *Enterococcus* spp. the HPr(His-P) mediated phosphorylation of two glycerol kinases results in an increased activity of both enzymes (4). In contrast, enzyme I / HPr(His-P) mediated phosphorylation of the lactose permease in *Streptococcus thermophilus* results in a reduced permease activity leading to a decreased uptake of sugar (26). In *B. subtilis*, the PTS regulates the expression of the levanase operon by HPr(His-P) mediated phosphorylation of the transcriptional regulator LevR resulting in activation of transcription (34). Apart from phosphorylation at residue His-15, a second phosphorylation site has been identified in HPr, the functionality of which has only been shown in Gram-positive bacteria. Phosphorylation of HPr at residue Ser-46 is catalyzed by an ATP-dependent protein kinase that is activated by fructose-1,6-diphosphate (FDP) (6, 32). Recently, the gene encoding the HPr(Ser) kinase has been cloned from *Bacillus subtilis*, and its involvement in the phosphorylation of HPr at residue Ser-46 was established (28). The seryl-phosphorylated form of HPr, designated HPr(Ser-P), interacts with several PTS and non-PTS sugar permeases, and this process, termed inducer exclusion, results in a reduced uptake of sugars (32). In addition, HPr(Ser-P) allosterically activates sugar-phosphate phosphatases in *Lactococcus lactis*, *E. feacalis* and *S. pyogenes*, that catalyze the dephosphorylation of various phosphorylated sugars, resulting in an efflux of the sugar from the cell, a process known as inducer expulsion (41, 44). Apart from these allosteric control systems, HPr(Ser-P) can also regulate the transcription of genes by an interaction with the catabolite control protein, CcpA (8, 16). The *in vitro* binding of CcpA to *cis*-acting operator sites *cre* (catabolite responsive element), located in the promoter region of genes controlled by CcpA, is reported to be enhanced by an interaction with HPr(Ser-P) (9, 13, 16). Deutscher *et al.* (7) demonstrated that expression of the gene encoding S46D HPr, that has a similar structure as HPr(Ser-P) leads to catabolite repression of the *B. subtilis* gluconate kinase gene even in the absence of glucose. In addition, they showed that the replacement of HPr with S46A HPr has the same effect as a mutation in the *ccpA* gene, since this rendered the gluconate kinase gene insensitive to catabolite repression.

Recently, we have shown the involvement of the *L. lactis* CcpA in the negative regulation of the expression of the genes involved in the galactose metabolism and the positive control of the *las* operon encoding phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase (24). Here, we report the cloning and analysis of the *L. lactis ptsHI* genes and the involvement of HPr in the catabolite repression of the galactose metabolism. Furthermore, we show for the first time the participation of HPr(Ser-P) in the CcpA-mediated transcriptional activation of the *las* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and transformation procedure. The *L. lactis* strains and plasmids used in this study (Table 1) were cultivated without aeration at 30°C in M17 broth supplemented with different carbon sources. *L. lactis* was transformed by electroporation as described by Holo and Nes (18). The *E. coli* strain MC1061 was used as a host for cloning experiments and grown in L-broth based medium with aeration at 37°C. Antibiotics were used in the following concentrations: ampicillin 50 μg/ml, chloramphenicol 5 μg/ml and erythromycin 2.5 μg/ml.

DNA techniques and DNA sequence analysis. All manipulations with recombinant DNA were carried out following standard procedures (33) and according to the specifications of the enzyme manufacturers (Gibco/BRL Life technologies or United States Biochemicals). Plasmid and chromosomal DNA of *L. lactis* was isolated as described previously (37). The DNA sequence of the *ptsHI* genes was determined on both strands using an ALF DNA sequencer (Pharmacia Biotech). PCR was performed in a total volume of 50 μl containing 10 mM Tris/HCl (pH8.8), 50 mM NaCl, 2 mM $MgCl_2$, 10 μg gelatin, 200 μM of each deoxynucleoside triphosphate, 1 U. *Taq* polymerase (GIBCO-BRL Gaithersburg, Md.), 10 pmol of each primer and 10 to 100 ng of template DNA. A small volume of mineral oil was added to prevent evaporation. PCR amplifications were performed in 25 cycles, each cycle consisting of a denaturation step at 95°C for 1 min, a primer annealing step at the appropriate temperature for 1 min, and a primer extension step at 72°C for 2.5 min, with a DNA Thermocycler (Perkin Elmer).

Table 1. *L. lactis* strains and plasmids used in this study.

Strain or plasmid	Relevant properties	References
Strains		
NZ9800		(21)
NZ9880	NZ9800 derivative; Δ*ptsH*	This work
NZ9881	NZ9800 derivative: Δ*ptsI*	This work
Plasmids		
pUC19E	pUC19 derivative containing an Ery^R gene	(23)
pNZ8030	Lactococcal cloning and expression vector	(30)
pNZ9279	pGEM-T containing a 0.8-kb PCR product derived from the *L. lactis ptsHI* operon	This work
pNZ9280	pUC19 containing a 4.5-kb *Sst*I-*Cla*I fragment	This work
pNZ9281	pUC19 containing a 3.5-kb *Eco*RV-*Bam*HI fragment	This work
pNZ9282	pNZ8030 containing the *L. lactis ptsH* gene translationally fused to the *nisA* promoter	This work
pNZ9283	pNZ8030 containing the *B. subtilis ptsH* gene translationally fused to the *nisA* promoter	This work
pNZ9284	pNZ8030 containing the S46A *ptsH* gene translationally fused to the *nisA* promoter	This work
pNZ9285	pNZ8030 containing the S46D *ptsH* gene translationally fused to the *nisA* promoter	This work
pNZ9286	pNZ8030 containing the *ptsH* and *ptsI* genes translationally fused to the *nisA* promoter	This work
pNZ9287	pNZ9286 derivative containing the *ptsI* gene transcriptionally fused to the *nisA* promoter	This work
pNZ9288	pNZ8030 containing S46A *ptsH* and *ptsI* translationally fused to the *nisA* promoter	This work
pNZ9289	pNZ8030 containing S46D *ptsH* and *ptsI* translationally fused to the *nisA* promoter	This work
pNZ9290	*ptsH* disruption construct	This work
pNZ9291	pUC19E derivative containing an internal *Cla*I-*Pst*I fragment of *ptsI*	This work

Construction of plasmids. Primers PTS1 (5'-AACWGGWATTCATGCWMGWCCWGC-3') and PTS2 (5'-GGTACCWCCAATATTWGTWACAAAWGC-3') were used in a PCR reaction with chromosomal DNA from *L. lactis* NZ9800 to amplify part of the *ptsHI* operon. The PCR product was cloned in the pGEM-T vector (Promega) yielding pNZ9279. The insert from pNZ9279 was used as a probe to clone a 4.5 kb *Sst*I – *Cla*I fragment from the chromosomal DNA of *L. lactis* into *Sst*I – *Acc*I digested pUC19 (40) resulting in pNZ9280. The same fragment was used as a probe to clone a 3.5 kb *Eco*RV – *Bam*HI fragment from the chromosomal DNA in *Sma*I – *Bam*HI digested pUC19 resulting in pNZ9281.

By the use of oligonucleotides HPRNCOI (5'- GAACCATAACCATGGCATCTAAAG-3') and HPRHINDIII (5'- GACAAGCAAGCTTGCCTTAGCTACTG –3') the *ptsH* gene was amplified by PCR with chromosomal DNA from *L. lactis* NZ9800 as template. Bases that were changed to introduce the *Nco*I and *Hin*dIII restriction sites are underlined. These sites were used to clone the obtained PCR product in *Nco*I – *Hin*dII digested pNZ8030 resulting in plasmid pNZ9282.

The *B. subtilis ptsH* gene (14) was amplified by PCR with oligonucleotides BSHPRNCO (5'- GGAGAATGATAACCATGGCACAAAAAAC-3') and BSHPRHIND (5'- GCAGAGATGTTAAGCTTTTCAACC – 3') and chromosomal DNA from *B. subtilis* 168 as template DNA. The obtained PCR product was digested with *Nco*I and *Hin*dIII and cloned into *Nco*I – *Hin*dIII digested pNZ8030 yielding pNZ9283.

Residue serine 46 of *L. lactis* HPr was changed to an alanine and an aspartic acid, respectively, by PCR-mediated megaprimer mutagenesis of the *ptsH* gene, as described previously (20) with the mutagenic primers HPR46A (3'-GTAAACCTTAAAGCAATCATGGGTGTTATGTC –3') and HPR46D (5'-GTAAACCTTAAAGATATCATGGGTGTTATGTC –3'), respectively. The substitutions needed to change codon 46 are underlined. Two amplification rounds were used, the first one with the mutagenic primers and the antiparallel primer PTSHINDIII. The second round was performed with the purified PCR products obtained from the first round as megaprimer, together with primer PTSHNCOI.
The obtained PCR products were digested with *Nco*I and *Hin*dIII and cloned into *Nco*I – *Hin*dIII digested pNZ8030 yielding pNZ9284 and pNZ9285 carrying the genes encoding S46A HPr and S46D HPr, respectively.

For the construction of a translational fusion between the *nisA* promoter and the entire *ptsHI* operon encoding either wild-type, S46A or S46D HPr together with enzyme I, a 3.2 kb *Dra*III – *Bam*HI fragment was isolated from pNZ9281. After treatment with Klenow DNA polymerase this fragment was cloned into either pNZ9282, pNZ9284 or pNZ9285 digested with *Dra*III and *Hin*dIII. The latter site was made blunt by treating it with Klenow DNA polymerase and the resulting plasmids were designated pNZ9286, pNZ9288 and pNZ9289, respectively.

Plasmid pNZ9287 was constructed by digesting plasmid pNZ9286 with *Nco*I and *Dra*III to remove the 5' end of the *ptsH* gene, treating the ends with Klenow and T4 DNA polymerase followed by self-ligation of the resulting DNA. To disrupt the *ptsH* gene a *Bam*HI site was introduced in the *ptsH* open reading frame by a PCR reaction using the primers HPRBAM2 (5'-GTGTTGGATCCCTCGGTGTTGGTCAAG–3'), corresponding to nucleotides 403 to 419, and primer PTS6 (5'-GATGGATTGTAAGGTTGATA-3'), complementary to nucleotides 1947 to 1967 of the *ptsHI* genes. The obtained PCR product was digested with *Bam*HI and *Hin*dIII and and the obtained 1.0-kb fragment was cloned into *Bam*HI – *Hin*dIII digested pUC19.

The resulting plasmid was digested with *Sst*I and *Bam*HI and a 1.2-kb *Sau*3A fragment from plasmid pIL253 carrying an erythromycin gene and a 3.5-kb *Sst*I – *Bcl*I fragment carrying the upstream region of the *ptsH* gene from plasmid pNZ9280 were cloned into these sites. The obtained constructs were verified by restriction analysis and one product was selected which was called pNZ9290 containing the erythromycin resistance gene in the same orientation as the *ptsI* open reading frame. Plasmid pNZ9291 was constructed by cloning a 0.9-kb *Cla*I – *Pst*I fragment from plasmid pNZ9281 the *Cla*I site of which was made blunt by Klenow DNA polymerase treatment into *Pst*I – *Bam*HI digested pUC19E the *Bam*HI site of which ha been filled in by use of Klenow DNA polymerase. The nucleotide sequences of all DNA fragments obtained via PCR were verified and found to be correct.

RNA analysis. RNA was isolated from *L. lactis* cultures as described previously (21). RNA was denatured and size fractionated on a 1-% agarose gel containing formaldehyde according to standard procedures (33). The RNA was stained by adding ethidium bromide to the sample buffer. As molecular weight markers the 0.24-9.5-kb RNA ladder from BRL was used. The gel was blotted to a nylon membrane (Gene Screen, New England Nuclear) as recommended by the manufacturer. Blots were probed with the following oligonucleotides: PEPTSH for *ptsH* and PTS6 for *ptsI*.

Primer-extension analysis. The oligonucleotide used for priming cDNA synthesis was PEPTSH (5'-CTGCAACGATGTGGAATTCTTTAG-3') complementary to nucleotides 254 to 278 in the coding strand of the *ptsH* gene. Primer extension reactions were performed by annealing 2 ng of oligonucleotide to 100μg of total RNA as described (21).

Enzyme assays. Pyruvate kinase and L-lactate dehydrogenase activities were determined according to standard methods (5, 17). Protein concentrations were assayed using bovine serum albumin as standard (3).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide sequence databases under the accession number Z97203.

Results

Cloning of the *ptsHI* operon. An alignment was made of the sequences of HPr proteins from different Gram-positive bacteria. Based on the conserved region TGIHARP, encompassing the catalytic phosphorylation site His-15 in several HPr sequences, oligonucleotide PTS1 was designed. The reported active site sequence of the L. lactis enzyme I (1) was used to design an anti-sense oligonucleotide, PTS2. Assuming that the gene order in *L. lactis* would be identical to that found in other bacteria, (*ptsHI*), primers PTS1 and PTS2 were used in a PCR reaction under non-stringent conditions using chromosomal DNA from *L. lactis* NZ9800 as template, which yielded a product of the expected size of 0.8 kb. Sequence analysis of this fragment showed that it contained two partial open reading frames encoding proteins with high sequence homology to HPr and enzyme I sequences.

This fragment was used as a probe and hybridized to a single DNA fragment of the *L. lactis* chromosome that was cloned as two overlapping fragments, resulting in plasmids pNZ9280 and pNZ9281.

Characterization of the *ptsH* and *ptsI* genes. Sequence analysis of the inserts in plasmids pNZ9280 and pNZ9281 revealed the presence of two complete open reading frames. The first designated *ptsH* (see below) could encode a protein of 88 amino acids that shows high sequence homology to HPr proteins from different bacteria with up to 91 % identical residues compared to the *S. salivarius* HPr protein (10). Ten nucleotides upstream of the ATG start codon a sequence (5'-AAGGAG-3') resembling a typical lactococcal ribosome binding sites was identified (38). The second open reading frame designated *ptsI* (see below) could encode a protein of 575 amino acids which shares high sequence similarity to enzyme I proteins of various bacteria with 83 % identity compared to the *S. salivarius* enzyme I (11). The putative ATG start codon of the *ptsI* gene, which is located eight nucleotides downstream of the *ptsH* gene, is preceded by a putative lactococcal ribosome binding site (5'-AAGGA-3') with an unusual spacing of fourteen base pairs. Two stem loop structures were identified in the *ptsHI* operon: one with a ΔG value of –6.4 kcal / mol located downstream of the *ptsH* gene (T_1) and a second with a ΔG value of –9.0 kcal / mol followed by an AT-rich stretch of nucleotides downstream of the *ptsI* gene (T_2) (Fig.1).

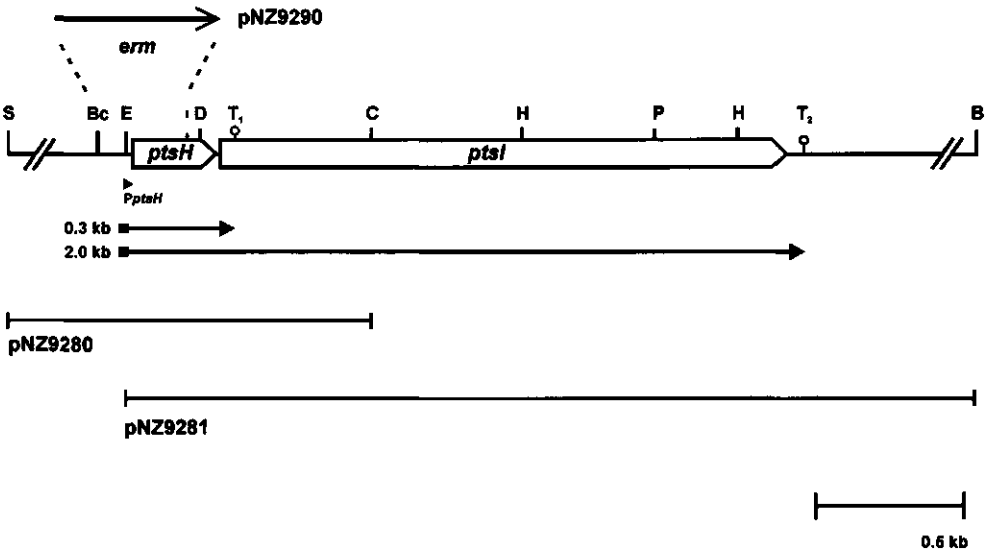

Figure 1. Transcriptional organisation of the *ptsHI* operon of *L. lactis* NZ9800. The genes (open arrows) connected by intergenic regions (thin lines) are shown with their products. The arrows denote the promoter and transcripts mapped with primer extension and Northern analysis, respectively. The putative terminators downstream of *ptsH* and *ptsI* are indicated (T_1 and T_2, respectively). Relevant restriction sites are S, *Sst*I; Bc, *Bcl*I; E, *Eco*RV; D, *Dra*III; C, *Cla*I; H, *Hin*dIII; P, *Pst*I; B, *Bam*HI.

Transcriptional analysis of the *ptsHI* operon. Primer extension analysis was performed using total RNA isolated from strain NZ9800 grown on glucose. Two equally intense labelled primer extension products were obtained indicating that transcription starts at two adjacent sites (Fig. 2). Upstream of these start sites a sequence corresponding to consensus *L. lactis* promoter sequences was identified (38). Northern analysis of the *ptsHI* genes with a *ptsH*-specific probe revealed the presence of two transcripts: one of 0.3 kb and a second of 2.0 kb (Fig. 3A). When the same blot was deprobed and reprobed with a *ptsI*-specific probe, only the 2.0 kb transcript hybridized (Figure 3A). RNA isolated from cells grown on either galactose or glucose was probed with a *ptsH*-specific probe and showed that the transcription of the *ptsHI* operon varies in response to the carbon source since at least tenfold higher transcription levels were observed in glucose-grown cells than in galactose-grown cells (Fig. 3B). This demonstrates that the expression of the *L. lactis ptsHI* operon is regulated at the transcriptional level in response to the carbon source.

