Transcriptional activation of the glycolytic las operon and catabolite repression of the gal operon in Lactococcus lactis are mediated by the catabolite control protein CcpA

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Summary
The Lactococcus lactis ccpA gene, encoding the global regulatory protein CcpA, was identified and characterized. Northern blot and primer extension analyses showed that the L. lactis ccpA gene is constitutively transcribed from a promoter that does not contain a cre sequence. Inactivation of the ccpA gene resulted in a twofold reduction in the growth rate compared with the wild type on glucose, sucrose and fructose, while growth on galactose was almost completely abolished. The observed growth defects could be complemented by the expression of either the L. lactis or the Bacillus subtilis ccpA gene. The disruption of the ccpA gene reduced the catabolite repression of the gal operon, which contains a cre site at the transcription start site and encodes enzymes involved in galactose catabolism. In contrast, CcpA activates the transcription of the cre-containing promoter of the las operon, encoding the glycolytic enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase, because its transcription level was fourfold reduced in the ccpA mutant strain compared with the wild-type strain. The lower activities of pyruvate kinase and L-lactate dehydrogenase in the ccpA mutant strain resulted in the production of metabolites characteristic of a mixed-acid fermentation, whereas the fermentation pattern of the wild-type strain was essentially homolactic.

Introduction
In many cases, the presence of a rapidly metabolizable carbon source in the growth medium of bacteria reduces the expression of genes involved in the utilization of other carbon sources. This phenomenon, termed carbon catabolite repression (CR), is well understood in Escherichia coli and other Gram-negative bacteria, in which the cytoplasmic glucose-specific enzyme IIA of the phosphotransferase system (PTS) acts as a general mediator of CR. In combination with the signal molecule cAMP and the cyclic AMP receptor protein (CRP), the glucose-specific enzyme IIA regulates the expression of several genes (Postma et al., 1993).

In Gram-positive bacteria, it has been established that CR is mediated via a negative regulatory mechanism (Hueck and Hillen, 1995). In Bacillus subtilis, the regulatory catabolite control protein CcpA has been shown to be involved in CR of the α-amylase gene (Henkin et al., 1991). CcpA belongs to the LacI/GalR family of bacterial regulator proteins, and disruption of the ccpA gene reduces CR of several genes involved in the carbohydrate metabolism. A cis-acting sequence, termed catabolite-responsive element (cre), present near the promoter of genes affected by CR, was found to be essential for mediating CR (Nicholson et al., 1987; Weickert and Chambliss, 1990).

Several groups have provided evidence that CcpA can bind to cre sites under different conditions in vitro. The binding of CcpA to cre sites is reported to be enhanced by elevated concentrations of early glycolytic intermediates such as glucose-6-P, which is an indicator of the energy state of the cell (Gössinger et al., 1997). Another signal involved in the activation of CcpA is the PTS phosphocarrier HPr. A metabolite-activated kinase has been shown to phosphorylate HPr on residue serine 46 at the expense of ATP (Deutscher and Saier, 1983) and, recently, the gene encoding the HPr(Ser) kinase has been cloned from B. subtilis, overexpressed and characterized (Reizer et al., 1998). This phosphorylated form of HPr [HPr(Ser-P)] interacts with CcpA, and this interaction enhances the binding of CcpA to cre sites located in the promoter region of the B. subtilis gluconate operon and the B. megaterium xyl operon (Deutscher et al., 1995; Fujita et al., 1995; Gössinger et al., 1997).

In B. subtilis, two genes, alsS and ackA, encoding α-acetolactate synthase and acetate kinase, respectively, have been reported to be positively regulated by CcpA (Grundy et al., 1993; Renna et al., 1993). The transcription
of the ackA and alsS genes is induced when glucose is present in the growth medium. This indicates that CcpA can act as both a negative and a positive regulator in B. subtilis and is involved in more regulatory mechanisms than CR. The involvement of CcpA in catabolite repression has also been established in Bacillus megaterium, Staphylococcus xylosus, Lactobacillus casei and Lactobacillus pentosus (Hueck et al., 1995; Egeter and Brückner, 1996; Lokman et al., 1997; Monedero et al., 1997). Disruption of ccpA genes in these organisms not only reduces the CR of several target genes but also decreases the growth rate, suggesting an involvement of CcpA in the regulation of other metabolic pathways. Other genes encoding proteins with a high sequence homology to CcpA proteins have been identified in Lactobacillus delbrueckii and Clostridium acetobutylicum, but evidence that they exert CR in the respective organisms is lacking (Davidson et al., 1995; Stucky et al., 1996). Using polyclonal antibodies raised against the purified CcpA protein from B. megaterium, it was possible to detect cross-reacting proteins in many Gram-positive bacteria, including L. lactis (Küster et al., 1996).

