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

PATRICK T. C. VAN DEN BOGAARD,* MICHEIL KLEEREBEZEM, OSCAR P. KUIPERS,† AND WILLEM M. DE VOS

Wageningen Centre for Food Sciences, NIZO Food Research, Department of Flavour and Natural Ingredients, 6710 BA Ede, The Netherlands

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*Corresponding author. Mailing address: Department of Flavour and Natural Ingredients, NIZO food research, Wageningen Centre for Food Sciences, P.O. Box 20, 6710 BA Ede, The Netherlands. Phone: (31) 318 659511, Fax: (31) 318 650400, E-mail: bogaard@nizo.nl.
† Present address: Molecular Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9750 AA Haren, The Netherlands.

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

Carbon catabolite repression (CR) in bacteria is the phenomenon of using a rapidly metabolizable carbon source in the growth medium by inhibiting utilization of other substrates. The mechanism underlying CR is best understood in enteric bacteria, where the glucose-specific enzyme IIa of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) modulates adenylate cyclase activity. Controlled by the level of cyclic AMP, the cyclic AMP receptor protein is a transcriptional regulator modulating expression of target genes (36, 38). In low-G+C gram-positive bacteria, the mechanism of CR is distinctly different. The catabolite control protein A (CcpA) is the central regulator of CR, as was shown first for *Bacillus subtilis*, in which it mediates glucose repression of the α-amylase gene (9). CcpA is a member of the LacI-GalR family of bacterial regulator proteins and appears to be widespread among low-G+C gram-positive bacteria (4, 12, 21, 29). Genes affected by CR typically contain a catabolite-responsive element (cre) near their promoter regions (44). CcpA has been shown to bind to these cre sites in vitro in a way that can be enhanced by indicators of a high energy state in the cell, e.g., glucose 6-phosphate (6, 27). Another important factor in this catabolite control mechanism is the PTS phosphocarrier HPr. In *B. subtilis*, high concentrations of the glycolytic intermediate fructose-1,6-diphosphate (FBP) trigger an ATP-dependent protein kinase that phosphorylates HPr at residue Ser-46. P-SerHPr subsequently enhances the binding of CcpA to cre and hence links glycolytic activity to CR (2, 5, 15). Catabolite control by CcpA involves not only repression of genes and operons but also activation. In *B. subtilis*, transcription of the alsS and ackA genes (encoding α-acetolactate synthase and acetate kinase, respectively) is activated by CcpA when glucose is present in the medium (7, 37). More direct evidence for a link between catabolite control and glycolytic activity was reported recently for *Lactococcus lactis*. In the presence of glucose in the medium, CcpA was found to be a transcriptional activator of the las operon, thus modulating glycolytic flux rates by controlling the production of the three key glycolytic enzymes, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase (22).

Although the mechanism of CR differs between gram-negative and low-G+C gram-positive bacteria, they have in common that a rapidly metabolizable PTS sugar reduces the expression of genes involved in the utilization of other PTS or non-PTS carbon sources. Glucose is the classical example of such a rapidly metabolizable PTS sugar in most bacteria. However, glucose is a non-PTS carbon source for *Streptococcus thermophilus* and is a poor substrate for growth (34). Lactose is also a non-PTS sugar for this organism but is a very good growth substrate on which growth is even more rapid than on a PTS sugar like sucrose. This indicates that *S. thermophilus*, a
homofermentative thermophilic lactic acid bacterium, is highly adapted to growth on lactose as the primary carbon and energy source. Together with other lactic acid bacteria, this organism is used as a starter culture for the production of yogurt and certain cheeses, where it mainly contributes to the rapid acidification of milk by conversion of lactose to lactic acid.

