Changes in Glycolytic Activity of *Lactococcus lactis* Induced by Low Temperature

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The effects of low-temperature stress on the glycolytic activity of the lactic acid bacterium *Lactococcus lactis* were studied. The maximal glycolytic activity measured at 30°C increased approximately 2.5-fold following a shift from 30 to 10°C for 4 h in a process that required protein synthesis. Analysis of cold adaptation of strains with genes involved in sugar metabolism disrupted showed that both the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) subunit HPr and catabolite control protein A (CcpA) are involved in the increased acidification at low temperatures. In contrast, a strain with the PTS subunit enzyme I disrupted showed increased acidification similar to that in the wild-type strain. This indicates that the PTS is not involved in this response whereas the regulatory function of 46-seryl phosphorylated HPr [HPr(Ser-P)] probably is involved. Protein analysis showed that the production of both HPr and CcpA was induced severalfold (up to two- to threefold) upon exposure to low temperatures. The *las* operon, which is subject to catabolite activation by the CcpA-HPr(Ser-P) complex, was not induced upon cold shock, and no increased lactate dehydrogenase (LDH) activity was observed. Similarly, the rate-limiting enzyme of the glycolytic pathway under starvation conditions, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was not induced upon cold shock. This indicates that a factor other than LDH or GAPDH is rate determining for the increased glycolytic activity upon exposure to low temperatures. Based on their cold induction and involvement in cold adaptation of glycolysis, it is proposed that the CcpA-HPr(Ser-P) control circuit regulates this factor(s) and hence couples catabolite repression and cold shock response in a functional and mechanistic way.

Lactic acid bacteria (LAB) are widely used to start industrial fermentations of foods, during which they face a variety of stress conditions. The adaptation responses of *Lactococcus lactis* to these stress conditions have been investigated (reviewed in references 22 and 24). Starter LAB are exposed to low temperatures during frozen storage, as well as during low-temperature fermentation. The survival and fermentation capacities of LAB under these conditions will determine the results of the fermentations. Many of the fermentations are stopped by storage at low temperature, and during this storage the fermentation may continue slowly, resulting in an overacidified product. For these reasons, it is of interest to study the cold-adaptive responses of LAB in relation to acidification characteristics.

Recent research on the low-temperature responses of various bacteria has resulted in the identification of a group of 7-kDa proteins that appear to represent the most highly induced proteins upon a rapid downshift in temperature and that are for that reason called cold shock proteins (CSPs). It has been shown that CSPs can function as RNA chaperones, transcriptional activators, and freeze-protective compounds in *Escherichia coli* and *Bacillus subtilis* (reviewed in references 6 and 29). Also, in *L. lactis* MG1363, a CSP family consisting of five members has been identified (28). Moreover, a variety of other cold-induced proteins (CIPs) have been characterized in several bacteria. In *E. coli* and *B. subtilis*, approximately 20 and 35 CIPs, respectively, have been observed, and these proteins are involved in a variety of cellular processes, such as chromosomal condensation, chemotaxis, general metabolism, transcription, and translation (7, 9, 10, 11). Strikingly, for *B. subtilis* cold induction was also observed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and HPr, both involved in glycolysis (7). *L. lactis* MG1363 showed induction of 17 CIPs, including β-phosphoglucomutase, a hypothetical signal transduction protein, ribosomal protein L9, and a histone-like protein (J. A. Wouters, H. Frenkiel, W. M. De Vos, O. P. Kuipers, and T. Abeel, submitted for publication).

For *L. lactis*, the main pathway for energy generation is glycolysis, in which two substrate-level phosphorylation reactions, involving phosphoglycerate kinase and pyruvate kinase, operate to yield energy. During the growth of *L. lactis* on glucose or lactose, more than 90% of the fermented sugar is converted into l-lactate (26). Pyruvate is the end product of glycolysis and is converted into either l-lactate (homolactic fermentation) or a mix of fermentation products, such as l-lactate, acetate, ethanol, or formate (mixed-acid fermentation), depending on the growth rate (5, 21). Glucose and lactose are transported in *L. lactis* by the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) that mediates the concomitant uptake and phosphorylation of these carbohydrates. This group translocation process is catalyzed by the non-sugar-specific enzymes I and HPr in combination with the sugar-specific enzyme II, which can consist of one or more proteins (17). The genes encoding phosphofructokinase (*pfk*), pyruvate kinase (*pyk*), and lactate dehydrogenase (LDH) (*ldh*) have been cloned and were shown to be located in the *las* (lactic acid synthesis) operon, which is under the control of a single promoter (15, 16). HPr is not only involved...
in sugar uptake but also plays a regulatory role in sugar metabolism and catabolite repression, depending on its phosphorylation. For *B. subtilis*, it has been reported that seryl phosphorylated HPr can form a complex with catabolite control protein A (CcpA) in the presence of glycolytic intermediates, such as fructose diphosphate or glucose-6-phosphate (4). Recently, it has been shown that the lactococcal 46-seryl phosphorylated HPr [HPr(Ser-P)] functions as a coactivator in the catabolite activation of the *pyk* and *ldh* genes in cooperation with CcpA (17). Furthermore, a role for the control of glycolysis in *L. lactis* has been assigned to GDPH, which has been shown to be rate limiting in the glycolytic activity of starved cells (19). The gene encoding GAPDH, *gap*, has been cloned and is expressed on a monocistronic transcript, while no other glycolytic-pathway genes were observed adjacent to *gap* (1).