Recently, we have shown that CcpA is involved in the catabolite repression of the galactose operon in *L. lactis*, most likely through an interaction with a *cre* site located in the promoter region of the *gal* operon (24). In order to investigate the role of HPr(Ser-P) in this regulatory process the mutant *ptsH* genes were also (over)expressed without the co-expression of *ptsI* in the wild-type strain NZ9800. Moderate overexpression of mutant *ptsH* genes encoding S46A HPr and S46D HPr did not show any effect on the growth rate of the cells (data not shown). However, overexpression of the mutant gene encoding S46D HPr resulted in a severe reduction of the growth on galactose, whereas the overexpression of the gene encoding S46A HPr did not affect the growth on galactose. The overexpression of the genes encoding S46A HPr or S46D HPr did not influence the growth rate on glucose and sucrose (Table 2).

Analysis of the role of HPr(Ser-P) in the regulation of the *las* operon

Previously, we showed that the activity of pyruvate kinase and L-lactate dehydrogenase varies in response to the carbon source provided (24). NZ9800 cells (wild-type) grown on glucose show higher activities of both enzymes than cells grown on galactose (Table 3). Furthermore, we have shown that the disruption of the *L. lactis ccpA* gene resulted in a reduction of the expression of the *las* operon which encodes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase, when the NZ9800 cells were grown on glucose. These results indicated that CcpA acts as an activator of the expression of the *las* operen most likely by an interaction with a *cre* site located in the promoter region of the *las* operon (24). Expression of the pNZ9285 located gene encoding S46D HPr in galactose-grown NZ9800 cells resulted in increased pyruvate kinase and L-lactate dehydrogenase activities compared to wild-type cells (Table 3). In contrast, the (over) production of S46A HPr did not affect both activities. When either of the mutant *ptsH* genes was expressed in cells grown on glucose the activities of pyruvate kinase and L-lactate dehydrogenase were not altered. Higher overexpression of the mutant genes by the addition of higher amounts of nisin A to the growth medium resulted in a lower activity of both pyruvate kinase and L-lactate dehydrogenase (data not shown). The disruption of the *ptsH* gene in strain NZ9880 resulted in a reduction of both the pyruvate kinase and L-lactate dehydrogenase activities to 70 % of the values measured in wild-type cells. Higher pyruvate kinase and L-lactate dehydrogenase activities were measured in strain NZ9880 (Δ*ptsH*) grown on glucose compared to cells grown on galactose, suggesting that the regulation of the expression of the *las* operon is not dependent on the presence of the *ptsH* gene. To assess the role of HPr(Ser-P) in the CcpA mediated activation of the *las* operon we expressed the S46D HPr gene in strain NZ9870, that carries a disrupted copy of the *ccpA* gene. Since strain NZ9870 does not grow on galactose the effects of the overproduction of S46D HPr could only be analyzed in cells grown on glucose.

Overexpression of the S46D HPr gene in strain NZ9870 (Δ*ccpA*) did not result in an increase of the activities to the levels measured in the wild-type strain NZ9800 grown on glucose (Table 3). These experiments indicate the necessity of an intact CcpA for the observed HPr(Ser-P) mediated activation of pyruvate kinase and L-lactate dehydrogenase.

Table 3. L-lactate dehydrogenase and pyruvate kinase activities of strains used in this study.

Strain	nisin A induction µg / ml	Carbon source	Pyruvate kinase µmol PEP/mg /min	L-lactate dehydrogenase µmol NADH/mg /min
NZ9800 (wild-type)	-	glucose	3.20 ± 0.57	14.2 ± 0.20
	-	galactose	1.67 ± 0.48	9.05 ± 0.31
NZ9800 + pNZ9284 (S46A *ptsH*)	$1x10^{-5}$	glucose	3.19 ± 0.43	13.84 ± 0.39
	$1x10^{-5}$	galactose	1.84 ± 0.06	9.12 ± 0.65
NZ9800 + pNZ9285 (S46D *ptsH*)	$1x10^{-5}$	glucose	2.95 ± 0.09	12.38 ± 0.23
	$1x10^{-5}$	galactose	2.85 ± 0.16	12.43 ± 0.9
NZ9870 (Δ*ccpA*)	-	glucose	0.79 ± 0.78	6.32 ± 0.55
NZ9870 + pNZ9285 (S46D *ptsH*)	$1x10^{-3}$	glucose	0.75 ± 0.26	6.42 ± 0.54
NZ9880 (Δ*ptsH*)	-	glucose	2.14 ± 0.53	9.87 ± 0.65
	-	galactose	1.45 ± 0.56	8.31 ± 0.32

Discussion

We have cloned and analyzed the *L. lactis ptsH* and *ptsI* genes using inactivation, mutation and overexpression studies in order to assess their roles in the phosphorylation cascade of the PTS and the global regulation of the carbohydrate metabolism. The genes are located in an operon structure with the gene order *ptsHI*. Two equally strong, adjacent transcriptional start sites were mapped, located downstream of a typical lactococcal promoter sequence. Transcriptional analysis revealed that the *ptsH* gene was located on two transcripts: a small transcript of approximately 0.3-kb and a larger a transcript of 2-kb. Probing with a *ptsI*-specific probe indicated that this gene is only located on the 2-kb transcript. These results indicate that the *ptsHI* genes are transcribed as a single transcript of 2-kb that terminates at the rho-independent terminator structure (T_2) located downstream of *ptsI* and that the stem-loop structure (T_1) located downstream of *ptsH* functions as a limited transcriptional terminator. This transcriptional organization most likely results in a higher expression of the *ptsH* gene, which is in accordance with the observation that a higher amount of HPr protein compared to enzyme I is found in several bacteria, including *Staphylococcus carnosus* (19).

The expression of the *L. lactis ptsHI* operon is regulated at the transcriptional level since tenfold higher mRNA levels were observed in glucose-grown cells than in galactose-grown cells. Induction of the *ptsHI* gene expression by glucose has also been reported for *E. coli* and *B. subtilis* (29, 31, 35). In *E. coli* this regulation is mediated via a regulatory mechanism that includes the transcriptional regulator FruR (31) and the cyclic AMP receptor protein (CRP) that also mediates catabolite repression (29). The glucose induction of the *B. subtilis ptsGHI* operon, that also contains the gene encoding the glucose-specific EII (*ptsG*), is mediated via the antiterminator protein GlcT (35). No *cis*-acting sequences like *cre* sites (39) involved in catabolite repression or RAT sequences (2) that play a role in antitermination mediated by proteins of the BglG/SacY family were detected at relevant positions in the DNA sequence of the *L. lactis ptsHI* operon. Further experiments are required to identify the elements involved in the transcriptional control of the *L. lactis ptsHI* operon in response to the carbon source.

The disruption of both the *ptsH* and *ptsI* genes resulted in strains that were affected in the growth on sucrose and fructose, indicating that these sugars are exclusively taken up via the PTS system. The growth on glucose was affected but not completely abolished, which is in accordance with a previous report that indicated that *L. lactis* can take up glucose via two transport systems: a permease and a PTS (36). Surprisingly, the growth on galactose and maltose was also affected by the *ptsH* and *ptsI* mutations since the growth rates were reduced to half of that observed for the wild-type. Both sugars are presumed to be taken up via non-PTS permeases in *L. lactis* (15, 22). Our results suggest that a functional PTS is also required for the efficient utilization of galactose and maltose in *L. lactis*. An activating role for the PTS component HPr(His-P) has been reported before in the case of the *B. subtilis* levanase regulator LevR (34) and the enterococcal GlpK proteins (4). In these examples the activation of the levanase or glycerol metabolism depends on the HPr(His-P) mediated phosphorylation of a regulatory or a structural protein, respectively. HPr(His-P) is absent in both NZ9880 (Δ*ptsH*) and NZ9881 (Δ*ptsI*) since phosphorylation at His-15 requires the presence of both HPr and enzyme I. A role for either HPr(Ser-P) or enzyme I in the observed reduction of the growth rate on non-PTS sugars in the PTS negative strains can be ruled out since these proteins are still present in strains NZ9881 (Δ*ptsI*) and NZ9880 (Δ*ptsH*), respectively. This suggests that HPr(His-P), the concentration of which increases in the absence of a PTS substrate, stimulates the utilization of galactose and maltose in *L. lactis*.

Further evidence for the functionality of the *ptsH* gene was obtained by the complementation of the *ptsH* mutation in strain NZ9880 by the expression of either the *L. lactis* or the *B. subtilis ptsH* gene under the control of the inducible *nisA* promoter. Induction of the *nisA* promoter with increasing concentrations of nisin A resulted only in a partial restoration of the growth defect observed in the *ptsH* mutant strain when grown on the PTS sugar sucrose, while the growth on galactose was not affected.

The readthrough from the promoter of the Ery^R gene is most likely not sufficient to allow full functional expression of the *ptsI* gene, since co-expression of the *ptsHI* genes did result in complete complementation of the growth defect. This result also demonstrates the dependence of *ptsI* expression on the promoter located upstream of the *ptsH* gene.

The *ptsI* mutation in strain NZ9881 was complemented by the expression of either the entire *ptsHI* operon or by the *ptsI* gene alone, transcriptionally fused to the *nisA* promoter on plasmid pNZ9287. Primarily, these results show the functionality of the *ptsI* gene, but they also establish the functionality of the RBS located upstream of the *ptsI* gene, despite its unusual spacing (14 bp) to the putative ATG start codon of the *ptsI* open reading frame.

In order to analyze the regulatory role of HPr in the metabolism of *L. lactis* we constructed site-directed mutants of the *L. lactis ptsH* gene encoding HPr proteins that are affected in the phosphorylation of residue Ser-46. Replacement of residue Ser-46 with an alanine (S46A HPr) has been shown to inactivate HPr as a regulatory molecule (7), while the introduction of an aspartate at residue 46 (S46D HPr) results in a permanently negatively charged residue 46 that resembles a phosphorylated serine. Production of S46D HPr in the wild-type strain NZ9800 resulted in a strong reduction of the growth rate on galactose, whereas no effects were observed when S46A HPr was overproduced. This can be explained by an enhanced repression of the *gal* gene expression by CcpA, in the presence of S46D HPr, but not of S46A HPr. S46DHPr has been shown to stimulate the binding of CcpA to *cre* sites, thus mediating catabolite repression (9, 13). The role of CcpA in this mechanism could not be further substantiated since a *ccpA* negative strain is impaired in the utilization of galactose (24). Moreover, the reduced growth on galactose after overexpression of the S46D HPr gene could be explained by the S46D HPr mediated activation of the inducer exclusion and expulsion mechanisms. These mechanisms that result in a reduction of the inducer concentration have been described in non-growing *L. lactis* and their activities are stimulated by the electroporation of *B. subtilis* S46D HPr in *L. lactis* vesicles (42, 43). The production of *L. lactis* HPr mutants in growing cells is a non-artificial system to study the inducer exclusion and expulsion mechanisms. Production of S46D HPr could result in a reduced uptake of galactose via an interaction with the galactose permease. In addition, S46D HPr mediated activation of the recently purified sugar-phosphate phosphatase (44) could lead to an increased dephosphorylation of galactose intermediates leading to a reduced growth rate.

Since it has been reported that CcpA can form a complex with HPr phosphorylated on residue serine 46 and that this complex enhances the binding of CcpA to *cre* sites (9, 13, 16), we were interested whether this complex also plays a role in the regulation of the *las* operon, known to be transcriptionally activated by CcpA (24). Production of S46D HPr but not S46A HPr in wild-type cells grown on galactose resulted in pyruvate kinase and L-lactate dehydrogenase activities which were elevated to the same level as found in the wild-type grown on glucose, suggesting that HPr(Ser-P) acts as a signal molecule that modulates the expression level of the pyruvate kinase and L-lactate dehydrogenase genes in response to the carbon source provided. To establish the role of CcpA in this mechanism, the gene encoding S46D HPr was also expressed in strain NZ9870 (Δ*ccpA*). Due to the inactivation of the *ccpA* gene the activities of both pyruvate kinase and L-lactate dehydrogenase are no longer activated by CcpA and therefore lower than those measured in the wild-type strain (24).

The fact that the activities of both pyruvate kinase and L-lactate dehydrogenase no longer respond to the production of S46D HPr indicates that CcpA is required for the observed S46D HPr-mediated activation of the activities in the wild-type strain. These results indicate that HPr(Ser-P) acts as a signal molecule involved in the CcpA-mediated activation of the transcription of the *las* operon. Remarkably, the disruption of the *ptsH* gene did not result in a strong reduction of the activities of pyruvate kinase and L-lactate dehydrogenase nor did it affect the carbon source dependent regulation. This finding suggests that additional signal molecules might be involved in the CcpA-dependent regulation of the *las* operon. Possible candidates could be glucose-6-P that has been shown to enhance the binding of *B. megaterium* CcpA to a *cre* site (13) or a lactococcal analogue of Crh, a HPr-like protein, that was recently identified in *B. subtilis* and that functions as a signal molecule in the catabolite repression of several genes (12).

The results presented here show the predominant role of the PTS in the uptake of various sugars in *L. lactis*. Furthermore, they show the regulatory functions of HPr in establishing a hierarchy in the preference for different sugars in *L. lactis*. Finally, we provide experimental evidence for the involvement of HPr(Ser-P) in the CcpA-mediated transcriptional activation of genes encoding glycolytic enzymes in *L. lactis*.

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CHAPTER 6

Summary and concluding remarks

Summary and concluding remarks

In view of the economic importance of fermented dairy products considerable scientific attention has been given to various steps of fermentation processes, including the L-lactate formation of lactic acid bacteria (de Vos, 1996). In particular, the carbohydrate metabolism of *L. lactis* has been the subject of extensive research and several genes encoding proteins involved in the central carbohydrate metabolism have been described (Llanos *et al.*, 1992; Llanos *et al.*, 1993; Cancilla *et al.*, 1995a; Cancilla *et al.*, 1995b; Qian *et al.*, 1997). Although several findings have established that the carbohydrate metabolism is subject to several forms of regulation, detailed information concerning this regulation and, in particular, the transcriptional control of the central carbohydrate metabolism is lacking (Collins and Thomas, 1974; Fordyce *et al.*, 1982; Hardman *et al.*, 1985; Garrigues *et al.*, 1997). A better understanding of the regulatory mechanisms involved would be an advantage for metabolic pathway engineering. Metabolic engineering is mainly aimed at the optimization of the metabolism and the diversion from L-lactate to other desired metabolites. The metabolite formation depends on the activity of enzymes of the central metabolic pathway and is therefore also subject to regulatory mechanisms in response to the carbon source provided. The research reported in this thesis has focussed on carbon catabolite repression (CCR), a global control system which regulates the transcription of genes involved in the carbohydrate metabolism depending on the carbon source availability (Hueck and Hillen, 1995). An overview of the present state of the art on CCR in Gram-positive bacteria is presented in **Chapter 1**. The aim of the work presented in this thesis was to investigate the elements involved in CCR in *L. lactis* and their effects on the carbohydrate metabolism. Several *cis*- and *trans*-acting elements involved in specific and global control systems were identified and their role in the transcriptional and allosteric control of carbohydrate metabolism was characterized. The salient features of their sequences are summarized in the Appendix.

Chapter 2 describes the transcriptional and functional analysis of Tn*5276*-located genes involved in sucrose metabolism in *L. lactis*. The observation that the transcription of the previously cloned *sacA* gene was subject to glucose repression and the identification of a *cre* element in the promoter region of the sucrose genes lead to the choice of the sucrose genes as a model system to study the effects of CCR. (Rauch and de Vos, 1992). In addition to the *sacA* gene, encoding a sucrose-6-phosphate hydrolase, three new complete genes were identified (Fig. 1). The *sacB* gene encodes a sucrose-specific EII protein of the phosphotransferase system (PTS) and its disruption resulted in the inability of the strain to utilize sucrose as carbon and energy source, thereby confirming the functionality of the *sacB* gene in the sucrose metabolism of *L. lactis*..

Downstream of the *sacB* gene the *sacK* gene was identified encoding a fructokinase. Partially overlapping *sacA*, the *sacR* gene was identified, the deduced protein sequence of which showed high homology to regulatory proteins of the LacI/GalR family. The *L. lactis* sucrose gene is the only gene cluster reported so far containing all three structural genes necessary for the complete catabolism of sucrose as well as a specific regulatory gene.

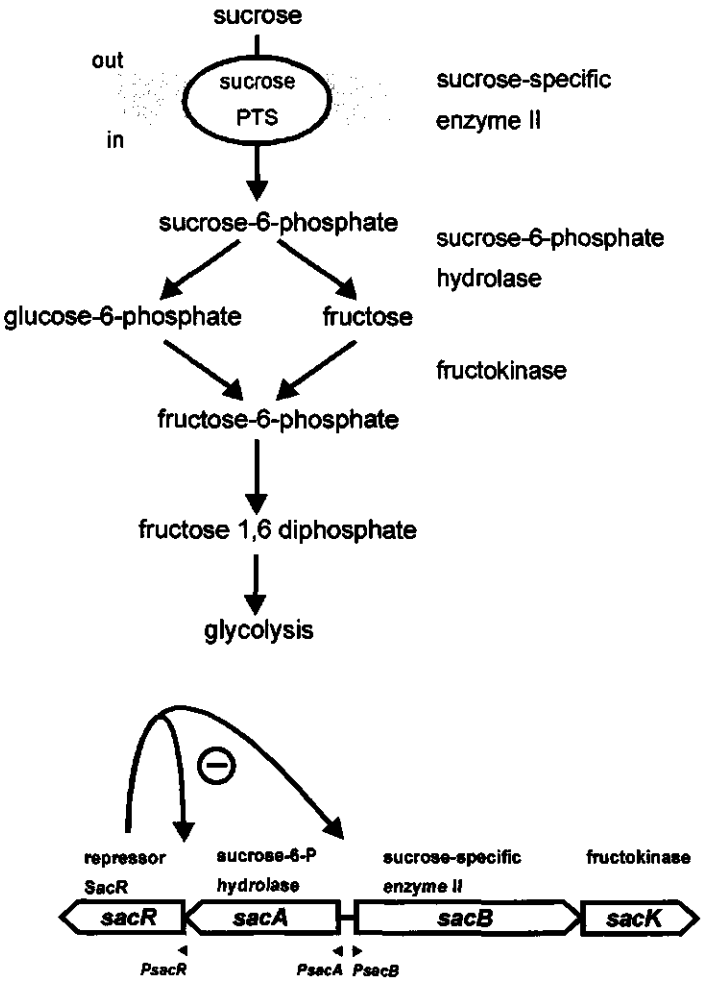

Figure 1. Overview of the proteins involved in the sucrose metabolism in *Lactococcus lactis* and the encoding genes. Following the uptake via the PTS the resulting sucrose-6-phosphate is hydrolyzed by the sucrose-6-phosphate hydrolase resulting in glucose-6-phosphate and fructose. After phosphorylation of the fructose by the fructokinase the fructose-6-phosphate can enter the glycolysis. The genes encoding the repressor protein SacR (*sacR*), the sucrose-6-phoshate hydrolase (*sacA*), the sucrose-specific EII protein (*sacB*) and the fructokinase (*sacK*) are organized as two operons under the control of three SacR-regulated promoters.