The *S. thermophilus lac* operon contains the genes encoding a lactose permease (*lacS*) and a β-galactosidase (*lacZ*) for the transport and hydrolysis of lactose, and its transcription is induced during growth on lactose (32, 40). Studies of the *lac* operon reveal a cis site located in the lacZ promoter, suggesting a possible involvement of CcpA in the regulation of this operon (34). The *S. thermophilus galm* and *gafG* genes, encoding enzymes of the Leloir pathway for galactose fermentation, were found upstream of this *lac* operon (33). The complete galkTE operon was recently identified in strain CNRZ302, which is unable to grow on galactose, like most *S. thermophilus* strains (E. E. Vaughan, P. T. C. van den Bogaard, P. Catzeddu, O. P. Kuipers, and W. M. de Vos, submitted for publication). From this strain, galactose-fermenting mutants were isolated, and their molecular characterization showed that these mutants were all *galK* promoter-up mutants. One of these mutants, used in this study, was designated NZ302G. Insertional mutagenesis studies of the *galR* gene located upstream of the galkTE operon, encoding a regulator protein of the LacI-GalR family of transcriptional regulators, showed that the GalR protein was an activator of the *galK* promoter (Vaughan et al., submitted). Transcription of this promoter was induced when cells were grown on medium containing lactose or galactose. Furthermore, GalR was also found to be a transcriptional activator of the *lac* operon, which is expressed at a basal level when cells are grown on glucose, while it is expressed at least twice as high in lactose- or galactose-grown cells.

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

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media, and culture conditions.** *S. thermophilus* was routinely grown at 42°C in M17 broth (Difco, Surrey, U.K.) containing 1% of the chosen carbon source unless stated otherwise. *Escherichia coli* was routinely grown at 42°C in M17 broth (Difco, Surrey, U.K.) containing 1% glucose broth with Em (28°C) or Am (42°C) at 40 μg/ml, and counterselected with chloramphenicol (Cm, 4 μg/ml), Cm (10 μg/ml), and Em (50 μg/ml). For cloning and disruption of the *S. thermophilus ccpA* gene, *E. coli* MC1061 was digested with Hinfl and fragments with sizes of between 3.0 and 3.5 kb were recovered, ligated with Hinfl- and *KpnI*-digested pUC19 (49), and transformed into *E. coli* MC1061. Clones carrying the *ccpA* gene were selected by colony bluing of the Ap-resistant colonies (39) onto chloramphenicol and the resistase-digested miniFb containing the radiolabeled *b. subtilis* *ccpA* gene. Sequence analysis of the positive clones confirmed that a 3.3-kb insert in *pUC19* contained a *ccpA*-like gene. This construct was designated pNZ6100 and used in a further experiment. pNZ6100 was digested with 398-bp internal Pfu restriction enzyme *ccpA* gene was generated from pNZ6100 as a template using primers CPAF9K (5'-CTGCTACTAGGATCTAGGG-3') and CCPARK (5'-AGTCAACATCGGATCTGG-3') and ligated into pGEM-T (Promega), yielding pNZ6101. Plasmids were then filled in using the Klenow polymerase followed by a second digestion with HindIII. The 1.3-kb fragment containing the *ccpA* gene was ligated in pNZ223 (30); and plasmid pNZ6103 was digested with Cm, which by digestion with HindIII, the promoter fragment (pNZ6103) harbors the *S. thermophilus ccpA* gene under the control of its own promoter. The *S. thermophilus* CNRZ302 lhd promoter was obtained by PCR using Pwo DNA polymerase (Boehringer Mannheim) and primers STLDH-F (5'-ACACTCATGGCATAATCGATA-3') and STLDH-R (5'-TCTTGGACGATACCTG-3') based on the sequence of the *ldh* locus of strain M-192 (14). The promoter fragment was admixed using Taq polymerase and ligated into pGEM-T (Promega), yielding pNZ6104.