In this paper, we report the cloning and molecular analysis of the L. lactis ccpA gene and the effects of its disruption on the catabolite repression of the galAMKTE genes involved in galactose catabolism (Grossiord et al., 1998). Furthermore, we show that CcpA can act as an activator of transcription of the las operon (Llanos et al., 1993), containing the plk, pyk and ldh genes encoding the key glycolytic enzymes phosphofructokinase, pyruvate kinase and L-lactate dehydrogenase, respectively, involved in energy production and lactic acid formation by L. lactis. The results indicate a pleiotropic function for the L. lactis CcpA, which not only represses the expression of genes involved in the uptake and utilization of galactose but also activates the central metabolism leading to an accelerated utilization of specific carbohydrates and enhanced production of end-products.

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. As the deduced protein sequence showed 48% identical residues compared with B. subtilis CcpA (Henkin et al., 1991), this gene was designated ccpA (see below; Fig. 1).

Disruption of the ccpA gene and its effects on growth

An erythromycin resistance (EryR) gene was introduced into a unique AccI 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 analyse the effect of CcpA on the carbohydrate metabolism, the growth rates of strain NZ9870 on different carbon sources were compared with 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%)

Fig. 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 by primer extension and Northern analysis respectively. Relevant restriction sites are indicated.
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 the addition of inducing concentrations of nisin A (Table 1). Similar results were obtained with plasmid pNZ9246, which contains the *B. subtilis* ccpA gene under the control of the nisA promoter (data not shown).

**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 analysed by Northern blot analysis. A band of approximately 1.2 kb hybridizing with *ccpA*-specific probe could be identified (Fig. 3). This suggests that transcription terminates at a rho-independent terminator structure, with a $\Delta G$ value of $-12.6$ kcal mol$^{-1}$, which was identified downstream of the *ccpA* open reading frame (ORF). 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).

**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). The *galK* gene was selected for analysis of the role of CcpA in the regulation of expression of the *gal* genes, as it encodes a galactokinase that is a key enzyme in the Leloir pathway. Total RNA (20 $\mu$g) 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

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**Table 1. Growth rates of strains used in this study.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Fructose</th>
<th>Galactose</th>
<th>Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ9800 (wild type)</td>
<td>1.42 ± 0.05</td>
<td>1.22 ± 0.04</td>
<td>0.92 ± 0.05</td>
<td>0.63 ± 0.04</td>
<td>0.51 ± 0.04</td>
</tr>
<tr>
<td>NZ9870 (ΔccpA)</td>
<td>0.71 ± 0.09</td>
<td>0.68 ± 0.08</td>
<td>0.63 ± 0.06</td>
<td>&lt;0.10 ± 0.10</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>NZ9870 + pNZ9245 (<em>L. lactis</em> ccpA)</td>
<td>1.36 ± 0.11</td>
<td>1.20 ± 0.07</td>
<td>0.88 ± 0.03</td>
<td>0.60 ± 0.05</td>
<td>0.51 ± 0.09</td>
</tr>
</tbody>
</table>

*Average values of at least two independent determinations including the error are given.*

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**Fig. 2.** Primer extension analysis of the ccpA promoter. The transcription start sites are indicated by arrows. The putative $-10$ region in the complementary strand is represented in bold. RNA was isolated from *L. lactis* strain NZ9800 grown on glucose.
glucose but, when the cells were grown on galactose, the transcription increased, indicating induction by a compound that 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 with 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 initiating from the galA promoter was not completely derepressed (Fig. 5). Similar results were obtained with different RNA concentrations (data not shown). As 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.