Despite increased knowledge of the cold shock response in recent years, knowledge of the physiological role of CIPs is still limited. In this work, we present data on glycolytic activity at low temperature, and we report on new CIPs involved in the glycolytic pathway. The glycolytic activity measured at 30°C shows a marked increase upon prior exposure of the cells to 10°C for several hours. This response seems to involve the regulatory CcpA-HPr(Ser-P) complex, and the role of this control circuit in the glycolytic pathway is discussed.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *L. lactis* NZ9800 (2) was used as the wild-type strain in this study and was cultured in M17 medium containing either 0.5% glucose or 0.5% maltose at 30°C or as otherwise indicated. Strains with the *ptsH* gene (L. lactis NZ9881) (17) or *cyaA* (L. lactis NZ9870) (16) gene disrupted were cultured on M17 broth containing 0.5% glucose, and a strain with the *ptsI* gene disrupted (L. lactis NZ9880) (17) was grown on M17 broth containing 0.5% maltose. The growth of *L. lactis* was monitored by measuring the optical density at 600 nm (OD$_{600}$). *L. lactis* cells were exposed to a cold shock treatment as described previously (28). In short, cultures were grown at 30°C to mid-exponential phase, after which they were spun down by centrifugation and resuspended in medium precooled to 10°C. After exposure of the cultures to 10°C for various periods, samples were taken.

**Determination of maximal glycolytic activity.** Glycolytic activity was assessed by measuring the initial rate of acidification essentially as described by Poolman et al. (19). In short, cells were cultured, centrifuged, and resuspended in 0.5 mM potassium morpholineethanesulfonic acid–50 mM KCl buffer (pH 6.5) to an OD$_{600}$ of 20. Subsequently, 0.4 ml of this suspension was added to 9 ml of the same buffer equilibrated at 30°C. The acidification of the medium was measured upon addition of glucose or maltose (0.5% final concentration) using a Schott pH electrode and a pH meter connected to a recorder. Changes in pH were converted into nanomoles of H$^+$ per minute per milligram of protein by calibration of the cell suspension with 10-µl portions of 100 mM NaOH. The protein content of the extract was determined by the bicinchoninic acid method as described by the supplier (Sigma Chemicals, St. Louis, Mo.). Using an Aminex ion exclusion column (Bio-Rad, Hercules, Calif.), the end products of the acidification reaction (lactate, acetate, and formate) were measured by high-performance liquid chromatography as described previously (25).

**mRNA analysis.** RNA was isolated and Northern blot analysis was performed as described previously (13). The RNA was denatured, and equal amounts of RNA were separated on 1% agarose gels containing formaldehyde according to the method of Sambrook et al. (23) and blotted onto GeneScreen Plus Membrane (Dupont, Wilmington, Del.). A 0.24- to 9.5-kb RNA ladder (GIBCO/BRL Life Technologies, Breda, The Netherlands) was used to determine the transcript size, and the RNA was stained with ethidium bromide. The blots were hybridized with a probe specific for *ptsH* (5'-CTGCAACGATGTGGAATCTTTAG-3'), *ptsI* (5'-GATGATTTGTTAAATGGCATTGTTTTCCA-3'), *cepa* (5'-GGTCCACATCATAAATTGTTGTTGTTG-3'), or *ldh* (5'-GATCAGATGTGCTGAG-3') (17, 18) that was end labeled with [γ-32P]ATP. For the detection of *gap* mRNA, a PCR fragment, obtained using the primers GAPFOR (5'-GGTGGTATGCTTTGTTGTTGTTGTTG-3') and GAPREV (5'-GATGATTTGTTAAATGGCATTGTTTTCCA-3'), was labeled with [γ-32P]ATP. The total amount of RNA loaded on the gels was analyzed using a 165 RNA probe (5'-ATCTAGGCTTTCAACCG CTCG-3') specific for *L. lactis* (14).

