Changes in Glycolytic Activity of Lactococcus lactis Induced by Low Temperature

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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), which is 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.
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 GAPDH, 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.

**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<sub>600</sub> 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 (ΔptsH) and *L. lactis* NZ9870 (Δacp4).** To further elucidate the mechanism of the increased maximum glycolytic activity of *L. lactis* cells exposed to low temperature, acidification rates were also determined for *L. lactis* NZ9880 (ΔptsH), *L. lactis* NZ9881 (ΔptsH), and *L. lactis* NZ9870 (Δacp4). For *L. lactis* NZ9880 (ΔptsH), 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 *L. lactis* NZ9880(ΔptsH) cells to low temperature, no increase in maximum glycolytic activity was observed (Fig. 2A). For *L. lactis* NZ9881(ΔptsI), 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 *L. lactis* NZ9881(ΔptsI) (Fig. 2B). Similar to wild-type *L. lactis*, an approximately twofold increase in acidification is also observed for *L. lactis* NZ9881(ΔptsI) upon exposure to 10°C after 2 to 3 h (Fig. 2B). The maximum glycolytic activity of *L. lactis* NZ9870(ΔccpA) 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 *las* operon. Also, for *L. lactis* NZ9870(ΔccpA), 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 *L. lactis* NZ9870(ΔccpA) 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 HPr 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 HPr(Ser-P) probably is involved.

**Analysis of ptsH, ptsI, and ccpA upon cold shock.** Using specific probes, the mRNA levels of *ptsH*, *ptsI*, and *ccpA* were analyzed in *L. lactis* NZ9880 after cold shock. The 2.0-kb *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 *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 *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 *ptsH*, *ccpA*, and *ptsI* was analyzed using cell extracts of *L. lactis* NZ9800 before and after cold shock (2 and 4 h). Based on 2D-EF gels for cell extracts of *L. lactis* NZ9870, *L. lactis* NZ9880, and *L. lactis* NZ9881 and based on the calculated MWs and pIs of HPr (MW, 9.1; pl, 4.9), enzyme I (MW, 62.6; pl, 4.6), and CcpA (MW, 36.6; pl 5.0), the spots representing the respective proteins could be determined for *L. lactis* NZ9800. HPr 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 HPr slightly increased upon cold shock for 2 or 4 h (1.5- to 2-fold [Fig. 3B]), which is in agreement with the increased *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 *Bacillus megaterium* CcpA antibody that revealed two- to threefold induction upon cold shock (data not shown).

**mRNA analysis of the *las* operon and *gap* and analysis of LDH and GAPDH activities.** No low-temperature-induced acidification is observed for strains with the genes encoding HPr and CcpA deleted. Hence, the complex that is assumed to be formed between HPr 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 *las* operon, which is known to be positively regulated by the putative CcpA-HPr(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 \textit{L. lactis} cells \footnote{19}. The monocistronic transcript of \textit{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 (\textit{ptsI}, \textit{ptsH}, \textit{ccpA}, and the las operon), the transcripts can hardly be detected at that time, conditions under which \textit{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 \textmu mol/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 \textit{L. lactis} MG1363 revealed the position of GAPDH, which appears to be a double spot, as previously reported \footnote{12}. Upon exposure to 10°C for 4 h, neither of these two spots was cold induced.

**DISCUSSION**

Since \textit{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 \footnote{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 \textit{L. lactis} was investigated, and it was revealed that upon exposure to low temperature the acidification rate of \textit{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. \footnote{20}. However, the possibility that the observed increased acidification is controlled by increased protein syn-
thesis as well as allosterical regulation by the concentration of different glycolytic intermediates cannot be excluded.

For *L. lactis* strains with the genes encoding HP 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 *ptsI*, 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 HP(Ser-P) is still observed, excluding a rate-limiting role for the PTS and also indicating that the regulatory function of HP(Ser-P) is probably involved. Strikingly, mRNA analysis revealed induction of *ccpA* and *ptsH*, as well as *ptsI* (encoding both HP and enzyme I), upon cold shock. *L. lactis* CcpA and HP were both cold induced at the protein level. Strikingly, the importance of HP in the cold-adaptive response is further stressed by the observation that *L. lactis* NZ9800(ΔptsH) is not able to grow at low temperature (J. A. Wouters, H. H. Kamphuis, and T. Abeel, unpublished data). It has been reported that the putative CcpA-HP(Ser-P) complex can either positively (e.g., the *las* operon) or negatively (e.g., the *gap* operon) control certain key steps in metabolic pathways (18, 19). However, no cold induction was observed for the transcripts of the *las* operon, and it was concluded that despite the increased level of CcpA and HP, the CcpA-HP(Ser-P) complex does not induce *las* operon expression under these conditions. Next, it was shown that LDH activity is not the rate-limiting step of glycolysis under these conditions. Poolman et al. (19) showed that GAPDH activity is the rate-limiting step in the glycolytic activity of starved *L. lactis* cells. Analysis of GAPDH at low temperatures revealed that neither the gap mRNA level nor GAPDH activity increased upon exposure to low temperatures. Furthermore, incubation of *L. lactis* cells at a low temperature in the presence of chloramphenicol revealed that GAPDH activity was also not rate limiting in glycolysis under these conditions. We speculate that CcpA and HP control several other steps of glycolysis by their specific interaction with the catabolite-responsive element. Catabolite-responsive elements are found throughout the *L. lactis* chromosome and differ in their homologies to the consensus sequence (17). It can be postulated that more of these elements are found in the genes of the glycolytic pathway, which would indicate an expanded regulatory role of HP and CcpA. Apparently, an unidentified factor(s) is required for increased glycolytic activity upon exposure to low temperatures, and we propose that the CcpA-HP(Ser-P) complex regulates the factor(s) required for this increase.

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 GAPDH, no induction was observed 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-HP(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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