Transcriptional analysis of the sucrose gene cluster lead to the identification of three sucrose-inducible transcripts. One of 3.2 kb containing *sacB* and *sacK* which initiates from the *sacB* promoter and is likely to terminate at the inverted repeat located downstream of the *sacK* gene. Another transcript of 3.4 kb, was shown to contain the *sacA* and *sacR* genes and initiates from the *sacA* promoter. Furthermore, a third sucrose-inducible transcript of 1.8 kb was identified, which contains the only the *sacR* gene and initiates from a promoter which was mapped upstream of the *sacR* gene.

Disruption of the *sacR* gene resulted in the constitutive transcription of the *sacBK* and *sacAR* transcripts suggesting that SacR acts as a negative regulator of transcription. Under non-induced circumstances SacR most likely binds to the putative operator sites that were identified in the three promoters of the sucrose operon, resulting in repression of transcription. The presence of an inducer molecule (most likely sucrose-6-P) may result in the dissociation of SacR from the operator leading to transcription of the sucrose genes. The *sacR* gene is subject to a negative autoregulatory mechanism that results in higher levels of the repressor protein under induced circumstances compared to the non-induced situation. This control system allows for a very tight control of the expression of all the sucrose genes and to fast adaptation to environmental changes and resembles the system identified for the transcriptional control of the galactose genes in *E. coli* (Weickert and Adhya. 1993).

The transcription of the *sacA* and *sacB* promoters in the wild-type strain is subject to glucose repression. The disruption of the *sacR* gene resulted in the complete absence of the glucose repression observed in the wild-type. This suggested that the glucose repression is dependent on SacR and is most likely due to a reduced induction resulting from lower concentrations of inducer molecules rather than the activity of a general regulatory mechanism like CcpA-mediated CCR. The concentration of inducer molecules may be affected by inducer control mechanisms like inducer exclusion and inducer expulsion (see below). The tight regulation of the expression of the *sac* genes by the operon-specific regulator SacR and the apparent independency of the chromosomally encoded CcpA-mediated CCR, may be a consequence of their location on a conjugative transposon of non-lactococcal origin that may be transferred to a variety of hosts.

Chapter 3 deals with the detection of CcpA-like proteins in different Gram-positive bacteria including *L. lactis*. Polyclonal antibodies raised against purified *Bacillus megaterium* CcpA were used to screen protein extracts of several Gram-positive bacteria of high and low GC content. The results indicate that cross-reacting proteins were present in all Gram-positive bacteria tested, and suggest that a CcpA-mediated regulatory mechanism, like CCR, is a wide-spread phenomenon.

In **Chapter 4** the cloning and analysis of the *L. lactis ccpA* gene is described. An *L. lactis* expression library was constructed and screened with the CcpA antiserum resulting in the isolation of the *L. lactis ccpA* gene. In contrast to the *Staphylococcus xylosus* and *Lactobacillus casei ccpA* genes, the expression level of the *L. lactis ccpA* gene does not vary significantly in response to the carbon source provided (Egeter and Brückner, 1996; Monedero *et al.*, 1997). The observed negative autoregulation of *ccpA* in *Staphylococcus xylosus* and *Lactobacillus casei* probably provides the cell with a mechanism controlling CCR by varying the level of CcpA protein. The observed regulation of the expression level of the *L. lactis ptsH* gene (see below) might allow a similar regulation of CCR activity in *L. lactis* because it affects the concentration of HPr(Ser-P), which functions as a coregulator. Inactivation of the *L. lactis ccpA* gene resulted in a reduced growth rate on all sugars tested, suggesting an involvement of CcpA in the regulation of a key metabolic pathway.

Because the sucrose gene cluster, despite the presence of a *cre* element, appeared to be independent of CcpA-mediated CCR, a new model system to study CCR was required. The recently identified galactose gene cluster, containing the genes involved in the catabolism of galactose via the Leloir pathway, contains a *cre* element in the promoter region and was therefore a likely candidate for CcpA-mediated CCR (Grossiord *et al.*, 1998). Disruption of the *ccpA* gene confirmed this involvement because the transcription of the *gal* operon in the resulting strain was partly relieved from CCR. However, the transcription of the *gal* operon was not completely relieved from CCR, suggesting that another regulatory mechanism was functional. Possible mechanisms for mediating the residual glucose repression are inducer exclusion and inducer expulsion.

The expression of the genes encoding the glycolytic enzymes pyruvate kinase and L-lactate dehydrogenase is subject to carbon source dependent regulation since higher activities of both enzymes were measured in cells grown on glucose compared to cells grown on galactose. The genes encoding pyruvate kinase and L-lactate dehydrogenase are located in an operon structure together with the gene encoding the glycolytic enzyme phosphofructokinase (Llanos *et al.*, 1992; Llanos *et al.*, 1993). This operon, designated *las* for lactic acid synthesis, contains a *cre* site in the promoter region and is therefore a likely candidate for CcpA-mediated regulation. The inactivation of the *ccpA* gene resulted in a four-fold reduction of the transcription of the *las* operon genes indicating that CcpA acts as a transcriptional activator. CcpA has been reported to act as a transcriptional activator of the *Bacillus subtilis alsS* and *ackA* genes encoding α-acetolactate synthase and acetate kinase, respectively (Grundy *et al.*, 1993; Renna *et al.*, 1993). However, the CcpA-mediated transcriptional activation of the *L. lactis las* operon is the first report of transcriptional control of genes encoding enzymes involved in the central carbohydrate metabolism in Gram-positive bacteria.

The lower transcription level of the *las* operon was reflected in reduced activities of pyruvate kinase and L-lactate dehydrogenase, resulting in a lower production of L-lactate. Furthermore, the fermentation pattern after growth on glucose had changed from almost homolactic, in case of the wild-type strain, to a more mixed-acid pattern in the *ccpA* knock out strain. The observation that the *B. subtilis ccpA* was capable of complementing the transcriptional activation, combined with the presence of *cre* sites in the promoter regions of glycolytic genes of different Gram-positive bacteria, strongly suggests that the observed transcriptional activation of glycolytic genes is not limited to *L. lactis*.

The analysis of the *L. lactis ptsHI* genes is described in **Chapter 5**. The *ptsHI* operon is transcribed as a 2.0-kb transcript from a single promoter mapped upstream of the *ptsH* gene. Furthermore, a 0.3-kb transcript was detected that contained only the *ptsH* gene. This transcript originates from the *ptsH* promoter and terminates at a stem-loop structure located downstream of the *ptsH* gene. This transcriptional organization most likely results in a higher expression of the *ptsH* gene, explaining the higher amount of HPr protein compared to enzyme I as observed in several bacteria, including *Staphylococcus carnosus* (Kohlbrecher *et al.*, 1992).

The expression of the *ptsHI* genes appeared to be regulated since lower transcription levels were observed when the cells were grown on the non-PTS sugar galactose compared to the PTS sugar glucose. Induction of the *ptsHI* expression by glucose has also been observed in *Bacillus subtilis* and allows the cell to control the activity of the PTS in response to the carbon source availability (Stülke *et al.*, 1997). The glucose induction of the *Bacillus subtilis ptsHI* genes is mediated via an antitermination mechanism and is dependent of a characteristic terminator structure located upstream of the *ptsH* gene. Because no obvious recognition sites for transcriptional regulators could be identified at relevant positions in the *L. lactis ptsHI* operon, the mechanism by which the transcriptional control of this operon operates remains to be clarified.

The disruption of both the *ptsH* and the *ptsI* genes resulted in the absence of growth on sucrose and fructose, indicating that these sugars are exclusively taken up by the PTS. The growth rate on glucose was severely reduced, suggesting that in addition to the PTS, another glucose uptake system is present. This finding is in agreement with the results of Thompson and coworkers who presented biochemical evidence that *L. lactis* uses the PTS and a non-PTS permease for the uptake of glucose (Thompson *et al.*, 1985). Complementation of the *ptsH* and *ptsI* genes with the appropriate *L. lactis* genes under the control of an inducible promoter confirmed the functionality of both genes. Furthermore, the growth rate on galactose and maltose, two sugars that are most likely taken up via a non-PTS system was reduced two-fold. This observation suggests an involvement of the PTS with either protein activities involved in the galactose and maltose catabolism or the regulation of the expression of the encoding genes.

In Gram-positive bacteria, the PTS has been reported to control catabolic pathways, like the *Bacillus subtilis* levanase or glycerol pathways (Stülke *et al.*, 1995; Charrier *et al.*, 1997) or the lactose uptake in *Streptococcus thermophilus* (Poolman *et al.*, 1995), by HPr(His-P)-dependent phosphorylation of either enzymes or regulatory proteins resulting in enhanced or reduced activities.

In order to analyze the regulatory role of HPr(Ser-P) in the sugar metabolism of *L. lactis*, mutant HPr proteins were constructed that were affected in the phosphorylation of residue Ser-46. Overproduction in a wild-type strain of HPr(S46D) where residue Ser-46 has been changed into an aspartic acid, that mimics a phosphorylated serine, resulted in a reduction of the growth rate on galactose, whereas the growth rate on glucose was not affected. These results suggested that HPr(Ser-P) is involved in the CCR of the galactose metabolism. Whether this regulation occurs in combination with the inducer control mechanisms or at the transcriptional level in combination with CcpA remains to be determined.

In addition to its role in the CCR of the genes involved in the galactose metabolism, HPr(Ser-P) is also involved in the positive regulation of the enzymes encoded by the *las* operon. Expression of the gene encoding S46D HPr in wild-type cells grown on galactose resulted in increased activities of both pyruvate kinase and L-lactate dehydrogenase. Since the positive effect of the production of S46D HPr on the activities of pyruvate kinase and L-lactate dehydrogenase depends on the presence of the *ccpA* gene, it is feasible that the regulation occurs at the transcriptional level.

These findings established the function of HPr(Ser-P) as a signal molecule in several allosteric and transcriptional metabolic control systems in *L. lactis* (Fig. 2). The occurrence of HPr(Ser-P) results in a reduced entry of new sugar phosphates into the glycolysis due to the inducer control mechanisms and CCR. In addition, the catabolite activation of the *las* operon results in an increased flux through the glycolysis. Consequently, the inducer control systems as well as the CcpA-mediated catabolite control can be seen as mechanisms to prevent the wasteful and possibly toxic accumulation of early glycolytic intermediates.

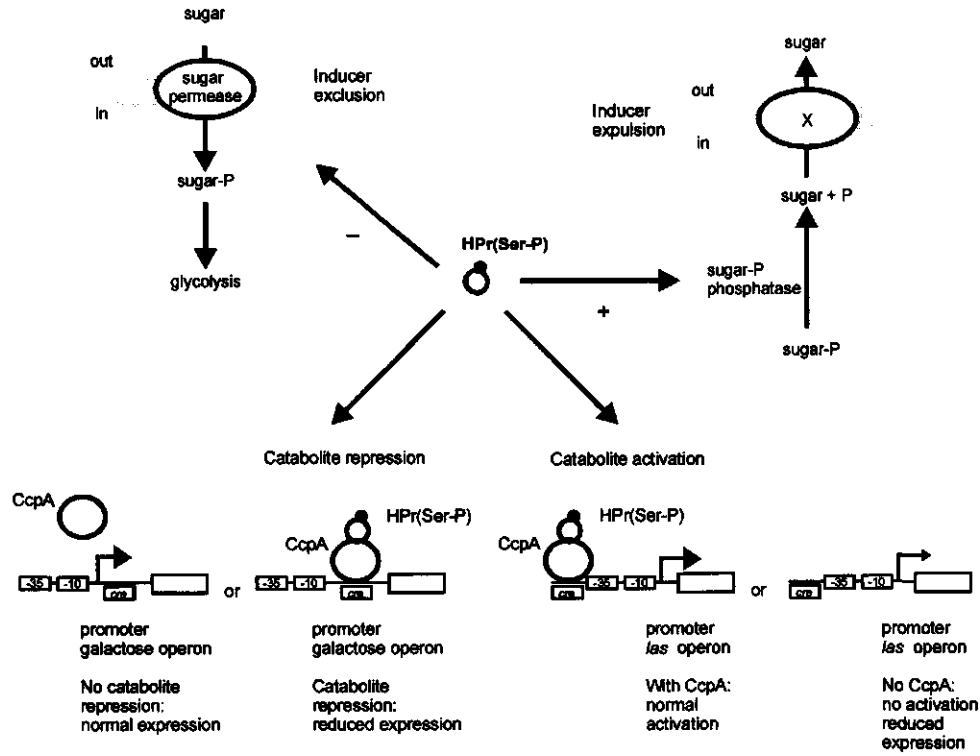

Figure 2. Overview of the pivotal role of HPr(Ser-P) in the control of the carbohydrate metabolism in *L. lactis*. During inducer exclusion the interaction of HPr(Ser-P) with sugar uptake proteins reduces the uptake of new sugar molecules in the cell. The influx of new sugar phosphates in the glycolysis is also reduced by inducer expulsion where HPr(Ser-P) activates a sugar phosphate phosphatase. This activation results in a dephosphorylation of sugar phosphates and the subsequent exit of the resulting sugar from the cell via an unknown channel prevents further catabolism of the sugar. An interaction of HPr(Ser-P) with CcpA results in the binding of this complex to *cre* elements leading to CCR of the *gal* operon, reducing further catabolism of galactose, and catabolite activation of the *las* operon resulting in a higher flux through the glycolysis.

The studies described in this thesis have resulted in the characterization of different regulatory mechanisms involved in the control of the carbohydrate metabolism in *L. lactis*. The regulation of the expression of the Tn*5276*-located sucrose genes appeared to be dependent on the operon-specific regulator, SacR. This apparent independence of chromosomally encoded global regulation might be a result of the fact that these genes are located on transposons, which can be conjugally transferred to other species and therefore require a host-independent transcriptional control system. The analysis of the *L. lactis ccpA* gene lead to the identification of two CcpA-dependent regulatory systems i.e. the CcpA-mediated CCR of the galactose operon and the transcriptional activation of the glycolytic *las* operon. The CCR of the galactose operon mediated by CcpA confirmed previous reports on the role of CcpA in other Gram-positive bacteria. So far, CcpA-mediated transcriptional activation of gene expression was only identified in *B. subtilis*. However, the observation that the *L. lactis* CcpA mediates the expression of genes encoding key enzymes of the glycolysis suggests that CcpA is involved in the global transcriptional control of the metabolic activity, in response to carbon source availability. The observation that the seryl-phosphorylated form of HPr is involved as coregulator in CCR as well as the CcpA-mediated transcriptional activation of gene expression established its important role as signal molecule reflecting the energy state of the cell.

The new information concerning the elements involved in the CcpA-mediated catabolite activation of the central carbohydrate metabolism can be used to accelerate the L-lactate formation in certains strains. The disruption of the *ccpA* gene most likely results in an increased intracellular concentration of early glycolytic intermediates like FDP, which might lead to an increased biosynthesis of e.g. extracellular polysaccharides since precursors thereof are derived from these metabolites. Data emerging from the *L. lactis* sequencing project (Bolotin *et al.*, 1998) in combination with new technologies like the microarray technology (de Saizieu *et al.*, 1998), allowing genome-wide monitoring of gene expression, and the knowledge of the global regulatory mechanisms presented in this thesis will facilitate the design of metabolic engineering strategies.

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CHAPTER 7

Nederlandstalige samenvatting

Nederlandstalige samenvatting

Melkzuur bacteriën.

Tijdens de productie van verschillende levensmiddelen, waaronder zuivelproducten, worden verschillende bacteriën gebruikt. Belangrijke bacteriën die in de zuivelindustrie gebruikt worden zijn de zogenaamde melkzuurbacteriën. De belangrijkste taak van deze bacteriën in deze productieprocessen is de vorming van melkzuur. De productie van melkzuur zorgt voor een verlaging van de zuurgraad van het uiteindelijke product en verhindert daardoor de groei van ongewenste bacteriën. Het mengsel van melkzuurbacteriën dat aan een product wordt toegevoegd, de startercultuur, bepaalt in belangrijke mate de uiteindelijke smaak, geur en textuur van het eindproduct. Tijdens de fermentatie van zuivelproducten zoals kaas en yoghurt wordt meestal een startercultuur gebruikt die bestaat uit een mengsel van verschillende melkzuurbacteriën die elk een bijdrage leveren aan de eigenschappen van het eindproduct. Een aantal van deze bacteriën zet tijdens de fermentatie de melksuiker lactose om in melkzuur en een aantal andere metabolieten. De verzuring levert een belangrijke bijdrage aan een betere conservering. Daarnaast draagt de verzuring in sommige producten, zoals yoghurt, ook voor een belangrijk deel bij aan de smaak. De afbraak van melkeiwitten zoals caseïne, door melkzuurbacteriën draagt tenslotte bij aan de smaak en de geur van het eindproduct.