**Bacterial isolation.** Northern blot and primer extension analysis. *S. thermophilus* strains were grown in M17 broth (30 ml) containing 1% galactose or lactose to an optical density at 600 nm (OD_{600}) of 1.0. Total RNA was isolated from the harvested cells using the Macaloid method as described by Kuipers et al. (18) with the following modifications. Prior to bead beating, the resuspended cells were incubated with lysozyme for 5 min on ice to increase RNA yield. Per sample, 4.5 μg of RNA was size separated on a 1.0% formaldehyde gel (39) and transferred to Gene Screen Plus membranes (DuPont) according to the protocols provided by the manufacturers. RNA size markers were obtained from Bethesda Research Laboratories. Hybridizations were performed at 65°C in a 0.5 M sodium phosphate buffer (pH 7.2) containing 1.0% bovine serum albumin (fraction V), 1 mM EDTA, and 7.0% sodium dodecyl sulfate (SDS), and subsequently, blots were washed at 55 to 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Internal PCR primers were used to label the *S. thermophilus* CNRZ302 chromosomal DNA as the template and primers based on published sequence data: lacS (5'-TACCACACAGGTGTTCAAGAAG-3'), lacSR (5'-GGTGACACGAACTCAGAAG-3'), and llhd (5'-GGTTTAGTTGGTACCGGAGT-3', and llhd (5'-ATGGATATGCTTC-3') (12). These fragments were glass matrix purified, labeled by nick translation with [α-32P]dATP (Amersham International plc), and subsequently used as hybridization probes (39). The images of the Northern blots were exposed to a Storage Phosphor Screen (Molecular Dynamics) and scanned using a STORM 840 PhosphorImager (Molecular Dynamics). The Northern signals were quantified using the ImageQuant 1.2 program (Molecular Dynamics). Per Northern blot, a final 16S rDNA probe, created by PCR with 16S-specific primers (NR7 [5'-GAAGACGCTGCT-3'] and NR19 [5'-GTGCTATGCCTGTA-3']) with *S. thermophilus* CNRZ302 chromosomal DNA as the template, was used to correct the gene-specific signals for the total amount of RNA loaded per sample, which never differed by more than 20%. Primer extension was performed as previously described (39) by annealing 20 ng of oligonucleotide PECPPA (5'-TGATCTTCAACAGTTACAAGG-3') and PELDH (5'-CGGACACCGTCACCAAG-3') (28) to 15 μg of total *S. thermophilus* CNRZ302 mRNA. The primer extension reaction was loaded on a 5% polyacrylamide gel together with a sequencing reaction obtained using the same oligonucleotide primer and appropriate template.

**β-Galactosidase and protein assays.** The *S. thermophilus* strains were grown to an OD_{600} of 1.0 in M17 broth containing 1% lactose, galactose, or glucose. For the preparation of cell extracts, cells were disrupted with zirconium glass beads in a Bead Beater (Biospec Products, Bartlesville, Okla.) by 3-min treatments, with intervals of 1 min in between on ice; cellular debris was removed by centrifugation. The extracts were kept on ice, and enzyme assays were performed within 2 h using 1 to 6 μg of protein per reaction. β-Galactosidase was assayed at 30°C by the method of Miller (38) and the lactate dehydrogenase activity was assayed at 30°C by the method of Hillier and Jago (11). All enzyme activity measurements presented are the means of at least two independent experiments. Protein concentrations were determined using the method of Lowry et al. (27) with bovine serum albumin as the standard.

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centrations were estimated by a dye-binding assay (1) using bovine serum albumin as the standard.

Small-scale sugar fermentations. The *S. thermophilus* strains were grown to an OD₆₀₀ of 1.0 in M17 broth containing 1% lactose or galactose, washed, and resuspended in a 4% β-glycerophosphate solution at a final OD₆₀₀ of 10.0. The cells were preincubated for 2 min at 42°C, and fermentation was started by addition of 20 mM lactose. Consecutive samples were taken at regular time intervals from the primary fermentation suspension and immediately transferred to −5°C in salted ice water to prevent further uptake and metabolic conversion.

The samples were centrifuged, and supernatants were analyzed by high-performance liquid chromatography (HPLC). Sugars were separated on a Polyspher C18 column (Merck) with water as the eluent. Organic acids were separated on a Rezex organic acid column (Phenomenex) using 5 mM sulfuric acid as the eluent. The separations were carried out on an isocratic pumping system (M6000; Perkin-Elmer) in combination with an automatic sample injector (717+; Waters) and a refractive index detector (M440, Waters).