**Protein extraction and protein analysis using 2D EF.** Proteins were extracted with an MSK cell homogenizer (B. Braun Biotech International, Melsungen, Germany) and zincium beads (0.1-mm diameter; Biospec Products, Bartlesville, Okla.). Protein analysis was performed by two-dimensional gel electrophoresis (2D EF) as described by Wouters et al. (27), and equal amounts (40 µg) of protein from the cell extracts were separated on an isoelectric point (pI) region from 4 to 7 and subsequently on 15% polyacrylamide homogenous sodium dodecyl sulfate gels together with a molecular weight (MW) marker. The proteins were visualized by silver staining, and the spots were analyzed with GEMINI software (Applied Imaging, Sunderland, England).

**LDH activity and GAPDH activity.** The LDH activities of cell extracts of *L. lactis* were analyzed by NADH consumption as described by Hillier and Jago (8). GAPDH activity was measured by analyzing the increase at 340 nm in a double-beam spectrophotometer as a result of NADH production, as described previously by Poolman et al. (19).

**RESULTS**

**Acidification rates of *L. lactis* cells incubated at low temperature.** To relate low-temperature incubation to physiological response, glycolytic activity was determined for *L. lactis* cultures grown under different conditions. Mid-exponential-phase cells (OD$_{600}$ 0.5) cultured at 30°C showed a maximal glycolytic activity of approximately 600 nmol/min/mg of protein, which was increased to approximately 1,600 nmol/min/mg of protein upon exposure to 10°C for several hours. This increase in glycolytic activity was maximal (2.3-fold) after 4 to 5 h of incubation at 10°C. Upon longer exposure, the maximum acidification rate decreased to approximately 900 nmol/min/mg of protein (Fig. 1). In the presence of chloramphenicol, which inhibits protein synthesis and consequently inhibits cell growth (data not shown), during cold incubation, cells did not show an increase in maximum glycolytic activity (Fig. 1). After prolonged incubation with chloramphenicol, the glycolytic activity was very low (60 nmol/min/mg of protein at 20 h after cold shock), indicating the necessity for constant protein synthesis to maintain glycolytic activity.

**No increased acidification for *L. lactis* NZ9880(Δ*ptsH*) and *L. lactis* NZ9870(Δ*accP*A).** To further elucidate the mechanism of the increased glycolytic activity of *L. lactis* cells exposed to low temperature, acidification rates were also determined for *L. lactis* NZ9880(Δ*ptsH*), *L. lactis* NZ9881(Δ*ptsI*), and *L. lactis* NZ9870(Δ*accP*A). For *L. lactis* NZ9880(Δ*ptsH*), the acidification rate is significantly reduced (nearly threefold) in mid-exponential-phase cells compared to that of wild-type cells in this growth phase (Fig. 2A), which can be explained by reduced sugar transport. Analysis of the end products revealed
that the production of acetic acid increased in comparison to that of wild-type cells, indicating characteristics of a mixed-acid fermentation. Upon exposure of \textit{L. lactis} NZ9880 (Δ\textit{ptsH}) cells to low temperature, no increase in maximum glycolytic activity was observed (Fig. 2A). For \textit{L. lactis} NZ9881 (Δ\textit{ptsI}), a fivefold reduction of the acidification rate was observed for cells grown at 30°C compared to wild-type cells, which is most likely explained by reduced sugar uptake by \textit{L. lactis} NZ9881 (Δ\textit{ptsI}) (Fig. 2B). Similar to wild-type \textit{L. lactis}, an approximately two-fold increase in acidification is also observed for \textit{L. lactis} NZ9881 (Δ\textit{ptsI}) upon exposure to 10°C after 2 to 3 h (Fig. 2B). The maximum glycolytic activity of \textit{L. lactis} NZ9870 (Δ\textit{ccpA}) cells was also strongly reduced at 30°C compared to that of wild-type cells (Fig. 2C), which might be explained by the reduced activity of the \textit{las} operon. Also, for \textit{L. lactis} NZ9870 (Δ\textit{ccpA}), an increased formation of acetic acid was observed, similar to that observed by Luesink et al. (18). Upon exposure to 10°C, no increased acidification is observed for \textit{L. lactis} NZ9870 (Δ\textit{ccpA}) cells compared to wild-type cells (Fig. 2C). High-performance liquid chromatography analysis revealed that the ratios of the products (lactate, acetate, and formate) formed by cells cultured at high and low temperatures were identical. In conclusion, these data indicate that both HP\textit{r} and CcpA are involved in increased acidification at low temperature, in contrast to enzyme I. This indicates that the PTS is not involved in this response, whereas the regulatory function of HP\textit{r}(Ser-P) probably is involved.