Eén van de best bestudeerde melkzuurbacteriën is *Lactococcus lactis*. Deze bacterie wordt onder andere gebruikt tijdens de bereiding van Goudse kaas. Tijdens de groei in melk haalt *Lactococcus lactis* het grootste deel van de energie- en de koolstofbehoefte uit lactose. Naast groei op lactose is *Lactococcus lactis* ook in staat een beperkt aantal andere suikers zoals glucose (druivesuiker), sucrose (biet- of rietsuiker) en galactose te gebruiken als koolstof- en energiebron. Om op deze suikers te kunnen groeien heeft *Lactococcus lactis* de beschikking over allerlei systemen om de suikers in de cel op te nemen en om te zetten in nuttige verbindingen. Na de opname wordt het merendeel van de suiker gebruikt om er energie uit te winnen. Dit gebeurt in de zogenaamde glycolyse. Dit is een verzameling van biochemische omzettingen die in het geval van *Lactococcus lactis* uiteindelijk resulteren in de vorming van energie en melkzuur.

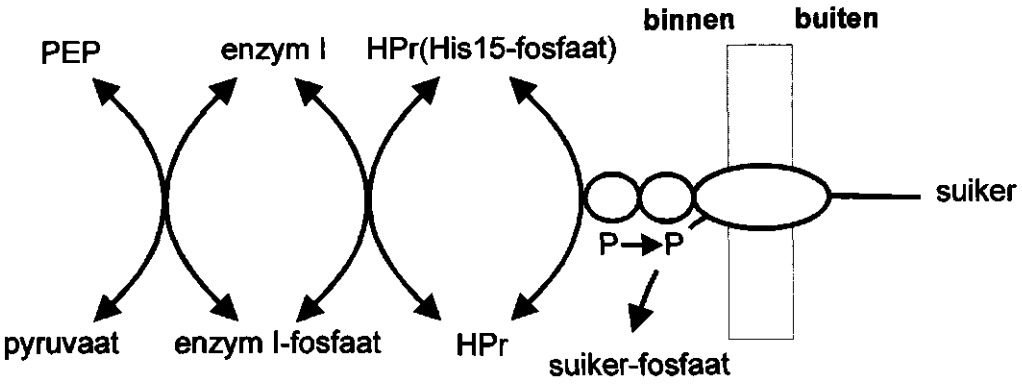
Genetische informatie.

De systemen die een bacterie nodig heeft voor transport en afbraak van suikers bestaan voornamelijk uit een verzameling eiwitten. Eiwitten zijn opgebouwd uit aminozuren en vervullen allerlei functies in de cel zoals het vergemakkelijken van allerlei chemische reakties (enzymatische functie). De informatie die de cel nodig heeft om deze eiwitten te maken ligt opgeslagen in het erfelijk materiaal, het DNA. DNA is opgebouwd uit vier verschillende bouwstenen, basen, die als de kralen aan een ketting achter elkaar liggen.

Het erfelijk materiaal van *Lactococcus lactis* is ongeveer 2,5 miljoen basen groot en bevat, naast de informatie voor de eiwitten voor het suikergebruik, ook informatie voor de aanmaak van andere eiwitten die de cel nodig heeft om te overleven. Het deel van het DNA dat de informatie bevat voor een bepaald eiwit wordt een gen genoemd. De volgorde van de vier bouwstenen in een gen bepaalt uiteindelijk de eigenschappen van alle eiwitten die de cel kan maken.
Het genetisch materiaal van *Lactococcus lactis* bevat waarschijnlijk tussen de 1500 en de 2000 genen die elk voor een eiwit kunnen coderen en deze bacterie is dus in staat om 1500 tot 2000 verschillende eiwitten te maken. Het tot expressie komen van een gen leidt tot de aanmaak van een eiwit; een hoger expressieniveau van een gen heeft een hogere eiwit concentratie in de cel tot gevolg. Niet alle genen moeten tegelijk tot expressie komen. Daarnaast kosthet tot expressie komen van een gen energie. Daarom heeft de cel allerlei methodes ontwikkeld om er voor te zorgen dat een gen alleen dan tot expressie komt wanneer dat nodig is voor het goed functioneren van de cel. Vele genen zijn daarom uitgerust met een schakelaar, een promoter, die aan- en uitgeschakeld kan worden. Door het aan- en uitschakelen van een promoter goed te regelen kan de cel voorkomen dat genen tot expressie komen en er eiwitten aangemaakt worden zonder dat dit nodig is. Nadat een promoter aangeschakeld is, wordt de genetische informatie van het gen gelezen door een eiwit, RNA-polymerase, en de informatie wordt gebruikt voor de aanmaak van RNA. RNA dat erg lijkt op DNA wordt op zijn beurt weer afgelezen door een ribosoom dat de informatie van het RNA gebruikt voor de productie van een eiwit. Nadat het RNA gebruikt is voor de productie van een eiwit wordt het na verloop van tijd afgebroken. RNA heeft dus, in tegenstelling tot DNA, een beperkte levensduur. Het aan en uitschakelen van een promoter kan op een aantal manieren gemeten worden. Allereerst kan er gekeken worden naar de aanwezigheid van het eiwit waarvoor het gen de informatie bevat, door de activiteit van dat eiwit te meten. Deze methode is niet altijd toepasbaar omdat niet alle eiwitten een meetbare activiteit hebben of omdat de activiteiten erg lastig te meten zijn. Een meer algemene methode om te bekijken of een promoter aangeschakeld staat of niet, is het meten van het RNA-niveau in de cel. Omdat het RNA een beperkte levensduur heeft, kan het RNA-niveau in de cel gezien worden als een afspiegeling van de activiteit van de promoter.

Dit proefschrift beschrijft een aantal regulatiemechanismen die er voor zorgen dat eiwitten die een rol spelen bij het gebruik suikers zo efficiënt mogelijk aangemaakt worden. Allereerst zal een regulatiemechanisme besproken worden dat specifiek betrekking heeft op de aanmaak van eiwitten voor het gebruik van een enkele suiker. Daarna zullen regulatiemechanismen behandeld worden die de aanmaak van een veel breder scala van eiwitten controleren en daardoor een globalere invloed op het suikergebruik van *Lactococcus lactis* hebben.

Specifieke regulatie.
De regulatie van de genen die een rol spelen in het gebruik van de suiker sucrose wordt beschreven in **Hoofdstuk 2**. Sucrose is een suiker die veelvuldig voorkomt in planten en omdat sommige *Lactococcus lactis* stammen op deze suiker kunnen groeien wordt vermoed dat deze bacterie in de natuur ook op plantenmateriaal kan groeien. *Lactococcus lactis* neemt sucrose op via het zogenaamde phosphotransferase systeem, afgekort tot PTS. De schematische opbouw van het PTS staat beschreven in Figuur 1 en bestaat uit twee algemene eiwitten HPr en enzym I die in Hoofdstuk 5 in meer detail besproken worden. HPr en enzym I spelen een rol in de overdracht van energie, in de vorm van een fosfaatmolecuul, van de energiedrager PEP (phosphoenolpyruvaat) naar een suiker-specifiek PTS-opname-eiwit. Tijdens de opname van een suiker via het PTS wordt de fosfaatgroep, die afkomstig is van PEP, via enzym I en HPr overgedragen naar het suiker-specifieke opname eiwit dat de fosfaat groep dan, tijdens de opname, aan het suikermolecuul koppelt.

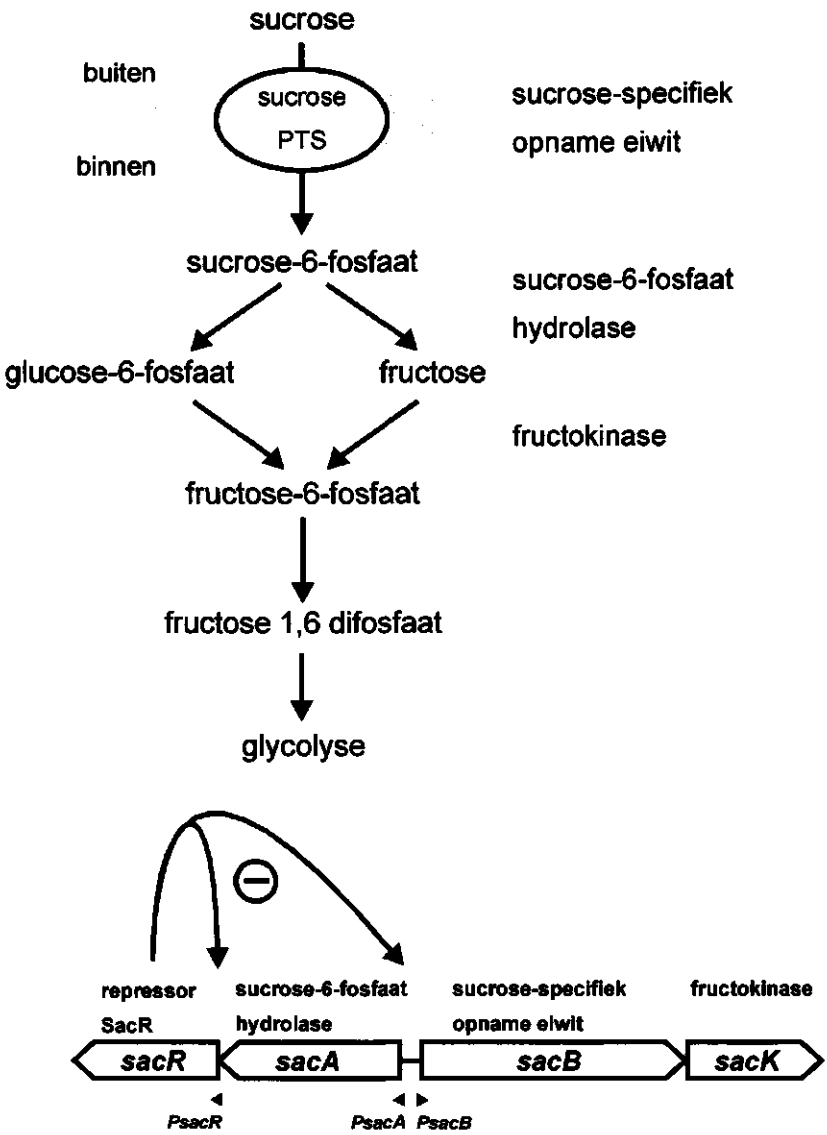


Figuur 1. Overzicht van het phosphotransferase systeem (PTS). Een fosfaat-groep afkomstig van PEP wordt via de algemene eiwitten enzym I en HPr overgedragen op een suiker-specifiek suikeropname-eiwit. Na de fosforylatie van het suiker-specifieke opname-eiwit is het in staat een suiker op te nemen en te fosforyleren.

Uit voorafgaand onderzoek was bekend dat *Lactococcus lactis* onder andere lactose, glucose en sucrose opneemt via het PTS. HPr en enzym I spelen een rol in opname van al deze suikers, terwijl er voor elke suiker apart een suiker-specifiek PTS-opname-eiwit aanwezig is. Opname van sucrose via het PTS resulteert in sucrose-6-fosfaat dat echter niet direct gebruikt kan worden om er energie uit te winnen. Hiervoor moeten er nog een aantal omzettingen plaatsvinden. De eerste stap is de splitsing van sucrose-6-fosfaat in de twee bouwstenen glucose-6-fosfaat en fructose, door een eiwit genaamd sucrose-6-fosfaat-hydrolase.

Glucose-6-fosfaat kan direct gebruikt worden om er energie uit te winnen maar fructose moet eerst voorzien worden van een fosfaatgroep voordat het gebruikt kan worden. Het derde enzym dat een rol speelt in het gebruik van sucrose is een fructokinase dat in staat is om een fosfaatgroep, afkomstig van een andere energiedrager ATP, over te dragen aan fructose, resulterend in fructose-6-fosfaat (zie overzicht in Figuur 2). Het gen (*sacA*) dat de genetische informatie voor het sucrose-6-fosfaat-hydrolase bevat was al in 1992 geisoleerd en beschreven. Tijdens het in dit proefschrift beschreven werk zijn ook de genen die de genetische informatie bevatten voor het sucrose-specifieke PTS-opname-eiwit en het fructokinase gevonden (*sacB* en *sacK*, respectievelijk). Na het uitschakelen van het *sacB*-gen was de resulterende stam niet meer in staat te groeien op sucrose. Dit bevestigde de rol van SacB-eiwit in de opname van sucrose. De drie sucrose genen, *sacABK*, bleken bij elkaar in de buurt te liggen in een zogenaamde operon structuur en twee genetische schakelaars (promoters) konden worden geidentificeerd. Naast het gen voor het sucrose-6-fosfaat hydrolase bleek nog een vierde gen te liggen, waarvan het voorspelde eiwit leek op eiwitten die een rol spelen in de regulatie van het aan- en uitschakelen van genen. De aanwezigheid van dit gen, dat de naam *sacR* kreeg, suggereerde dat het afgeleide eiwit SacR een rol speelt in het aan- en uitschakelen van de sucrose genen. Ook voor het *sacR* gen kon een promoter geidentificeerd worden. Door het meten van de RNA-niveaus bleek dat de genen voor het gebruik van sucrose alleen in aanwezigheid van sucrose aangeschakeld stonden. Bij groei op andere suikers, zoals glucose, bleek er geen RNA gemaakt te worden, suggererend dat de schakelaars van de sucrose-genen niet aangeschakeld waren. Verder kon uit de analyse van het RNA geconcludeerd worden dat het aan- en uitschakelen van de sucrose genen afhankelijk was van de activiteit van drie promoters, die alledrie op een gelijke manier reageren op de aanwezigheid van sucrose in het groei medium.

Om de rol van het SacR-eiwit in de regulatie van de sucrose genen te analyseren werd het *sacR*-gen uitgeschakeld. Omdat dezelfde hoeveelheid RNA werd gemeten in SacR-deficiënte cellen die op glucose en op sucrose gegroeid waren, werd de conclusie getrokken dat SacR verantwoordelijk was voor het suiker-afhankelijke aan- en uitschakelen van de sucrose-genen. Tijdens de groei op glucose, bindt het SacR eiwit waarschijnlijk aan de sucrose-schakelaars en verhindert daardoor de aanmaak van RNA en dus de productie van de eiwitten voor het gebruik van sucrose. Op het moment dat er sucrose in het groeimedium aanwezig is en in geringe mate de cel binnen komt, verandert waarschijnlijk de vorm van het SacR-eiwit zodanig dat het niet meer in staat is te binden aan de sucrose schakelaars en daardoor begint de productie van de eiwitten voor het effiënte gebruik van sucrose.



Figuur 2. Overzicht van het sucrose-metabolisme in *Lactococcus lactis* (boven) en de genen die coderen voor de betrokken eiwitten (onder). Na de opname via het PTS wordt het resulterende sucrose-6-fosfaat gesplitst door het eiwit sucrose-6-fosfaat hydrolase resulterend in glucose-6-fosfaat en fructose. Na fosforylering van het fructose door een fructokinase kan het fructose-6-fosfaat gebruikt worden in de glycolyse. De genen coderend voor de repressor SacR (*sacR*), het sucrose-6-fosfaat hydrolase (*sacA*), het sucrose-specifieke opname-eiwit (*sacB*) en de fructokinase (*sacK*) liggen naast elkaar en de drie promoters (*PsacA*, *PsacB* en *PsacR*) worden door het SacR repressor eiwit aan- en uitgeschakeld.

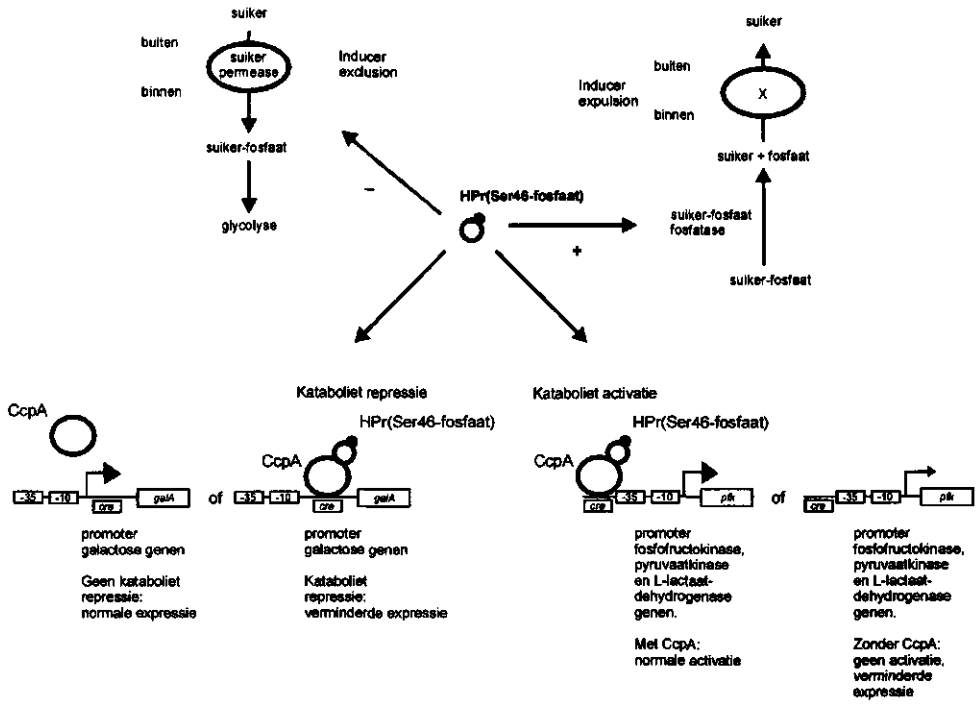
Globale regulatie.
De hierboven beschreven experimenten zijn een vereenvoudiging van de situatie die de cel onder normale omstandigheden, dat wil zeggen in de natuur, tegenkomt. De hoeveelheid suiker waarover de cel kan beschikken is variabel en bovendien kunnen niet één maar meerdere suikers tegelijk beschikbaar zijn. Omdat de groeisnelheid van een bacterie op sommige suikers hoger is dan op andere, hebben bacteriën regulatiesystemen ontwikkeld die er voor zorgen dat, als er meerdere suikers beschikbaar zijn, de suiker die leidt tot de hoogste groeisnelheid meestal het eerst wordt gebruikt. Dit wordt katabolietrepressie genoemd omdat een suiker (kataboliet) de aanmaak van eiwitten voor het gebruik van een andere suiker onderdrukt. Deze repressie vindt onder andere plaats op het niveau van de aanmaak van RNA.