Western blot analysis. For CcpA detection, cells were grown to an OD₆₀₀ of 1.0 in M17 broth containing 1% lactose or glucose. For the preparation of cell extracts, cells were disrupted with zirconium glass beads in a Bead Beater (Biospec Products) by three treatments of 1 min, interspersed by 1 min of cooling of the samples on ice; cellular debris was removed by centrifugation. For LacS analysis, portions of the cells that were used for the small-scale sugar fermentations were protoplasted by extensive treatment with a combination of lysozyme (2 mg/ml) and mutanolysin (25 U/ml) in THMS buffer (30 mM Tris [pH 8.0], 100 mM NaCl, 20% (vol/vol) glycerol, and 0.5% (wt/vol) Triton X-100) to solubilize the LacS protein (16). The suspensions were mixed, and after 20 min of incubation at 4°C, the insoluble material was removed by centrifugation.

Protein concentrations were estimated by a dye-binding assay (1). Samples were separated and separated by SDS–12.5% polyacrylamide gel electrophoresis.

The separated proteins were transferred to Gene Screen Plus membranes (Du- pont) using electroblot equipment (LKB 2051 Midget Multiblot). CcpA proteins were detected using antibodies raised against *Bacillus megaterium* CcpA that were cloned, expressed, and purified in E. coli and detected by Western blot analysis using antibodies raised against the COOH terminus of LacS (35). These antibodies were detected using goat anti-rabbit immunoglobulin peroxidase-conjugated antibodies (Gibco-BRL) as described by the manufacturer.

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

**RESULTS**

Cloning, characterization, and disruption of the *S. thermophilus* ccpA gene. To determine the mechanism of CR in *S. thermophilus* CNRZ302, its ccpA gene was identified on a 3.3-kb chromosomal fragment on basis of its hybridization with the *B. subtilis* ccpA gene and subsequently cloned, resulting in plasmid pNZ6100. Nucleotide sequence analysis showed that this fragment to contain two open reading frames (ORFs). Trans-
grown cells (data not shown), indicating a preference for lactose as a carbon and energy source. The primary effects of ccpA disruption were a prolonged lag time and reduced growth rate on all sugars tested. In addition, lactose-grown NZ6150 cells reached a significantly lower OD_{600} than wild-type cells, which was not observed for growth on the other sugars (data not shown). To rule out pleiotropic effects of the insertion of the integration vector, NZ6150 was complemented with plasmid pNZ6103, expressing the S. thermophilus ccpA gene. Wild-type growth characteristics could be restored to NZ6150, showing that disruption of the ccpA gene was responsible for the observed impaired growth (data not shown).

Sugar uptake and utilization of lactose in ccpA disruption strains. To obtain insight on the kinetics of lactose fermentation in the ccpA disruption mutant compared to the wild-type strain, small-scale fermentations were performed using resting cells in a strongly buffered system (Fig. 3). The wild-type cells show a very rapid initial uptake of lactose accompanied by the appearance of an equimolar amount of galactose in the buffer, as was observed in previous S. thermophilus studies (13, 32) (Fig. 3A). The values for lactose internalization and galactose expulsion are probably somewhat overestimated in the first 1.5 min due to the high initial transport and hydrolysis that continued for several seconds on ice during sampling. Hence, the later samples were used to calculate the rates of lactose internalization and galactose expulsion, as the overestimation decreases greatly with the decrease in transport rate. For these samples, the rates agree well with the nonlinear kinetic model for lactose uptake by the S. thermophilus LacS transporter (32). The wild-type strain consumed half of the added amount of lactose in 10 min, whereas the ccpA disruption mutant achieved this in 2.5 min, consuming almost all added lactose within 20 min (Fig. 3B). Remarkably, after a very short lag period (30 s), glucose appeared in the fermentation medium of the ccpA disruption strain and remained after 20 min to two-thirds of the internalized lactose. This indicates that the ccpA disruption strain ferments only one-third of the glucose derived from the internalized lactose, whereas the wild-type strain ferments this completely. Moreover, no detectable β-galactosidase activity was found in the fermentation buffer of either strain, which rules out the possibility that the difference in lactose consumption and appearance of galactose or glucose in the fermentation buffer was caused by release of β-galactosidase due to differential lysis of the ccpA disruption strain. The time and amount of lactate production agreed with the influx of lactose-derived glucose that was strongly reduced in the ccpA disruption strain. No end products other than lactate could be detected in the fermentation buffer, in contrast to what was found in an L. lactis ccpA disruption mutant, which showed a mixed acid fermentation (22). In a similar experiment with NZ6151 cells fermenting galactose, no apparent differences were observed in the sugar consumption rate compared to NZ302G cells, indicating that the CcpA effect on galactoside uptake is lactose specific (data not shown).