\textbf{Analysis of \textit{ptsH}, \textit{ptsI}, and \textit{ccpA} upon cold shock.} Using specific probes, the mRNA levels of \textit{ptsH}, \textit{ptsI}, and \textit{ccpA} were analyzed in \textit{L. lactis} NZ9880 after cold shock. The 2.0-kb \textit{ptsH} transcript (17) appeared to be induced upon cold shock to 10°C (a maximum of twofold after 4 h). Using a probe specific for \textit{ptsH}, two transcripts of 2.0 and 0.3 kb, as described by Luesink et al. (17), were detected that were also induced upon exposure to 10°C (a maximum of 2- and 1.5-fold, respectively, after 4 h). Next, the expression of the 1.2-kb \textit{ccpA} transcript (18) was slightly induced upon cold shock (a maximum of 1.5-fold at 4 h) (Fig. 3A).

The effect of exposure to low temperature on the levels of the proteins encoded by \textit{ptsH}, \textit{ccpA}, and \textit{ptsI} was analyzed using cell extracts of \textit{L. lactis} NZ9800 before and after cold shock (2 and 4 h). Based on 2D-EF gels for cell extracts of \textit{L. lactis} NZ9870, \textit{L. lactis} NZ9880, and \textit{L. lactis} NZ9881 and on the calculated MWs and pIs of HP\textit{r} (MW, 9.1; pI, 4.6), enzyme I (MW, 62.6; pI, 4.6), and CcpA (MW, 36.6; pI 5.0), the spots representing the respective proteins could be determined for \textit{L. lactis} NZ9880. HP\textit{r} is one of the most copiously produced proteins (5% of the total visualized proteins on the 2D-EF gels) in mid-exponential-phase cells. The quantity of HP\textit{r} slightly increased upon cold shock for 2 or 4 h (1.5- to 2-fold [Fig. 3B]), which is in agreement with the increased \textit{ptsH} mRNA level. For enzyme I, no induction was observed upon exposure to low temperature (Fig. 3B). Cold induction was also observed for CcpA (Fig. 3B), which was confirmed by use of a \textit{Bacillus megaterium} CcpA antibody that revealed two- to threefold induction upon cold shock (data not shown).

\textbf{mRNA analysis of the \textit{las} operon and \textit{gap} and analysis of LDH and GAPDH activities.} No low-temperature-induced acidification is observed for strains with the genes encoding HP\textit{r} and CcpA deleted. Hence, the complex that is assumed to be formed between HP\textit{r} and CcpA might play a role in increased acidification upon incubation at 10°C by inducing specific genes. To investigate this assumption, the mRNA level of the \textit{las} operon, which is known to be positively regulated by the putative CcpA-HP\textit{r}(Ser-P) complex (17), was monitored upon exposure to cold shock. None of these transcripts (4, 3, and 1 kb) were induced by cold shock (Fig. 4A), and the LDH activity also did not increase upon exposure to cold shock. In the presence of chloramphenicol, a significant reduction in LDH activity was measured at 0.5, 2, and 4 h after cold shock,
indicating that de novo protein synthesis is required to maintain LDH activity (Fig. 4B). This also indicates that LDH cannot be the rate-limiting factor in glycolysis, since the maximum glycolytic activity in these cells stays at a constant level during this period (Fig. 1).

The conversion of glyceraldehyde-3-P to 1,3-diphosphoglycerate, catalyzed by GAPDH, was previously identified as the rate-limiting step in the glycolysis of starved L. lactis cells (19). The monocistronic transcript of gap has a size of 1.3 kb and was constant during the first hours after cold shock (Fig. 4A). Strikingly, the transcript is induced at 20 h after cold shock (approximately threefold), whereas for the other genes analyzed here (ptsI, ptsH, ccpA, and the las operon), the transcripts can hardly be detected at that time, conditions under which L. lactis is probably starved. Upon cold shock for 0.5, 2, and 4 h, the GAPDH activity was identical to the activity prior to cold shock (approximately 4 μmol/min/mg of protein [Fig. 4B]).