Uit het werk van andere onderzoeksgroepen was duidelijk geworden dat in bacteriën die lijken op *Lactococcus lactis*, de aanwezigheid van glucose in het groeimedium het gebruik van andere suikers duidelijk reduceerde en dat het zogenaamde CcpA-eiwit daarin een belangrijke rol speelde. De aanwezigheid van glucose in het groeimedium leidt tot een verhoging van de concentratie in de cel van allerlei intermediairen (tussenprodukten) van het suikermetabolisme, waaronder ook fructose-1,6-di-fosfaat. Een verhoogde concentratie fructose-1,6-di fosfaat leidt direct of indirect tot een vormverandering van het CcpA-eiwit, waardoor dit in staat is aan het DNA van verschillende genen te binden en de aanmaak van eiwit te beïnvloeden. De plaats waar het CcpA-eiwit aan het DNA kan binden wordt een *cre*-element genoemd en wordt meestal gevonden in de buurt van de promoter van een gen.

De **Hoofdstukken 3 en 4** beschrijven de isolatie en karakterisatie van het *ccpA*-gen uit *Lactococcus lactis*. Naast de isolatie van het *ccpA*-gen was het nodig een gen of meerdere genen te vinden die beinvloed worden door CcpA en die dus een *cre*-element in het DNA bevatten. De groeisnelheid van *Lactococcus lactis* op galactose is ongeveer de helft van de groeisnelheid die gemeten wordt op glucose. Bovendien was uit voorafgaande experimenten duidelijk geworden dat de aanmaak van de eiwitten voor het gebruik van galactose sterk gereduceerd werd als er glucose in het groeimedium aanwezig was. De genen voor het gebruik van galactose zijn geïsoleerd en bleken een *cre*-element te bevatten in de buurt van de genetische schakelaar. Hierdoor waren de galactose-genen een ideaal model om de katabolietrepressie in *Lactococcus lactis* te bestuderen. Allereerst werd gekeken of er daadwerkelijk een verminderde aanmaak van RNA plaatsvond in de aanwezigheid van galactose en glucose vergeleken met de aanwezigheid van galactose alleen. Omdat er geen RNA van de galactose-genen kon worden aangetoond onder deze omstandigheden werd de conclusie getrokken dat katabolietrepressie een rol speelt in de regulatie van deze genen, naast een operon-specifieke regulatie van de galactose-genen.

Om de rol van CcpA in de katabolietrepressie vast te stellen is het *ccpA*-gen uitgeschakeld en zijn de effecten van de deze mutatie op de aanmaak van RNA van de galactose-genen bestudeerd. Het uitschakelen van het *ccpA*-gen resulteerde in een reductie van de katabolietrepressie van de galactose-genen; dat wil zeggen dat er, in vergelijking met de uitgangsstam, duidelijk meer aanmaak van RNA van de galactose-genen was tijdens de groei op een mengsel van galactose en glucose. Hiermee was de rol van CcpA in de katabolietrepressie van de galactose-genen bewezen. De observatie dat er niet evenveel RNA werd gemeten vergeleken met het niveau van de uitgangsstam op galactose alléén, suggereerde dat er nog een ander regulatiemechanisme functioneel zou kunnen zijn.

Naast het verminderen van de katabolietrepressie van de galactose-genen resulteerde het uitschakelen van het *ccpA*-gen ook in een vermindering van de groeisnelheid op verschillende suikers. Dit fenomeen was ook in andere bacteriën geobserveerd en verschillende onderzoekers hebben gespeculeerd dat CcpA een rol zou kunnen spelen in de produktie van eiwitten die van belang zijn voor de aanmaak van energie. In *Lactococcus lactis* blijken de genen coderend voor drie belangrijke eiwitten fosfofructokinase, pyruvaatkinase en L-lactaatdehydrogenase, die een rol spelen in de aanmaak van energie, bij elkaar in de buurt te liggen in een zogenaamde operon-structuur. De aanmaak van deze eiwitten is afhankelijk van één enkele promoter. Nauwkeurige analyse van deze promoter leidde tot de identificatie van een *cre*-element suggererend dat het CcpA-eiwit een rol speelt in de regulatie van de aanmaak van deze eiwitten. De hoeveelheid RNA afgeleid van de genen die coderen voor fosfofructokinase, pyruvaatkinase en L-lactaatdehydrogenase was met een factor vier verminderd in de stam waar het *ccpA* gen was uitgeschakeld, vergeleken met de uitgangsstam. Bovendien had het uitschakelen van het *ccpA* gen een twee- tot viervoudige reductie van de activiteiten van pyruvaatkinase en L-lactaatdehydrogenase tot gevolg. Deze resultaten tonen aan dat het CcpA-eiwit een rol speelt in het aanschakelen van deze belangrijke genen. De algemene conclusie van **Hoofdstuk 4** is dat het CcpA-eiwit in staat is om op twee verschillende manieren de aanmaak van RNA te beïnvloeden. De aanmaak van RNA van de galactose-genen wordt negatief beinvloed als gevolg van de aanwezigheid van glucose, terwijl de aanmaak van RNA van de fosfofructokinase, pyruvaatkinase en L-lactaatdehydrogenase genen gestimuleerd worden door CcpA (overzicht in Figuur 3).



Figuur 3. Overzicht van de centrale rol van HPr(Ser46-fosfaat) in de regulatie van het koolstofmetabolisme in *Lactococcus lactis*. Tijdens inducer exclusion resulteert de interactie van HPr(Ser46-fosfaat) met een opname-eiwit tot een sterk gereduceerde opname van suiker. De verdere instroom van suikers in de glycolyse wordt ook voorkomen door inducer expulsion waarin de interactie van HPr(Ser46-fosfaat) met een suikerfosfatase een defosforylering van suiker-fosfaten tot gevolg heeft, waarna de zo ontstane suikers de cel verlaten via een nog niet-geidentificeerd transport-kanaal (X). Het complex gevormd tussen HP(Ser46-fosfaat) en het CcpA-eiwit kan binden aan verschillende *cre*-elementen en deze binding kan de aanmaak van RNA remmen zoals bij de galactose-genen of, in het geval van de genen coderend voor fosfofructokinase, pyruvaatkinase en L-lactaatdehydrogenase, de aanmaak van RNA juist stimuleren.

De analyse van de *Lactococcus lactis ptsH-* en *ptsI*-genen coderend voor de eiwitten HPr en enzym I van het PTS staat beschreven in **Hoofdstuk 5**. Het uitschakelen van beide genen had tot gevolg dat de resulterende stam niet meer in staat bleek te groeien op sucrose en fructose. Bovendien was de groei op glucose sterk verminderd. Hieruit kon de conclusie worden getrokken dat het PTS een belangrijk opnamesysteem is voor deze suikers in *Lactococcus lactis*.
Uit het werk van andere onderzoeksgroepen was bekend dat het HPr-eiwit naast de suikeropname ook een rol speelt in katabolietrepressie. Om deze functie te vervullen bleek de aanwezigheid van een fosfaatmolecuul op het serine-residu op positie 46 in het HPr-eiwit, resulterend in HPr(Ser-46-fosfaat), essentieel te zijn.

De aanwezigheid van deze negatief-geladen fosfaatgroep verandert de lading van het HPr-eiwit en hierdoor kan het HPr-eiwit interacties aangaan met andere eiwitten. HPr(Ser46-fosfaat) kan een interactie aangaan met het eerder genoemde CcpA-eiwit en het complex van deze twee eiwitten kan binden aan *cre*-elementen van genen die gevoelig zijn voor katabolietrepressie.

Daarnaast kan HPr(Ser46-fosfaat) tijdens een proces met de naam inducer exclusion (het buitensluiten van een koolstofbron) een interactie aangaan met een suikeropname-eiwit en deze interactie vermindert de suikeropname door dit eiwit. Bij een ander proces genaamd inducer expulsion (het naar buiten sluizen van een koolstofbron), gaat HPr(Ser46-fosfaat) een interactie aan met een eiwit dat er voor zorgt dat een aantal in de cel aanwezige suikers hun lading verliezen en daarna via een tot nog toe onbekende weg de cel verlaten. Om de rol van het HPr-eiwit in de katabolietrepressie, inducer exclusion, en inducer expulsion te onderzoeken is het aminozuur-residu serine-46 veranderd in een asparaginezuur. Asparaginezuur is een aminozuur, net als serine, maar dit aminozuur is onder de omstandigheden in de cel altijd negatief geladen; hierdoor lijkt het heel erg op een serine-residu met een fosfaatgroep. Het mutante eiwit HPr(Ser46Asp) waarin deze mutatie is aangebracht lijkt daarom op het HPr(Ser46-fosfaat) eiwit en de productie van dit eiwit in de cel kan dus leiden tot het aanschakelen van de katabolietrepressie, inducer exclusion, en inducer expulsion. Productie van het mutante eiwit HPr(Ser46Asp) in *Lactococcus lactis* leidde tot een sterk verminderde groei op galactose. Deze remming van de groei kan veroorzaakt worden door het aanschakelen een of meerdere van de bovenstaande mechanismen. Verdere experimenten zijn nodig om te bepalen welke mechanismen in welke mate bij dragen aan de verminderde groei op galactose.

Omdat uit de bovenstaande resultaten duidelijk geworden was dat CcpA een rol speelt in het stimuleren van de aanmaak van de fosfofructokinase, pyruvaatkinase en L-lactaatdehydrogenase eiwitten, is ook de rol van HPr(Ser46-fosfaat) in deze stimulatie bekeken. Ook in deze experimenten is het mutante eiwit HPr(Ser46Asp) geproduceerd en zijn de effecten van dit eiwit op de aanmaak van pyruvaatkinase en L-lactaatdehydrogenase geanalyseerd.

Wanneer de uitgangsstam gegroeid werd op galactose bleken de activiteiten van de pyruvaatkinase en L-lactaatdehydrogenase lager dan wanneer de cellen gegroeid waren op glucose.

Productie van HPr(Ser46Asp) in cellen gegroeid op galactose resulteerde in een duidelijke verhoging van de activiteiten van pyruvaatkinase en L-lactaatdehydrogenase vergeleken met de groei op galactose zonder productie van dit eiwit. Bovendien was de activiteit van beide eiwitten verhoogd tot het niveau dat normaal in glucose-gegroeide cellen gemeten werd. Omdat de productie van HPr(Ser46Asp) in cellen gegroeid op glucose geen effect had op de gemeten activiteiten werd aangenomen dat dit niveau het maximaal haalbare productieniveau was.

Het mutante HPr(Ser46Asp)-eiwit werd ook geproduceerd in een stam met een uitgeschakeld *ccpA*-gen om te onderzoeken of HPr een rol speelt in de CcpA-afhankelijke regulatie van het expressie-niveau van de genen coderend voor pyruvaatkinase en L-lactaatdehydrogenase. Omdat de activiteiten van pyruvaatkinase en L-lactaatdehydrogenase niet veranderden als reactie op het produceren van HPr(Ser46Asp) werd de conclusie getrokken dat HPr(Ser46-fosfaat) een rol speelt in de *Lactococcus lactis* cel als signaal in de CcpA-afhankelijke stimulatie van de aanmaak van deze twee eiwitten.

Uit het bovenstaande is duidelijk dat het HPr-eiwit een erg belangrijke rol speelt in de controle van het suikermetabolisme van *Lactococcus lactis*. Naast de rol van HPr in de opname van suikers zorgt het ontstaan van HPr(ser46-fosfaat) voor een verminderde toevoer van verschillende suikers in de glycolyse. Dit gebeurt door de rol die HPr(Ser46-fosfaat) speelt in de inducer exclusion, inducer expulsion en in de katabolietrepressie. Daarnaast zorgt de stimulatie van de aanmaak van de fosfofruktokinase, pyruvaatkinase en L-lactaatdehydrogenase door HPr(Ser46-fosfaat) voor een verhoogde glycolytische activiteit (zie het overzicht in Figuur 3). De bovenstaande HPr(Ser46-fosfaat)-afhankelijke mechanismen voorkomen de mogelijk giftige ophoping van intermediairen van de glycolyse.

Het onderzoek beschreven in dit proefschrift heeft geleid tot de identificatie van een aantal specifieke en globale regulatie-mechanismen die een rol spelen in de controle van het centrale metabolisme van *Lactococcus lactis*. De sucrose-genen van *Lactococcus lactis* blijken door de operon-specifieke regulator SacR gereguleerd te worden terwijl de galactose-genen naast een nog niet-geïdentificeerde specifieke regulator ook onder de controle van de globale regulatie-mechanismen staan. Het CcpA-eiwit dat een rol speelt in de katabolietrepressie van de galactose-genen blijkt daarnaast ook de aanmaak van de belangrijke eiwitten fosfofruktokinase, pyruvaatkinase en L-lactaatdehydrogenase te stimuleren. HPr(Ser46-fosfaat) waarvan reeds bekend was dat het een rol als signaaleiwit in de katabolietrepressie speelde, blijkt in *Lactococcus lactis* ook in de positieve regulatie van de genen coderend voor fosfofruktokinase, pyruvaatkinase en L-lactaatdehydrogenase een rol te spelen.

De resultaten die beschreven staan in dit proefschrift laten zien dat de regulatie van het centrale metabolisme van *Lactococcus lactis* afhankelijk is van meerdere mechanismen die zowel de aanmaak als de activiteiten van verschillende eiwitten controleren. Het onderzoek heeft bijgedragen aan een beter inzicht in deze regulatie-mechanismen en de verkregen informatie kan gebruikt worden als basis voor studies gericht op het verbeteren van stammen van *Lactococcus lactis*.

Appendix

```
TCATTTTTTTTCCTCATTTACTAAATTATATATATTTATGATAAACGTTTTTTATAAAAAAATCAATATAAATAAGTATAAAAAAA

TAAAAAATAAAAAAAGTGTGGCAAACGCTTGACATATATCAAAAAAATGATAAAATAACTTCTGTAAGCGAAATCATTTTATTTGA
                          -35                  -10
GGAGAAAATTATGAATCATAAGCAGGTAGCTGAACGCATTTTAAATGCAGTTGGGCGTGATAACATTCAAGGAGCTCGACATTGTG
          M  N  H  K  Q  V  A  E  R  I  L  N  A  V  G  R  D  N  I  Q  G  A  R  H  C
CGACACGACTACGTTTAGTCCTAAAAGATACTGGCGTTATTGATCAAGAGGCTCTAGATAATGACCCAGATCTTAAGGGGACTTTT
A  T  R  L  R  L  V  L  K  D  T  G  V  I  D  Q  E  A  L  D  N  D  P  D  L  K  G  T  F
GAAGCAGCTGGTCAATATCAAATTATTGTAGGGCCAGGTGATGTTAACACTGTATACGAAGAATTTATTAAACTTACAGGAATAAG
 E  A  A  G  Q  Y  Q  I  I  V  G  P  G  D  V  N  T  V  Y  E  E  F  I  K  L  T  G  I  S
TGAGGCATCAACTGCAGATCTCAAAGAGATTGCAGGAAGTCAAAAAAAACAAAATCCAGTAATGGCACTTGTAAAGCTGCTTTCTG
  E  A  S  T  A  D  L  K  E  I  A  G  S  Q  K  K  Q  N  P  V  M  A  L  V  K  L  L  S
ATATTTTTGTTCCTTTGATACCAGCTCTTGTAGCTGGTGGTCTGTTAATGGCTCTTAATAATGCGCTAACAGCTGAACATCTTTTC
D  I  F  V  P  L  I  P  A  L  V  A  G  G  L  L  M  A  L  N  N  A  L  T  A  E  H  L  F
GCGACGAAATCACTTGTTGAAATGTTTCCGATGTGGAAAGGATTTGCTGATATTGTTAATACTATGAGTGCCGCACCATTTACCTT
 A  T  K  S  L  V  E  M  F  P  M  W  K  G  F  A  D  I  V  N  T  M  S  A  A  P  F  T  F
TATGCCAATTTTAATAGGTTATTCTGCCACAAAAAGATTTGGCGGAAATCCATATTTAGGTGCTGTTGTAGGTATGATTATGGTGA
  M  P  I  L  I  G  Y  S  A  T  K  R  F  G  G  N  P  Y  L  G  A  V  V  G  M  I  M  V
TGCCTGGACTTATTAATGGATATAATGTTGCTGAAGCAATCTCTAATCATACAATGACTTATTGGGATATTTTTGGTTTTAAAGTT
M  P  G  L  I  N  G  Y  N  V  A  E  A  I  S  N  H  T  M  T  Y  W  D  I  F  G  F  K  V
GCCCAGGCTGGATATCAAGGACAAGTTCTTCCTGTAATTGGAGTGGCTTTTATCCTTGCTAAACTTGAGAGATTCTTTCATAAATA
 A  Q  A  G  Y  Q  G  Q  V  L  P  V  I  G  V  A  F  I  L  A  K  L  E  R  F  F  H  K  Y
CCTTAACGATGCTATAGACTTCACATTTACTCCGTTACTTTCAGTCATCATAACTGGATTTCTCACATTCACTATTGTGGGACCAG
  L  N  D  A  I  D  F  T  F  T  P  L  L  S  V  I  I  T  G  F  L  T  F  T  I  V  G  P
CACTTCGTTTTGTATCAAATGGCTTGACAGATGGTTTGGTTGGACTTTATAACACATTAGGGGCACTAGGAATGCTTGTGTTTGGA
A  L  R  F  V  S  N  G  L  T  D  G  L  V  G  L  Y  N  T  L  G  A  L  G  M  L  V  F  G
GGCTTCTACTCGGCAATAGTAGTAACTGGATTACATCAGAGTTTTCCGGCCATTGAAACAATGTTAATCACAAACTATCAACACAG
 G  F  Y  S  A  I  V  V  T  G  L  H  Q  S  F  P  A  I  E  T  M  L  I  T  N  Y  Q  H  S
TGGTATTGGTGGAGACTTTATTTTTCCAGTTGCGGCCTGCGCAAATATGGCTCAAGCTGGTGCAACTTTTGCAATTTTATTTGTTA
  G  I  G  G  D  F  I  F  P  V  A  A  C  A  N  M  A  Q  A  G  A  T  F  A  I  L  F  V
CTAAAAACATTAAGACAAAAGCTCTTGCAGCTCCAGCTGGTGTATCTGCTATTCTAGGTATTACAGAACCTGCGTTATTTGGGATT
T  K  N  I  K  T  K  A  L  A  A  P  A  G  V  S  A  I  L  G  I  T  E  P  A  L  F  G  I
AATCTAAAACTAAAATATCCGTTCTTTATTGCTCTTGGGGCTTCAGCAATTGGTTCATTATTTATGGGATTATTCCATGTCCTTGC
 N  L  K  L  K  Y  P  F  F  I  A  L  G  A  S  A  I  G  S  L  F  M  G  L  F  H  V  L  A
GGTTAGTCTGGGATCCGCAGGATTAATTGGCTTTATCTCTATTAAAGCTGGGTATAACTTACAATTTATGATTTCGATATTTATTA
  V  S  L  G  S  A  G  L  I  G  F  I  S  I  K  A  G  Y  N  L  Q  F  M  I  S  I  F  I
GCTTTCTTATTGCCTTTGTTGTTACCTCAATCTATGGTCGGCGGATGGAAGCTAAATCTATTACGAAAGAAAAAAATAAACAGAAT
S  F  L  I  A  F  V  V  T  S  I  Y  G  R  R  M  E  A  K  S  I  T  K  E  K  N  K  Q  N
GCAACAACTCAATACCAACCTGAGAAAGTTATTATCGATCCAGTTAAAAGTGGCGAACTCCTTGCTCCGATAAATGGATTTGTGAT
 A  T  T  Q  Y  Q  P  E  K  V  I  I  D  P  V  K  S  G  E  L  L  A  P  I  N  G  F  V  I
TCCTCTGTCTGATGTAAGTGACCCTGTTTTCTCAAAAGAAATTATGGGAAAAGGTATTGCAATCAAGCCTAAATCTGGAGAACTTT
  P  L  S  D  V  S  D  P  V  F  S  K  E  I  M  G  K  G  I  A  I  K  P  K  S  G  E  L
TCTCACCAGCCGACGGAGAAATTATTATTGCTTATGAAACAGGTCACGCTTATGGTATAAAAACAAAAAATGGAGGTGAAGTTCTT
F  S  P  A  D  G  E  I  I  I  A  Y  E  T  G  H  A  Y  G  I  K  T  K  N  G  G  E  V  L
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 L  H  I  G  I  D  T  V  S  M  N  G  N  G  F  I  Q  N  V  K  V  G  Q  K  V  K  A  G  D
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  L  L  G  S  F  D  K  E  E  I  K  K  S  G  L  D  D  T  V  I  I  V  I  T  N  S  A  S
ACAATGAGATTTTGCCATTGAGTGAAAATGTTGATATCAAAGTTGGAGAAAAAATTCTATTATTGAACTAGAGGGAGCGAACTATG
Y  N  E  I  L  P  L  S  E  N  V  D  I  K  V  G  E  K  I  L  L  L  N  -
TCCGTATACTATGGATCAATTGAGGCTGGTGGTACAAAATTTGTACTTGC (4900)
```