Regulation of the lac operon by CcpA. The efficiency of the S. thermophilus lacS promoter in CNRZ302 is induced during growth on lactose and galactose as a consequence of GalR activity (Vaughan et al., submitted). This lac promoter contains a cre site overlapping the −10 box and the transcriptional start site, identical in sequence and location to that previously published for strain A147 (Fig. 4) (34). To study the effect of CcpA as an additional transcriptional regulator of the lac operon, total RNA was isolated from the ccpA disruption and wild-type strains grown on various carbon sources. An internal fragment of the lacS gene was used in Northern blots to detect the single 5.2-kb lacSZ messenger (Fig. 5A). Lactose-grown wild-type cells showed a twofold increase in lacSZ expression relative to glucose-grown cells, due to GalR activity. In contrast, this increase was sevenfold in the ccpA disruption strain (NZ6150). Galactose-grown NZ302G cells showed a high
amount of lacSZ transcript, even relative to lactose-grown NZ6150 cells. This did not differ significantly in strain NZ6151, indicating that CcpA-mediated repression of the lacSZ promoter during growth on galactose did not occur (data not shown). The basal level of lacSZ transcription in cells grown on glucose was not significantly affected by the loss of a functional CcpA. To further substantiate the effect of the ccpA gene disruption on the expression of the lac operon, the lacZ gene of this operon was used as a reporter (Fig. 5B). Lactose-grown wild-type cells showed 1.5-fold-higher β-galactosidase activity than cells grown on glucose. This induction was approximately threefold in the ccpA disruption mutant grown on lactose, while the β-galactosidase activity of glucose-grown cells was not significantly affected by the loss of a functional CcpA. The absolute induction values were lower relative to those of the transcriptional analysis but showed the same tendencies. Galactose-grown NZ302G cells showed a twofold-increased β-galactosidase activity relative to CNRZ302 cells grown on lactose, which was not increased further by the disruption of ccpA in this strain. NZ302G cells grown on a combination of galactose and glucose showed only a slightly lower β-galactosidase activity than galactose-grown NZ302G. Introduction of pNZ6103 in the ccpA disruption strain (NZ6150) was found to restore the wild-type β-galactosidase activity levels.

Since the ccpA disruption strain was able to transport lactose with a significantly higher rate than the wild-type strain, the amount of LacS protein in these strains was compared. Total protein was isolated from parent (CNRZ302 and NZ302G) and ccpA disruption (NZ6150 and NZ6151) strains grown on lactose or galactose. Using antibodies raised against LacS, protein bands of the expected molecular weights were detected (16). Significantly higher amounts of LacS protein were detected for both ccpA disruption strains NZ6150 and NZ6151 grown on lactose compared to their parent strains (data not shown), indicating relief of a repressing effect by CcpA on LacS production. In analogy with the β-galactosidase results, the galactose-grown NZ302G cells contained high amounts of LacS protein compared to lactose-grown wild-type cells, which was not significantly affected by the ccpA disruption. These results indicate that the repression of the lacSZ promoter during growth on lactose is relieved by the loss of a functional CcpA and is not occurring during growth on galactose. This strongly suggests that the glucose moiety of lactose is responsible for this CcpA-mediated repression.