Similar to the LDH activity, the GAPDH activity was reduced upon cold shock in the presence of chloramphenicol, indicating that the GAPDH activity is also not a rate-limiting step under these conditions. Comparison of the 2D-EF gels of Fig. 3B with a gel of L. lactis MG1363 revealed the position of GAPDH, which appears to be a double spot, as previously reported (12). Upon exposure to 10°C for 4 h, neither of these two spots was cold induced.

**DISCUSSION**

Since L. lactis is extensively used in dairy fermentations, it is of great importance to be able to control its metabolic pathways. In recent years, metabolic engineering has proved to be a valuable tool for the optimization of fermentation processes and the design of novel fermentation pathways (3). Expanding our knowledge of the stress response in this respect will contribute to the benefits of these new approaches. In this report, the relationship between the glycolytic pathway and the cold stress response of L. lactis was investigated, and it was revealed that upon exposure to low temperature the acidification rate of L. lactis cells increases. At low temperature, enzyme-catalyzed reaction rates are known to decrease, and it is assumed that under these conditions induction of certain factors is required to compensate for this loss in activity. It is conceivable that exposure to 10°C results in induction of glycolytic enzymes to compensate for an overall lower glycolytic capacity. In the presence of chloramphenicol during exposure to low temperature, no increased acidification is observed, indicating that protein synthesis is required. This observation also excludes the possibility of deregulation of glycolysis at low temperature by uncoupling of regulatory mechanisms, as described by Poolman et al. (20). However, the possibility that the observed increased acidification is controlled by increased protein syn-

**FIG. 3.** Analysis of the cold induction of HPr, enzyme I, and CcpA. (A) mRNA levels of the ptsH, ptsI, and ccpA genes in L. lactis NZ9800 were analyzed by Northern blotting prior to cold shock and upon exposure to cold shock for several periods. Total RNA was extracted at 0, 1, 2, 3, 4 and 24 h after cold shock from 30 to 10°C of L. lactis NZ9800. The blots were hybridized with the specific ptsHI, ccpA, and 16S rRNA probes. The transcript sizes are about 2.0, 0.3, 1.2, and 1.5 kb for the ptsHI, ptsH, and ccpA genes and 16S rRNA, respectively, and are indicated by arrows. (B) 2D-EF gels of cell extracts of L. lactis NZ9800 isolated prior to cold shock (30°C in mid-exponential phase [top]) and at 2 h (middle) and 4 h (bottom) after cold shock to 10°C. Equal amounts of protein were loaded on the gels, and the proteins were visualized by silver staining. Molecular size marker bands are indicated on the left, and a pI scale is given at the bottom. The putative spots representing HPr (H), enzyme I (I), CcpA (C), and GAPDH (G1 and G2) are indicated. t, time (in hours).
thesis as well as allosterical regulation by the concentration of different glycolytic intermediates cannot be excluded.

For *L. lactis* strains with the genes encoding HPp and CcpA, two important regulators of the glycolytic activity, deleted, no increase in maximal glycolytic activity is observed upon exposure to low temperature. In the absence of *psl*, which encodes the enzyme I subunit of the PTS, an increased acidification is still observed, excluding a rate-limiting role for the PTS and also indicating that the regulatory function of HPp(Ser-P) is probably involved. Strikingly, mRNA analysis revealed induction of *ccpA* and *ptsH*, as well as *psl* (encoding both HPp and enzyme I), upon cold shock. *L. lactis* CcpA and HPp were both cold induced at the protein level. Strikingly, the importance of HPp in the cold-adaptive response is further stressed by the observation that *L. lactis* NZ9800(*ΔptsH*) is not able to grow at low temperature. In contrast, *ptsH* is not deleted in the isolate *NZ9800* but is deleted in the isolate *NZ9880*.

In conclusion, the maximal glycolytic activity measured at 30°C showed a marked increase upon incubation of *L. lactis* cells at 10°C for several hours. However, for the rate-limiting steps of glycolysis, i.e., the activities of the enzymes encoded by the *las* operon and GDPDH, no induction was observed upon cold shock. This indicates that a factor other than LDH or GDPDH is rate determining for the increased glycolytic activity upon exposure to low temperatures. Based on their cold induction and involvement in cold adaptation of glycolysis, it is proposed that the CcpA-HPp(Ser-P) control circuit regulates this factor(s) and hence couples catabolite repression and cold shock response in a functional and mechanistic way.

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