Figure 1. The nucleotide sequence of the *L. lactis sacB* gene. The promoter hexamers are underlined and the ribosome binding site is presented in bold. The number in brackets correspond to the numbering of the nucleotide data submitted to the EMBL, Genbank and DDBJ Nucleotide sequence databases under the accession number Z97015.

```
L.l. SacB   M-NHKQVAERILNAVG-RDNIQGARHCATRLRLVLKDTGVIDQEALDNDP    48
S.m. ScrA   M-DYSKVASEVITAVG-KDNLVAAAHCATRLRLVLKDDSKVDQKALDKNA    48
St.x.ScrA   M-NYKKSAENILQALGGEDNVEAMTHCATRLRLVLKDEGLVDEKALGDMD    49
B.s. SacP   MGELNKSARQIVEAVGGAENIAAATHCVTRLRFALIDESKVDQEMLDQID    50
K.p. PtsB   M-DFEQISRSLLPLLGGKENIASAAHCATRLRLVLVDDALADQQAIGKID    49
S.t. PtsB   M-DFEQISCSLLPLLGGKENIASAAHCATRLRLVLVDDSLADQQAIGKVE    49
            * . .. .  ..  .*  .*. .  **.****..* *..  *.. ...

L.l. SacB   DLKGTFEAAGQYQIIVGPGDVNTVYEEFIKLTGISEASTADLKEIAGSQK    98
S.m. ScrA   DVKGTFKTDGQYQVIIGPGDVNFVYDEIIKQTGLTEVSTDDLKKIAASGK    98
St.x.ScrA   VVKGTFSTGGQYQVIIGSGTVNKVFSELEKITGKEASSVSEVKT--QGTK    97
B.s. SacP   VVKGSFSTNGQFQVVIGQGTVNKVYAELVKETGIGESTKDEVKK--ASEK    98
K.p. PtsB   GVKGCFRNAGQMQIIFGTGVVNKVYAAFIQAAGISESSKSEAADLAA--K    97
S.t. PtsB   GVKGCFRNAGQMQIIFGTGVVNKVYAAFTQAAGISESSKSEAADIAA--K    97
              .** * ..** *.. * * ** *.... . .* .. . ..  .     *

L.l. SacB   KQNPVMALVKLLSDIFVPLIPALVAGGLLMALNNALTAEHL-FATKSLVE   147
S.m. ScrA   KFNPIMALIKLLSDIFVPIIPALVAGGLLMALNNFLTSEGL-FGTKSLVQ   147
St.x.ScrA   NMNPFQRFVKMLSDIFVPIIPAIVAGGLLMGINNILTAPGIFYDNQSLIE   147
B.s. SacP   NMNPLQRAVKTLADIFIPILPAIVTAGLLMGINNILTAEGISFSTKSIVQ   148
K.p. PtsB   KLNPFQRIARLLSNIFVPIIPAIVASGLLMGLLGMVKTYGWVDPSNALYI   147
S.t. PtsB   KLNPFQRIARLLSNIFVPIIPAIVASGLLMGLLGMVKTYGWVDPGNAIYI   147
            . **     . *..**.*..**.*..****.. . ...      ....

L.l. SacB   MFPMWKGFADIVNTMSAAPFTFMPILIGYSATKRFGGNPYLGAVVGMIMV   197
S.m. ScrA   QFPIIKGSSDMIQLMSAAPFWFLPILVGISAAKRFGANQFLGASIGMIMV   197
St.x.ScrA   VQNQFSGLAEMINIFANAPFTLLPILIGFSAAKRFGGNAYLGAALGMILV   197
B.s. SacP   VYPQWADLANMINLIAGTAFTFLPALIGWSAVKRFGGNPLLGIVLGVMLV   198
K.p. PtsB   ----------MLDMCSSAAFIILPILIGFTAAREFGGNPYLGATLGGILT   187
S.t. PtsB   ----------MLDMCSSAAFIILPILIGFTAAREFGGNPYLGATLGGILT   187
                     ...  ....* ..* *.* .*.. **.*. **  .* ...

L.l. SacB   MPGL--INGYNVAEAISNHTMT-----YWDIFGFKVAQAGYQGQVLPVIG   240
S.m. ScrA   APGAANIIGLAANAPISKAATIGAYTGFWNIFGLHVTQASYTYQVIPVLV   247
St.x.ScrA   HPELMSAYDYPKALEAGKEIPH------WNLFGLEINQVGYQGQVLPMLV   241
B.s. SacP   HPDLLNAWGYGAAEQSG-EIPV------WNLFGLEVQKVGYQGQVLPILL   241
K.p. PtsB   HPALTNAWGVAAGFHTMN------------FFGIEVAMIGYQGTVFPVLL   225
S.t. PtsB   HPALTNAWGVAAGFHTMN------------FFGFEIAMIGYQGTVFPVLL   225
             *.     .   .                  .**...   .*   *.*..

L.l. SacB   VAFILAKLERFFHKYLNDAIDFTFTPLLSVIITGFLTFTIVGPALRFVSN   290
S.m. ScrA   AVWLLSILEKFFHKRLPSAVDFTFTPLLSVIITGFLTFIVIGPVMKEVSD   297
St.x.ScrA   ATYILATIEKGLRKVIPTVLDNLLTPLLAILSTGFITFSFVGPLTRTLGY   291
B.s. SacP   ASYMLAKIEVFLTKRTPEGIQLLVVAPITLLLTGFASFIIIGPITFAIGN   291
K.p. PtsB   AVWFMSMVEKRLRRVIPDALDLILTPFLTVIISGFIALLLIGPAGRALGD   275
S.t. PtsB   AVWFMSIVEKQLRRAIPDALDLILTPFLTVIISGFIALLIIGPAGRALGD   275
            . .... .*  . .   . ..   .. .... .** ..  .**    ..

L.l. SacB   GLTDGLVGLYNTLGALGMLVFGGFYSAIVVTGLHQSFPAIETMLITNYQH   340
S.m. ScrA   WLTNGIVWLYDTTGFLGMGVFGALYSPVVMTGLHQSFPAIETQLISAFQN   347
St.x.ScrA   WLSDGLTWLYEFGGAIGGLIFGLLYAPIVITGMHHSFIAIETQLIA--DS   339
B.s. SacP   VLTSGLISVFGSFAALGGLLYGGFYSALVITGMHHTFLAVDLQLIG----   337
K.p. PtsB   GISFILSTLISHAGWLAGLLFGGLYSVIVITGIHHSFHAIEAGLLG---N   322
S.t. PtsB   GISFVLSTLISHAGWLAGLLFGGLYSVIVITGIHHSFHAVEAGLLG---N   322
             .. .  . .  . ..  ..* .*. .*.**.*..* *..  *..
```

```
L.l. SacR   M-IKLEDVANKAGVSVTTVSRVINRKGYLSDATISKVEKAMQDLHYIPNA    49
B.s. DegA   MKTTIYDVAKAAGVSITTVSRVINNTGRISDKTRQKVMNVMNEMAYTPNV    50
B.m. CcpA   MNVTIYDVAREASVSMATVSRVVNGNPNVKPSTRKKVLETIERLGYRPNA    50
E.c. RbsR   M-ATMKDVARLAGVSTSTVSHVINKDRFVSEAITAKVEAAIKELNYAPSA    49
H.i. PurR   M-ATIKDVAKMAGVSTTTVSHVINKTRFVAKDTEEAVLSAIKQLNYSPSA    49
B.s. RbsR   M-ATIKDVAGAAGVSVATVSRNLNDNGYVHEETRTRVIAAMAKLNYYPNE    49
            *  .. ***  *.** .***. .* .  .   .  *  ..  . * *.

L.l. SacR   AARSLQGKSLKLIGLVFPTIKNIFYAELIEKIEQALFIRGYKAMLATTE-    98
B.s. DegA   HAAALTGKRTNMIALVAPDISNPFYGELAKSIEERADELGFQMLICSTD-    99
B.m. CcpA   VARGLASKKTTTVGVIIPDISNIFYAELARGIEDIATMYKYNIILSNSD-    99
E.c. RbsR   LARSLKLNQTHTIGMLITASTNPFYSELVRGVERSCFERGYSLVLCNTEG    99
H.i. PurR   VARSLKVNTTKSIGMIVTTSEAPYFAEIIHSVEEHCYRQGYSL-FCVTHK    98
B.s. RbsR   VARSLYKRESRLIGLLLPDITNPFFPQLARGAEDELNREGYRLIFGNSDE    99
             * .*  .    .... .. .. .. ..    *       .   .  ..

L.l. SacR   HDEQKERDYLALLLSNQVDGIIYGSHNLKAH------DYIAIEAPIVAFD   142
B.s. DegA   YDPKKETKYFSVLKQKKVDGIIFATGIESHDSMSALEEIASEQIPIAMIS   149
B.m. CcpA   QNQDKELHLLNNMLGKQVDGIIFMSGNVTEEHV---EELKKSPVPVVLAA   146
E.c. RbsR   -DEQRMNRNLETLMQKRVDGLLLLCTETHQPSREIMQRYPT--VPTVMMD   146
H.i. PurR   MDPEKVKNHLEMLAKKRVDGLLVMCSEYTQDSLDLLSSFST--IPMVVMD   146
B.s. RbsR   -ELKKELEYLQTFKQNHVAGII------AATNYPDLEEYSGMNYPVVFLD   142
             . ..    .  .  ..*.*..                    * .  .

L.l. SacR   RL-LTPETTVVSSDNFEGGILATKALINSGSKKIAIFTGNDNTNSPTYLR   191
B.s. DegA   QDKPLLPMDIVVIDDVRGGYEAAKHLLSLGHTNIACIIGD-GSTTGEKNR   198
B.m. CcpA   SIESTNQIPSVTIDYEQAAFDAVQSLIDSGHKNIAFVSGTLEEPINHAKK   196
E.c. RbsR   WAPFDGDSDLIQDNSLLGGDLATQYLIDKGHTRIACITGPLDKTPARL-R   195
H.i. PurR   WGP-NANTDVIDDHSFDGGYLATKHLIECGHKKIGIICGELNKTTART-R   194
B.s. RbsR   RT--LEGAPSVSSDGYTGVKLAAQAIIHGKSQRITLLRGPAHLPTAQ-DR   189
                      .  .   .   *.. ..     .*.   *          .

L.l. SacR   RDGYLLELERNQLK-PHIIKIPSQWTLLRKKVEIKKILENNDF-DGVFCT   239
B.s. DegA   IKGFRQAMEEAGVPIDESLIIQTRFSLESGKEEAGKLLDRNA-PTAIFAF   247
B.m. CcpA   VKGYKRALTESGLPVRDSYIVEGDYTYDSGIEAVEKLLEEDEKPTAIFVG   246
E.c. RbsR   LEGYRAAMKRAGLNIPDGYEVTGDFEFNGGFDAMRQLLSHPLRPQAVFTG   245
H.i. PurR   YEGFEKAMEEAKLTINPSWVLEGAFEPEDGYECMNRLLTQEKLPTALFCC   244
B.s. RbsR   FNGALEILKQAEVDFQV--IETASFSIKDAQSMAKELFASYPATDGVIAS   237
             .*    .. . .          . ..           ...      ...

L.l. SacR   DDLTAILV----KDLASNLKKSLNVVGFDGTEFIENYYPNLTTIKQPIND   285
B.s. DegA   NDVLACAAIQAARIRGIKVPDDLSIIGFDNTILAEMAAPPLTTVAQPIKE   297
B.m. CcpA   TDEMALGVIHGAQDRGLNVPNDLEIIGFDNTRLSTMVRPQLTSVVQPMYD   296
E.c. RbsR   NDAMAVGVYQALYQAELQVPQDIAVIGYDDIELASFMTPPLTTIHQPKDE   295
H.i. PurR   NDVMALGAISALTEKGLRVPEDMSIIGYDDIHASRFYAPPLTTIHQSKLR   294
B.s. RbsR   NDIQAAAVLHEALRRGKNVPEDIQIIGYDDIPQSGLLFPPLSTIKQPAYD   287
            .*  *  .       . .. ... ..*.*..       * *... *.

L.l. SacR   LAELLVDLIIRKIDGDNIDITYQLPVQLHYGID----------   318
B.s. DegA   MGAERHRTAGRSNRGKRKAKQ---KIVLPPELVVRHSTSPLNT   337
B.m. CcpA   IGAVAMRLLTKYMNKETVDSS---IVQLPHRIEFRQSTK----   332
E.c. RbsR   LGELAIDVLIHRITQPT--LQQ-QRLQLTPILMERGSA-----   330
H.i. PurR   LGRQAINILLERITHKDEGVQQYSRIDITPELIIRKSVKSI-L   336
B.s. RbsR   MGKEAAKLLLGIIKKQPLAETA---IQMPVTYIGRKTTRKED-   326
            ..                        . .
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Figure 6. Multiple sequence alignment of *L. lactis* SacR, *Bacillus subtilis* DegA (P37947), *Bacillus megaterium* CcpA (P46828), *Escherichia coli* RbsR (P25551), *Haemophilus influenzae* PurR (P46456) and *Bacillus subtilis* RbsR (P36944). Residues which are perfectly conserved are indicated with an asterisk and homologous residues are indicated with a point.

	L.l. SacR	*B.s.* DegA	*B.m.* CcpA	*E.c.* RbsR	*H.i.* PurR	*B.s.* RbsR
***L.l.* SacR**		26	26	25	25	22
***B.s.* DegA**			25	30	27	29
***B.m.* CcpA**				27	26	23
***E.c.* RbsR**					25	26
***H.i.* PurR**						44
***B.s.* RbsR**						

Table 3. Sequence identity percentages between *L. lactis* SacR, *Bacillus subtilis* DegA (P37947), *Bacillus megaterium* CcpA (P46828), *Escherichia coli* RbsR (P25551), *Haemophilus influenzae* PurR (P46456) and *Bacillus subtilis* RbsR (P36944).