Expression of the ldh gene. The observation that the ccpA disruption strain grown on lactose produced less lactate than the wild-type strain (Fig. 3) could indicate that glycolysis was affected by the disruption of the ccpA gene. The production of lactate from pyruvate by lactate dehydrogenase is an essential step in homolactate fermentative lactic acid bacteria to reoxidize NADH that is generated during glycolysis. The lac operon of L. lactis, comprising the pfk, pyk, and ldh genes, was found to be transcriptionally activated by CcpA on glucose (22). In S. thermophilus, the ldh gene (14) and the pfk/pyk operon (F. Crispie, J. Anba, P. Renault, S. D. Ehrlich, G. F. Fitzgerald, and D. van Sinderen, unpublished data) are located at distinct chromosomal locations. Sequence analysis of the ldh promoter region revealed a cre site upstream of the −35 region (Fig. 4). This promoter was isolated from CNRZ302, and sequence analysis confirmed the presence of this cre site. Therefore, the involvement of CcpA in the regulation of transcription of the ldh gene was analyzed by Northern blot analysis using an internal fragment of the ldh gene as a probe (Fig. 6A). The ldh gene showed a single transcript of 1.0 kb, of which the amount was twofold.
higher in lactose-grown compared to glucose-grown wild-type cells. Galactose-grown NZ302G cells showed an even lower amount of \( ldh \) transcript than glucose-grown wild-type cells (data not shown). This sugar-dependent regulation of \( ldh \) expression was completely lost in the \( ccpA \) disruption strains. In analogy, lactate dehydrogenase activity was highest in wild-type cells grown on lactate but significantly lower in the \( ccpA \) disruption strains. In contrast, the gene encoding lactate dehydrogenase was found to be transcriptionally activated by CcpA. Western blot analysis showed that CcpA production was sugar source dependent, with more than a twofold-higher amount found in glucose-grown cells than in lactose-grown cells. The observed regulation of \( S. \) thermophilus CcpA production could be regulated at the transcriptional level as negative autoregulation, as has been found for some but not all other \( ccpA \) genes (4, 25, 29). Inspection of the \( ccpA \) sequence showed the presence of two cre-like elements. One putative cre (\( 5’\)-TGTAAACCATGATT-\( 3’ \)) is located at \( -150 \) relative to the transcription start site of the \( ccpA \) promoter and is thus more closely linked to the divergently transcribed \( pepQ \) gene (located at \( -100 \) relative to its putative promoter), suggesting its involvement in regulation of \( pepQ \) expression rather than \( ccpA \) autoregulation. The second putative cre (\( 5’\)-ATTC ACCGTTCACA-\( 3’ \)) is located within the coding sequence of the \( ccpA \) gene (+873 bp), which could suggest a role in transcriptional control. An internal cre site has also been found in the coding region of the \( S. \) lactis \( ccpA \) gene, but autoregulation could not be established in this organism (22). An alternative mechanism for the observed autoregulation involving the two cre sites could be that CcpA binds to these sites to form a DNA loop, thereby inhibiting \( ccpA \) promoter activity. The large distance found between the two sites could then explain the small magnitude of the observed regulatory effect. The restoration of sugar source-dependent regulation of CcpA production observed in the \( ccpA \) disruption strain when complemented with the complete \( ccpA \) gene in trans indicates that the regulation mechanism is not impaired by the presence of multiple \( ccpA \) gene copies, supporting the suggested autoregulation of CcpA. Nevertheless, some form of posttranslational regulation of CcpA production cannot be excluded on the basis of our experiments. Disruption of the \( ccpA \) gene seriously impaired the growth of \( S. \) thermophilus, as has also been observed for other gram-positive bacteria (4, 12, 29). On both PTS (sucrose) and non-PTS (glucose, lactate, and galactose) sugars, growth of the \( ccpA \) disruption mutants showed a prolonged lag phase and a reduced maximum growth rate. In contrast to the other sugars tested, the \( ccpA \) disruption mutant NZ6150 grown on lactose reached a significantly lower final cell density in the stationary phase compared to the wild-type cells. It is likely that growth ceased because all lactose in the medium was depleted by the high-lactose transport and hydrolysis capacity. Apparently, growth of NZ6150 does not continue on the expelled glucose.