```
TAAATTCTCCTGAAAATAAAAAACACTATATATCCATTCTACGCTATTTTTATTGAAAAATCACGTAAAAAAACACACTTGAAGGT

TGAAAAGGTTTACAGTTCATGATATAATTTGTAAATGAAAATGGTTTTCATAAATACTATGAATAAAAAGAAAAGAAAGGATAG
 -35          -10
CCAAATATGGTAGAATCAACAACAACAATTTATGATGTGGCACGTGTCGCCGGAGTCTCAATGGCAACCGTTAGTCGCGTTGTGAA
        M  V  E  S  T  T  T  I  Y  D  V  A  R  V  A  G  V  S  M  A  T  V  S  R  V  V  N
TGGAAATGCAAATGTGAAGGAAAAGACGCGCCAGAAGGTTTTAGAAGCTATTGCTGAGCTTGACTATCGTCCTAACGCTGTGGCAC
  G  N  A  N  V  K  E  K  T  R  Q  K  V  L  E  A  I  A  E  L  D  Y  R  P  N  A  V  A
GTGGACTTGCAAGTAAACGGACAACAACAGTCGGTGTTATCTTGCCAACAATCACTTCAACATACTTCGCAGCTATTACCCGCGGA
R  G  L  A  S  K  R  T  T  T  V  G  V  I  L  P  T  I  T  S  T  Y  F  A  A  I  T  R  G
GTTGATGATATCGCTTCCATGTATAAATACAACATGATTTTAGCTAATAGTGATAATGATGTTGAAAAAGAAGAAAAAGTTTTAGA
 V  D  D  I  A  S  M  Y  K  Y  N  M  I  L  A  N  S  D  N  D  V  E  K  E  E  K  V  L  E
AACTTTCTTGTCAAAACAAGTAGACGGAATTGTTTATATGGGTTCATCATTAGATGAAAAAATTAGAACTTCCCTTAAAAATTCAA
  T  F  L  S  K  Q  V  D  G  I  V  Y  M  G  S  S  L  D  E  K  I  R  T  S  L  K  N  S
GAACACCTGTTGTTTTAGTAGGAACAATTGATGGGGATAAAGAAATTCCATCAGTTAATATTGATTACCATTTGGCTGCTTACCAA
R  T  P  V  V  L  V  G  T  I  D  G  D  K  E  I  P  S  V  N  I  D  Y  H  L  A  A  Y  Q
TCAACAACTAAATTAATCGAAAATGGAAATAAGAAAATTGCTTACATCATGGGTTCATTGAAAGATGTTGAAAACACTGAACGTAT
 S  T  T  K  L  I  E  N  G  N  K  K  I  A  Y  I  M  G  S  L  K  D  V  E  N  T  E  R  M
GGTTGGTTATCAAGAAGCTTTACTTGAAGCAAATATTGAATTTGATGAAAATCTTGTTTTTGAAGGTAATTATAGCTACGAACAAG
  V  G  Y  Q  E  A  L  L  E  A  N  I  E  F  D  E  N  L  V  F  E  G  N  Y  S  Y  E  Q
GCAAATCACTTGCTGAACGCTTACTTGAAAGAGGAGCAACCTCTGCAGTTGTTTCACATGATACGGTAGCCGTTGGCCTCTTGTCT
G  K  S  L  A  E  R  L  L  E  R  G  A  T  S  A  V  V  S  H  D  T  V  A  V  G  L  L  S
GCGATGATGGATAAAGAAGTTAAAGTTCCTGAAGAATTTGAAATCATTTCTGGTGCAAACTCACCAATTACTCAATATACTTATCC
 A  M  M  D  K  E  V  K  V  P  E  E  F  E  I  I  S  G  A  N  S  P  I  T  Q  Y  T  Y  P
AACATTAACTTCTGTTAATCAACCGCTTTATGATTTAGGGGCTGTAGCAATGCGTCTTTTGACAAAATTAATGCTTAAAGAAGATG
  T  L  T  S  V  N  Q  P  L  Y  D  L  G  A  V  A  M  R  L  L  T  K  L  M  L  K  E  D
TTGAGCAAAACCAATTAGTTTTGGATCATGAAATCATTTCTCGTCGCTCAACTAAATAAAAATTAAGCCCAATTAGGGGCTTTTTT
V  E  Q  N  Q  L  V  L  D  H  E  I  I  S  R  R  S  T  K  -
TATTTAAAGTAAAAAGTATTTGACATTTACAAGAAAAGTCGCAATAATAGATAGACAATAGAAAATGACTTTAT (1278)
```

Figure 7. The nucleotide sequence of the *L. lactis ccpA* gene. The promoter hexamers are underlined and the ribosome binding site is presented in bold. The number in brackets correspond to the numbering of the nucleotide data submitted to the EMBL, Genbank and DDBJ Nucleotide sequence databases under the accession number L07920.

```
L.l. CcpA   MV----ESTTTIYDVARVAGVSMATVSRVVNGNANVKEKTRQKVLEAIAE    46
B.m. CcpA   M-------NVTIYDVAREASVSMATVSRVVNGNPNVKPSTRKKVLETIER   43
B.s. CcpA   M------SNITIYDVAREANVSMATVSRVVNGNPNVKPTTRKKVLEAIER   44
E.c. CytR   MKAKKQETAATMKDVALKAKVSTATVSRALMNPDKVSQATRNRVEKAARE   50
H.i. RbsR   M--------ATMKDIARLAQVSTSTVSHVINGSRFVSDEIREKVMRIVAE   42
H.i. GalR   M--------STIRDVAKLANVSVATVSRVLNHSLSVSENTRLVVEQAIAQ   42
            *         *. *.*  * ** .***...     *.  .*  *

L.l. CcpA   LDYRPNAVARGLASKRTTTVGVILPTITSTYFAAITRGVDDIASMYKYNM    96
B.m. CcpA   LGYRPNAVARGLASKKTTTVGVIIPDISNIFYAELARGIEDIATMYKYNI   93
B.s. CcpA   LGYRPNAVARGLASKKTTTVGVIIPDISSIFYSELARGIEDIATMYKYNI   94
E.c. CytR   VGYLPQPMGRNVKRNESRTILVIVPDICDPFFSEIIRGIEVTAANHGYLV   100
H.i. RbsR   LNYTPSAVARSLKVRETKTIGLLVTATNNPFFAEVMAGVEQYCQKNQYNL   92
H.i. GalR   LAYQPNANAQALAVQNTDTIGVVVTDVTDAFFAILVKAVDKVAEAHQKTI   92
            ..* * . .... . . *. ..... . ... .  ...          .

L.l. CcpA   ILANSDNDVEKEEKVLETFLSKQVDGIVYMGSSLDEKIRTSLKNSRTPVV   146
B.m. CcpA   ILSNSDQNQDKELHLLNNMLGKQVDGIIFMSGNVTEEHVEELKKSPVPVV   143
B.s. CcpA   ILSNSDQNMEKELHLLNTMLGKQVDGIVFMGGNITDEHVAEFKRSPVPIV   144
E.c. CytR   LIGDCAHQNQQEKTFIDLIITKQIDGMLLLGSRLPFDASIEEQRNLPPMV   150
H.i. RbsR   IIATTGGDAKRLQQNLQTLMHKQVDGLLLMCGDSRFQADIELAISLPLVV   142
H.i. GalR   LIGIGYHHAEKEREAINTLLRKRCSSLVVHSKALSDDELSHYLNTVQGMV   142
            ...    . ..    .. .. *. ....        .        .  .*

L.l. CcpA   LVGTIDGDKEIPSVNIDYHLAAYQSTTKLIENGNKKIAYIMGSLKD-VEN   195
B.m. CcpA   LAASIESTNQIPSVTIDYEQAAFDAVQSLIDSGHKNIAFVSGTLEEPINH   193
B.s. CcpA   LAASVEEQEETPSVAIDYEQAIYDAVKLLVDKGHTDIAFVSGPMAEPINR   194
E.c. CytR   MANEFAPELELPTVHIDNLTAAFDAVNYLYEQGHKRIGCIAGPEEMPLCH   200
H.i. RbsR   MDWWFT-ELNADKILENSALGGYLATKALIDAGHRKIGIITGNLKKSVAQ   191
H.i. GalR   IINRVIKGYEHRCVSLDNQKGTYLATEMLIRYGHQHIAYI-GSNHAIFDE   191
            .        .   .  .   . . ..  *   *.  *. . *

L.l. CcpA   TERMVGYQEALLEANIEFDENLVFEGNYSYEQGKSLAERLL--ERGATSA   243
B.m. CcpA   AKKVKGYKRALTESGLPVRDSYIVEGDYTYDSGIEAVEKLLEEDEKPTAI   243
B.s. CcpA   SKKLQGYKRALEEANLPFNEQFVAEGDYTYDSGLEALQHLMSLDKKPTAI   244
E.c. CytR   Y-RLQGYVQALRRCGIMVDPQYIARGDFTFEAGSKAMQQLLDLPQPPTAV   249
H.i. RbsR   N-RLQGYKNALSEAKIALNPHWIVESHFDFEGGVLGIQSLLTQSSRPTAV   240
H.i. GalR   VERRNGYLAALKDHNYPIIEQAITLNSPDFEGGEKAMIDLLSYNKNLTAV   241
              .  **  **             .  .  ... *      *.      *.

L.l. CcpA   VVSHDTVAVGLLSAMMDKEVKVPEEFEIISGANSPITQYTYPTLTSVNQP   293
B.m. CcpA   FVGTDEMALGVIHGAQDRGLNVPNDLEIIGFDNTRLSTMVRPQLTSVVQP   293
B.s. CcpA   LSATDEMALGIIHAAQDQGLSIPEDLDIIGFDNTRLSLMVRPQLSTVVQP   294
E.c. CytR   FCHSDVMALGALSQAKRQGLKVPEDLSIIGFDNIDLTQFCDPPLTTIAQP   299
H.i. RbsR   FCCSDTIAVGAYQAIQQQGLRIPQDLSIMGYDDIELARYLSPPLSTICQP   290
H.i. GalR   VAYNDSMAAGAISVLNENNISVPSQFSIIGFDDMPIARYLIPKLTTIRYP   291
                * .* *        .....* ...*.. ..  ..    * *...  *

L.l. CcpA   LYDLGAVAMRLLTKLMLKEDVEQNQLVLDHEIISRRSTK---    332
B.m. CcpA   MYDIGAVAMRLLTKYMNKETVDSSIVQLPHRIEFRQSTK---    332
B.s. CcpA   TYDIGAVAMRLLTKLMNKEPVEEHIVELPHRIELRKSTKS--    334
E.c. CytR   RYEIGREAMLLLLDQMQGQHVGSGSRLMDCELIIRGSTRALP    341
H.i. RbsR   KAELGKLAVETLLQRIKNPNENYRTLVLEPTCVLRESIYSLK    332
H.i. GalR   IDLMATYAAKLALSLTDEKIITPPVVQFNPTLVRRFSVES-R    332
              ..  *          .     .      * *.
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Figure 8. Multiple sequence alignment of *L. lactis* CcpA, *Bacillus megaterium* CcpA (P46828), *Bacillus subtilis* CcpA (P25144), *Escherichia coli* CytR (P06964), *Haemophilus influenzae* RbsR (P44329) and *Haemophilus influenzae* GalR (P31766). Residues which are perfectly conserved are indicated with an asterisk and homologous residues are indicated with a point.

	L.l. CcpA	*B.m.* CcpA	*B.s.* CcpA	*E.c.* CytR	*H.i.* RbsR	*H.i.* GalR
***L.l.* CcpA**		47	47	30	28	27
***B.m.*CcpA**			76	34	25	27
***B.s.* CcpA**				34	28	28
***E.c.* CytR**					29	32
***H.i.* RbsR**						29
***H.i.* GalR**						

Table 4. Sequence identity percentages between *L. lactis* CcpA, *Bacillus megaterium* CcpA (P46828), *Bacillus subtilis* CcpA (P25144), *Escherichia coli* CytR (P06964), *Haemophilus influenzae* RbsR (P44329) and *Haemophilus influenzae* GalR (P31766).

```
TTAAAAGCTGTCAAAACTGACAGTTTTTTTCAATTTTAAAGAGTATAAAAGTAGTTTAAAAAATATTAATTTGATCAGGAGATGAA
GCGGATAGTAGTAAGGCTTCAAAAGTAGCCCTGAAAAAAAACTTGAAAATTTAACAGCAGATATTGCAAACCCTTTCGTTTGTGG
                                                                    -35
TACAATTTCAAGAGTGATAGATATTTTAGATATCGTCAATAAAAATGAAAAAACATCTAAGGAGAACCATAAAAATGGCATCTAAA
 -10                                                                          M  A  S  K
GAATTCCACATCGTTGCAGAAACTGGTATCCATGCACGTCCAGCTACATTGCTTGTGCAAACAGCTTCAAAATTCACATCAGAAAT
 E  F  H  I  V  A  E  T  G  I  H  A  R  P  A  T  L  L  V  Q  T  A  S  K  F  T  S  E  I
CACTTTGGAATACAAAGGTAAATCAGTAAACCTTAAATCAATCATGGGTGTTATGTCACTCGGTGTTGGTCAAGGCGCTGACGTTA
  T  L  E  Y  K  G  K  S  V  N  L  K  S  I  M  G  V  M  S  L  G  V  G  Q  G  A  D  V
CTATTTCAGCTGAAGGTGCAGATGCTGACGACGCAATCGCAACAATCGCTGAAACAATGACTAAAGAAGGACTCGCAGAATAATCA
T  I  S  A  E  G  A  D  A  D  D  A  I  A  T  I  A  E  T  M  T  K  E  G  L  A  E  -
TGACAACTATGCTTAAAGGTATCGCCGCATCATCAGGTGTAGCAGTAGCTAAGGCATACTTGCTTGTCCAACCAGATTTGTCTTTT
M  T  T  M  L  K  G  I  A  A  S  S  G  V  A  V  A  K  A  Y  L  L  V  Q  P  D  L  S  F
GAAACAAAGACAATTGCTGATACAGCTAATGAAGAAGCTCGCCTTGATGCAGCACTTGCTACATCGCAAAGCGAGCTTCAACTTAT
 E  T  K  T  I  A  D  T  A  N  E  E  A  R  L  D  A  A  L  A  T  S  Q  S  E  L  Q  L  I
TAAGGACAAAGCAGTAACAACCCTTGGTGAAGAAGCAGCATCTGTATTTGACGCTCATATGATGGTCCTTGCTGACCCAGATATGA
  K  D  K  A  V  T  T  L  G  E  E  A  A  S  V  F  D  A  H  M  M  V  L  A  D  P  D  M
CTGCTCAAATCAAAGCAGTAATAAATGACAAAAAAGTTAACGCTGAATCAGCTCTTAAAGAAGTAACTGATATGTTTATCGGTATC
T  A  Q  I  K  A  V  I  N  D  K  K  V  N  A  E  S  A  L  K  E  V  T  D  M  F  I  G  I
TTTGAAGGAATGACTGACAATGCTTATATGCAAGAACGTGCAGCTGATATTAAAGACGTTACAAAACGTGTTTTAGCTCACCTTCT
 F  E  G  M  T  D  N  A  Y  M  Q  E  R  A  A  D  I  K  D  V  T  K  R  V  L  A  H  L  L
TGGCGTTAAATTGCCAAGTCCAGCACTCATCGATGAAGAAGTAATCATCGTTGCTGAAGATTTGACACCATCTGATACAGCTCAAT
  G  V  K  L  P  S  P  A  L  I  D  E  E  V  I  I  V  A  E  D  L  T  P  S  D  T  A  Q
TGGACAAGAAATTCGTAAAAGCCTTTGTTACTAACATTGGTGGACGTACTTCTCACTCTGCAATTATGGCTCGTACTTTGGAAATT
L  D  K  K  F  V  K  A  F  V  T  N  I  G  G  R  T  S  H  S  A  I  M  A  R  T  L  E  I
CCAGCAGTTCTTGGAACAAATAATATTACTGAACTTGTTTCTGAAGGTCAACTTTTGGCTGTTTCAGGATTGACAGGTGAAGTAAT
 P  A  V  L  G  T  N  N  I  T  E  L  V  S  E  G  Q  L  L  A  V  S  G  L  T  G  E  V  I
TCTTGACCCATCTACTGAACAACAAAGCGAATTCCATAAAGCTGGTGACGCTTATGCTGCTCAAAAAGCAGAATGGGCTGCTCTTA
  L  D  P  S  T  E  Q  Q  S  E  F  H  K  A  G  D  A  Y  A  A  Q  K  A  E  W  A  A  L
AAGACGCTGAAACTGTTACAGCTGATGGACGTCATTATGAGCTTGCTGCTAATATCGGTACACCTAAAGACGTTGAAGGTGTTAAC
K  D  A  E  T  V  T  A  D  G  R  H  Y  E  L  A  A  N  I  G  T  P  K  D  V  E  G  V  N
GATAATGGTGCTGAAGCAATTGGTCTTTATCGTACAGAATTCTTGTACATGGATGCACAAGATTTCCCAACAGAAGATGACCAATA
 D  N  G  A  E  A  I  G  L  Y  R  T  E  F  L  Y  M  D  A  Q  D  F  P  T  E  D  D  Q  Y
TGAAGCTTACAAAGCTGTACTTGAAGGAATGAATGGTAAACCAGTTGTTGTCCGTACAATGGACATCGGTGGTGACAAAACATTGC
  E  A  Y  K  A  V  L  E  G  M  N  G  K  P  V  V  V  R  T  M  D  I  G  G  D  K  T  L
CTTACTTTGATCTTCCTAAAGAAATGAACCCATTCCTCGGATGGCGTGCACTTCGTATCAGCCTTTCAACAGCTGGTGACGGAATG
P  Y  F  D  L  P  K  E  M  N  P  F  L  G  W  R  A  L  R  I  S  L  S  T  A  G  D  G  M
TTCCGTACACAATTGCGTGCGCTCTTGCGTGCTTCTGTACACGGACAACTTCGTATCATGTTCCCAATGGTTGCTCTCGTAACTGA
 F  R  T  Q  L  R  A  L  L  R  A  S  V  H  G  Q  L  R  I  M  F  P  M  V  A  L  V  T  E
GTTCCGTGCAGCTAAAAAGATTTATGATGAAGAAAAATCTAAATTGATTGCAGAAGGTGTTCCAGTCGCAGAAGGTATCGAAGTTG
  F  R  A  A  K  K  I  Y  D  E  E  K  S  K  L  I  A  E  G  V  P  V  A  E  G  I  E  V
GTATCATGATTGAAATTCCAGCAGCAGCAATGCTTGCAGACCAATTTGCAAAAGAAGTTGATTTCTTCTCAATTGGTACAAACGAC
G  I  M  I  E  I  P  A  A  A  M  L  A  D  Q  F  A  K  E  V  D  F  F  S  I  G  T  N  D
CTCATCCAATATACAATGGCTGCAGACCGTATGAATGAGCAAGTTTCTTACCTTTATCAACCTTACAATCCATCAATCCTTCGTTT
 L  I  Q  Y  T  M  A  A  D  R  M  N  E  Q  V  S  Y  L  Y  Q  P  Y  N  P  S  I  L  R  L
GATTAACAATGTAATCAAAGCAGCTCACGCTGAAGGTAAATGGGCTGGTATGTGTGGTGAAATGGCCGGCGACCAAACTGCTGTAC
  I  N  N  V  I  K  A  A  H  A  E  G  K  W  A  G  M  C  G  E  M  A  G  D  Q  T  A  V
CATTGCTTATGGGTATGGGGCTTGACGAATTCTCAATGTCAGCAACATCAGTACTCCAAACACGTTCACTTATGAAACGTTTGGAT
P  L  L  M  G  M  G  L  D  E  F  S  M  S  A  T  S  V  L  Q  T  R  S  L  M  K  R  L  D
TCTAAGAAAATGGAAGAATTGTCTAGCAAAGCTTTGAGCGAATGTGCAACAATGGAAGAAGTTATTGCCCTCGTTGAAGAATATAC
 S  K  K  M  E  E  L  S  S  K  A  L  S  E  C  A  T  M  E  E  V  I  A  L  V  E  E  Y  T
TAAATAATATTTTCAATTAGTTTCGAATGATAAGCCTGTCAATTAATGACAGGTTTTTTTAAATAGTCTGTTTAGCTATAATAGAT
  K  -
TTTTTAGAAGTAAAAAAACTTATCCACGGGGATAAGTTTTTTTCTATCAAATGAATTTGAAAAGAGTATAATAATAAG (2400)
```