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

CcpA-mediated CR in low-G+C gram-positive bacteria is
dependent on the intracellular amounts of FBP, as relatively high concentrations of this glycolytic intermediate stimulate the HPr kinase in *B. subtilis* to convert HPr to P-Ser-HPr (2). The LacS permease of *S. thermophilus* constitutes a very fast and efficient system for lactose uptake that facilitates high influx of glucose into glycolysis. At the maximal growth rate of *S. thermophilus*, P-Ser-HPr appears to be the dominant phosphorylated species, whereas P-His-HPr dominates in the stationary phase (8). This reflects a relatively high intracellular FBP concentration that subsequently induces CR of the lacS promoter. Galactose metabolism by the relatively slow LeuE pathway probably yields insufficient intracellular FBP concentrations for induction of CcpA-mediated repression. CR of the lac operon in *S. thermophilus* may not be so much carbon source dependent as determined by the rate of glycolysis relative to sugar uptake, in which the FBP concentration may act as the intracellular indicator of this glycolytic flux. Small-scale fermentation experiments substantiated the negative regulation of CcpA on the uptake and utilization of lactose, but also showed involvement of this regulator in the central metabolism of *S. thermophilus*. In the absence of a functional CcpA, the cells not only take up lactose and expel galactose at least four times faster than the wild-type cells but also show a significant reduction in the amount of lactate produced. The increased lactose uptake by the *ccpA* disruption strain does not result in an increased growth rate. Moreover, glucose was expelled into the fermentation medium by the *ccpA* disruption mutant, and its amount correlates with the amount of internalized lactose and that of lactate produced, closing the carbon balance. Obviously, this glucose is derived from lactose, since not only the amount of LacS transporter, and hence its transport capacity, was increased in the *ccpA* disruption mutant, but also the β-galactosidase activity. Since this glucose is expelled with a short lag time, whereas galactose is expelled instantaneously and in equimolar amounts with lactose uptake, it is likely that the additional amount of glucose entering glycolysis (from the increased uptake of lactose) in the *ccpA* disruption mutant cannot be processed by glycolysis and is expelled.

*S. thermophilus* *ldh* expression is sugar regulated and mediated by CcpA. The *cre* site found in the *ldh* promoter region is situated upstream of the −35 box, agreeing with positive control by CcpA (10). *ldh* induction is highest during growth on lactose and decreased during growth on glucose and galactose, the order of which correlates with the growth rates observed. The activating effect of CcpA is presumably also mediated by P-Ser-HPr, explaining the high induction of *ldh* transcription on lactose and lower induction on glucose and galactose. However, as lactate dehydrogenase catalyzes the last step in homolactic fermentation, it is unlikely that this is the sole glycolytic step regulated by CcpA, causing the massive glucose expulsion. The *ccpA* disruption mutant of strain CNRZ302 still produces only lactate as its end product, although several strains of *S. thermophilus* have been reported to produce other end products, such as acetoin, α-acetolactate, and diacetyl (42). Apparently, no massive accumulation of the pyruvate pool occurs in this mutant, indicating that glycolysis indeed is falling at additional steps, similar to what has been reported for *L. lactis* (22; E. Janat, C. Delorme, S. D. Ehrlich, A. Bolotina, A. Sorokin, and P. Renault, Proc. 6th Symp. Lactic Acid Bacteria, abstr. HS8, 1999). In the small-scale lactose fermentations, the internalization of lactose was four times faster in the *ccpA* disruption strain compared to the wild-type strain, while lactate expulsion was reduced twofold. This indicates that during exponential growth, *S. thermophilus* has a lactose transport capacity that exceeds the maximal glycolysis rate by at least twofold, suggesting that glycolysis tunes down the total lactose transport capacity to meet maximal glycolytic flux. This is in contrast to the situation in various other bacteria, where uptake of a PTS substrate is the principal rate-limiting factor in sugar metabolism (36). During late exponential and stationary growth, P-His-HPr becomes the predominant phosphorylated form of HPr, which indicates that lactose transport probably becomes rate limiting (8).

CcpA has been studied in many low-G+C gram-positive bacteria, where it mediates CR when cells are grown on PTS carbon sources, of which glucose is the most preferred. To the best of our knowledge, the other catabolic systems have not been reported in which non-PTS carbon sources induce CR at the transcriptional level. Lactose, a non-PTS sugar in which *S. thermophilus* is highly adapted for growth, causes not only repression of the lac operon but also activation of glycolysis, both events being mediated by CcpA. Glucose, also a non-PTS sugar for *S. thermophilus*, is not able to repress the lac operon, and the activation of glycolysis is not as strong as that induced by lactose. In conclusion, CcpA simultaneously tunes the uptake of lactose and the capacity for glycolysis to yield optimal glycolytic flux and growth rate of *S. thermophilus*.

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