Figure 9. The nucleotide sequence of the *L. lactis ptsHI* genes. The promoter hexamers are underlined and the ribosome binding site is presented in bold. The number in brackets correspond to the numbering of the nucleotide data submitted to the EMBL, Genbank and DDBJ Nucleotide sequence databases under the accession number Z97203.

```
L.l. HPr    MASKEFHIVAETGIHARPATLLVQTASKFTSEITLEYKGKSVNLKSIMGV    50
S.s. HPr    MASKDFHIVAETGIHARPATLLVQTASKFASDITLDYKGKAVNLKSIMGV    50
S.m. HPr    MASKDFHIVAETGIHARPATLLVQTASKFASDITLDYKGKAVNLKSIMGV    50
E.f. HPr    MEKKEFHIVAETGIHARPATLLVQTASKFNSDINLEYKGKSVNLKSIMGV    50
B.st.HPr    MAEKTFKVVSDSGIHARPATILVQTASKFNSEIQLEYNGKTVNLKSIMGV    50
B.s. HPr    MAQKTFKVTADSGIHARPATVLVQTASKYDADVNLEYNGKTVNLKSIMGV    50
            *. *.*......********.*******..... *.*.**.*********

L.l. HPr    MSLGVGQGADVTISAEGADADDAIATIAETMTKEGLAE    88
S.s. HPr    MSLGVGQGADVTISAEGADADDAIVAIAETMTKEGLA-    87
S.m. HPr    MSLGVGQGADVTITAEGADADDAIAAINETMTKEGLA-    87
E.f. HPr    MSLGVGQGSDVTITVDGADEAEGMAAIVETLQKEGLAE    88
B.st.HPr    MSLGIPKGATIKITAEGADAAEAMAALTDTLAKEGLAE    88
B.s. HPr    MSLGIAKGAEITISASGADENDALNALEETMKSEGLGE    88
            ****. .*....*...***..... .. .*. .***.
```

Figure 10. Multiple sequence alignment of *L. lactis* HPr, *Streptococcus salivarius* HPr (P24366), *Streptococcus mutans* HPr (P45596), *Enterococcus faecalis* HPr (P07515), *Bacillus stearothermophilus* HPr (P42013) and *Bacillus subtilis* HPr (P08877). Residues which are perfectly conserved are indicated with an asterisk and homologous residues are indicated with a point.

	L.l. **HPr**	***S.s.*** **HPr**	***S.m.*** **HPr**	***E.f.*** **HPr**	***B.st.*** **HPr**	***B.s.*** **HPr**
L.l. **HPr**		91	90	80	68	64
S.s. **HPr**			97	77	66	64
S.m. **HPr**				79	68	63
E.f. **HPr**					68	60
B.st. **HPr**						70
B.s. **HPr**						

Table 5. Sequence identity percentages between *L. lactis* HPr, *Streptococcus salivarius* HPr (P24366), *Streptococcus mutans* HPr (P45596), *Enterococcus faecalis* HPr (P07515), *Bacillus stearothermophilus* HPr (P42013) and *Bacillus subtilis* HPr (P08877).

```
L.l.  EI    MTTMLKGIAASSGVAVAKAYLLVQPDLSFETKT-IADTANEEARLDAALA   49
S.s.  EI    MTEMLKGIAASDGVAVAKAYLLVQPDLSFETVT-VEDTSAEEARLDAALA   49
S.m.  EI    MTEMLKGIAASDGVAVAKAYLLVQPDLTFETVS-VTDTQAEEARLDAALE   49
B.s.  EI    M-QELKGIGASAGIAIAKAYRLEEPDLTVEKKN-ISDSEAEVSRFDEAIA   48
St.c. EI    MAKQIKGIAASDGVAIAKAYLLVEPDLSFD-NESVTDTDAEVAKFNGALN   49
St.a. EI    MSKLIKGIAASDGVAIAKAYLLVEPDLTFDKNEKVTDVEGEVAKFNSAIE   50
            *   .***.**.*.*.**** * .***. .  . ..*  .* .....*..

L.l.  EI    TSQSELQLIKDKAVTTLGEEAASVFDAHMMVLADPDMTAQIKAVINDKKV   99
S.s.  EI    ASQDELSVIREKAVESLGEEAAAVFDAHLMVLADPEMTGQIKETIRAKQV   99
S.m.  EI    ASQNELSLIRQKAVDTLGEEAAAVFDAHLMVLADPEMIGQIKETIRTKEV   99
B.s.  EI    RSKEELEKIKEHALKELGQDKADIFSAHLLVLSDPELLNPVKEKISTDSV   98
St.c. EI    KSKVELTKIRNNAEKQLGADKAAIFDAHLLVLEDPELIQPIEDKIKNESV   99
St.a. EI    ASKVELTKIRNNAEVQLGADKAAIFDAHLLVLDDPELIQPIQDKIKNENA  100
             *. **  *...*   **.. *..*.**..**.**..  .... *... .

L.l.  EI    NAESALKEVTDMFIGIFEGMTDNAYMQERA-ADIKDVTKRVLAHLLGVKL  148
S.s.  EI    NAEAALTEVTDMFIAIFEGMEDNPYMQERA-ADIRDVTKRVLANLLGKKL  148
S.m.  EI    NAESALKEVTDMFVTLFENMEDNPYMQERA-ADIRDVAKRVLAHLLGVEL  148
B.s.  EI    NAEFALKETSSMFVTMFESM-DNEYMKERA-ADIRDVTKRVTGHLLGVEI  146
St.c. EI    NAAQALTDVSNQFITIFESM-DNEYIAERAKADIRDVSKRVLAHILGVEL  148
St.a. EI    NAATALTDVTTQFVTIFESM-DNEYMKERA-ADIRDVSKRVLSHILGVEL  148
            **. **..... *...**.* ** *. *** ***.**.*** ...** ..

L.l.  EI    PSPALIDEEVIIVAEDLTPSDTAQLDKKFVKAFVTNIGGRTSHSAIMART  198
S.s.  EI    PNPATINEESIVVAHDLTPSDTAQLNKKYVKAFVTNIGGRTSHSAIMART  198
S.m.  EI    PNPATISEESIVIAHDLTPSDTAQLDANYVKAFVTNIGGRTSHSAIMART  198
B.s.  EI    PNPSMISEEVIIVAEDLTPSDTAQLNREFVKGFTTDIGGRTSHSAIMARS  196
St.c. EI    PNPSIVDESVVIIGNDLTPSDTAQLNKEYVQGFVTNIGGRTSHSAIMSRS  198
St.a. EI    PNPSMIDESVVIVGNDLTPSDTAQLNKEFVQGFATNIGGRTSHSAIMSRS  198
            *.*. ..*. .....**********. ..*..*.*.*********.*.

L.l.  EI    LEIPAVLGTNNITELVSEGQLLAVSGLTGEVILDPSTEQQSEFHKAGDAY  248
S.s.  EI    LEIAAVLGTNNITELVKDGDILAVSGITGEVVINPTEEQIAEFKAAGEAY  248
S.m.  EI    LEIAAVLGTNDITERVKNGDIVAVNGITGQVIINPTEDQIAEFKAAGETY  248
B.s.  EI    LEIPAVVGTKAATGTIQNGVTVIVDGINGDVIIDPSAETVKEYEEKHNAY  246
St.c. EI    LEIPAVVGTKSITEEVEAGDTIVVDGMTGDVLINPSDEVIAEYQEKRENF  248
St.a. EI    LEIPAIVGTKSITQEVKQGDMIIVDGLNGDVIVNPTEDELIAYQDKRERY  248
            ***.*..**.. *  . .*  . *.*..*.*...*...   ...   . .

L.l.  EI    AAQKAEWAALKDAETVTADGRHYELAANIGTPKDVEGVNDNGAEAIGLYR  298
S.s.  EI    AKQKAEWALLKDAQTVTADGKHFELAANIGTPKDVEGVNDNGAEAVGLYR  298
S.m.  EI    AKQKAEWALLKDAETVTADGKHFELAANIGTPKDVEGVNNNGAEAVGLYR  298
B.s.  EI    LAQKAEWAKLVNEPTVSKDGHHVELAANIGTPDDVKGVLENGGEAVGLYR  296
St.c. EI    FKDKQELQKLRDAESVTADGHHVELAANIGTPNDLPGVIENGAEGIGLYR  298
St.a. EI    FADKKELQKLRDADTVTVDGVHAELAANIGTPNDLPGVIENGAQGIGLYR  298
              .* * . * .. .*. ** * **********.*. ** .**....****

L.l.  EI    TEFLYMDAQDFPTEDDQYEAYKAVLEGMNGKPVVVRTMDIGGDKTLPYFD  348
S.s.  EI    TEFLYMDSQDFPTEDDQYEAYKAVLEGMNGKPVVVRTMDIGGDKELPYFD  348
S.m.  EI    TEFLYMDSQDFPTEDEQYEAYKAVLEGMNGKPVVVRTMDIGGDKELPYFD  348
B.s.  EI    TEFLYMGRDQLPTEDEQFDAYKTVLERMEGKSVVVRTLDIGGDKELPYLQ  346
St.c. EI    TEFLYMGRDQMPTEEEQFEAYKAVLEAMKGKRVVVRTLDIGGDKELPYLD  348
St.a. EI    TEFLYMGRDQMPTEEEQFEAYKEVLEAMGGKRVVVRTLDIGGDKELSYLN  348
            ******. ...***..*..***.*** * **.*****.******.*.*..
```

```
L.l.  EI    LPKEMNPFLGWRALRISLSTAGDGMFRTQLRALLRASVHGQLRIMFPMVA   398
S.s.  EI    LPKEMNPFLGYRALRISISETGNQMFRTQLRALLRASVHGKLRIMFPMVA   398
S.m.  EI    LPKEMNPFLGFRALRISISETGNQMFRTQLRALLRASVHGQLRIMFPMVA   398
B.s.  EI    LPKEMNPFLGYRAIRLCLEEQ--EIFRTQLRALLRASTYGNLKIMFPMIA   394
St.c. EI    LPEEMNPFLGYRAIRLCLDQP--EIFRPQLRALLRASVFGKLNIMFPMVA   396
St.a. EI    LPEEMNPFLGYRAIRLCLAQQ--DIFRPQLRALLRASVYGKLNIMFPMVA   396
            **.*******.**.*....     .**.*********. *.*.*****.*

L.l.  EI    LVTEFRAAKKIYDEEKSKLIAEGVPVAEGIEVGIMIEIPAAAMLADQFAK   448
S.s.  EI    LLTEFRTAKGILEEEKAKLVAEGVAVADDIEVGIMIEIPAAAMLADQFAK   448
S.m.  EI    LLNEFRKAKGILEEEKANLKAEGVAVSDDIQVGIMIEIPAAAMLADQFAK   448
B.s.  EI    TVNEFKEAKAILLEEKEKLVKAGQAVSDDIEVGMMVEIPSTAVIADQFAK   444
St.c. EI    TIQEFRDAKAFLKKNVLTLKMKAMKVADDIELGIMVEIPSTAALADIFAK   446
St.a. EI    TINEFREAKAILLEEKENLKNEGHDISDDIELGIMVEIPATAALADVFAK   446
             . **. ** .  ..  .*   .  ....*..*.*.***..* .** ***

L.l.  EI    EVDFFSIGTNDLIQYTMAADRMNEQVSYLYQPYNPSILRLINNVIKAAHA   498
S.s.  EI    EVDFFSIGTNDLIQYTMAADRMNEQVSYLYQPYNPSILRLINNVIKAAHA   498
S.m.  EI    EVDFFSIGTNDLIQYTMAADRMNEQVSYLYQPYNPSILRLVDHVVKAAHA   498
B.s.  EI    EVDFFSIGTNDLIQYTMAADRMNERVSYLYQPYNPAILRLITLVIEAAHK   494
St.c. EI    EVDFFSIGTNDLIQYTMAADRMSERVSYLYQPYISNFSFLVKQVIEASHA   496
St.a. EI    EVDFFSIGTNDLIQYTLAADRMSERVSYLYQPYNPSILRLVKQVIEASHK   496
            ****************.*****.*.******** ...  *.. *..*.*

L.l.  EI    EGKWAGMCGEMAGDQTAVPLLMGMGLDEFSMSATSVLQTRSLMKRLDSKK   548
S.s.  EI    EGKWAGMCGEMAGDQTAVPLLVGMGLDEFSMSATSVLRTRSLMKKLDTAK   548
S.m.  EI    EGKWAGMCGEMAGDQTAVPLLVGIGLDEFSMSATSVLRTRSLMKKLDTAK   548
B.s.  EI    EGKWVGMCGEMAGDEIAIPILLGLGLDEFSMSATSILPARTQISKLSKQE   544
St.c. EI    EGKWTGMCGEMAGDQTAIPLLLGLGLDEFSMSATSILKARVLIRSLNESE   546
St.a. EI    EGKWTGMCGEMAGDETAIPLLLGLGLDEFSMSATSILKARRQINGLSKNE   546
            ****.*********..*.*.*.*.*.***********.* .*  .. *.. .

L.l.  EI    MEELSSKALSECATMEEVIALVEEYTK--   575
S.s.  EI    MEEYANRALTECSTMEEVLELSKEYVNVD   577
S.m.  EI    MQELAQRALTECATMEEVLELEKEYIDFD   577
B.s.  EI    AESFKEKIL-SMSTTEEVVAFVKETFK--   570
St.c. EI    MKELSERAV-QCATSEEVVDLVEEYTKNA   574
St.a. EI    MTELANRAV-DCATQEEVIELVNNYVK--   572
              .   . .   .* ***... ..  .
```

Figure 11. Multiple sequence alignment of *L. lactis* enzyme I, *Streptococcus salivarius* enzyme I (P30299), *Streptococcus mutans* enzyme I (P45595), *Bacillus subtilis* enzyme I (P08838), *Staphylococcus carnosus* enzyme I (P23533), and *Staphylococcus aureus* enzyme I (P51183). Residues which are perfectly conserved are indicated with an asterisk and homologous residues are indicated with a point.

	L.l. EI	*S.s.* EI	*S.m.* EI	*B.s.* EI	*St.c.* EI	*St.a.* EI
***L.l.* EI**		83	80	59	58	57
***S.s.* EI**			89	59	60	58
***S.m.* EI**				59	60	60
***B.s.* EI**					64	67
***St.c.* EI**						79
***St.a.* EI**						

Table 6. Sequence identity percentages between *L. lactis* enzyme I, *Streptococcus salivarius* enzyme I (P30299), *Streptococcus mutans* enzyme I (P45595), *Bacillus subtilis* enzyme I (P08838), *Staphylococcus carnosus* enzyme I (P23533), and *Staphylococcus aureus* enzyme I (P51183).

Curriculum vitae.

Evert Jan Luesink werd geboren op 11 januari 1969 te Hengelo Gld. Na het behalen van het VWO diploma in 1988 is hij begonnen aan een studie Biologie aan de Landbouw Universiteit in Wageningen, na de propaedeuse koos hij voor de orientatie celbiologie. Een afstudeervak Microbiologie (dr. S. Kengen) werd gevolgd door een afstudeervak Bacteriële Genetica (dr. R. Eggen) beiden bij de vakgroep Microbiologie in Wageningen. Een stage werd uitgevoerd bij de afdeling Biotechnology van Ciba-Geigy, Basel, Zwitserland (dr. P. Fuerst). Hierop volgde een tijdelijk aanstelling als wetenschappelijk medewerker bij de afdeling Biophysische Chemie van het het Nederlands Instituut voor Zuivel Onderzoek te Ede, waar onder leiding van dr. O.P. Kuipers en Prof. dr. W.M. de Vos het in dit proefschrift beschreven promotie-onderzoek is verricht.

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