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 polymerase chain reaction (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, as the enzyme activities in galactose-grown cells were reduced to 50% and 65%, respectively, compared with glucose-grown cells (Table 2). Disruption of the ccpA gene resulted in a two- to fourfold reduction in both the pyruvate kinase and L-lactate dehydrogenase activities in cells grown on glucose (Table 2). The introduction of plasmid pNZ9245 into strain NZ9870 (ΔccpA) and the 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 the control of the nisA promoter was also able to restore the pyruvate kinase and L-lactate dehydrogenase activities in strain NZ9870.

Table 2. Lactate dehydrogenase and pyruvate kinase activities of strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Carbon source</th>
<th>Pyruvate kinase ( \mu \text{mol NADH mg}^{-1} \text{min}^{-1} )</th>
<th>Lactate dehydrogenase ( \mu \text{mol NADH mg}^{-1} \text{min}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ9800 (wild type)</td>
<td>Glucose</td>
<td>3.20 ± 0.19</td>
<td>14.20 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>1.67 ± 0.13</td>
<td>9.05 ± 0.72</td>
</tr>
<tr>
<td>NZ9800 + pNZ9245 (L. lactis ccpA)</td>
<td>Glucose</td>
<td>2.91 ± 0.42</td>
<td>13.55 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>2.81 ± 0.21</td>
<td>13.31 ± 0.71</td>
</tr>
<tr>
<td>NZ9870 (ΔccpA)</td>
<td>Glucose</td>
<td>0.79 ± 0.08</td>
<td>6.32 ± 0.55</td>
</tr>
<tr>
<td>NZ9870 + pNZ9245 (L. lactis ccpA)</td>
<td>Glucose</td>
<td>2.72 ± 0.13</td>
<td>11.48 ± 1.02</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td>2.58 ± 0.48</td>
<td>11.57 ± 0.66</td>
</tr>
<tr>
<td>NZ9870 + pNZ9246 (B. subtilis ccpA)</td>
<td>Glucose</td>
<td>2.65 ± 0.13</td>
<td>10.74 ± 0.34</td>
</tr>
</tbody>
</table>

Average values of at least two independent determinations as well as the error are given.

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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 analyse 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.

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 analysed 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

Fig. 4. A. Schematic representation of the L. lactis gal and las operons. The mapped promoters are indicated by arrowheads. The proposed processing sites of the 4 kb transcript of the las operon are indicated by arrows. These structures with a free energy of $-4.6$ kcal mol$^{-1}$ and $-3.0$ kcal mol$^{-1}$ are centred 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 by arrows. The putative cre sites are aligned with the consensus sequence.

Fig. 5. Slot-blot analysis of 20 μg of total RNA isolated from strains NZ9800 (wild-type) and NZ9870 (ΔccpA) grown under different circumstances after hybridization with a galK-specific probe.
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 led 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 scintillation counter. Based on the ratio between the gene-specific and the 16S-derived signals, the relative mRNA levels were calculated. The reduction in the transcription levels in strain NZ9870 (ΔccpA) compared with 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, as 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 had occurred in L-lactate production from 50 mM in the wild-type strain to 37 mM in the ccpA mutant, 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.

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 analysed. 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 in the growth rate on both PTS and non-PTS sugars, as has also been 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 the control of the inducible nisA promoter leads, after the addition of inducing

### Table 3. Product formation of L. lactis strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glucose consumption (mM)</th>
<th>Concentration of end-products (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L-lactate</td>
</tr>
<tr>
<td>NZ9800 (wild type)</td>
<td>33.1 ± 0.9</td>
<td>50.1 ± 2.5</td>
</tr>
<tr>
<td>NZ9870 (ΔccpA)</td>
<td>33.2 ± 0.2</td>
<td>37.3 ± 0.3</td>
</tr>
</tbody>
</table>

Average values of two independent determinations as well as the error are given. ND, not detectable.
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, as 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, as 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 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, which have been described in *L. lactis* and have been proposed as playing an important role in the regulation of transcription of the lactose gene cluster by regulating the level of inducer (Ye et al., 1994a,b). 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, because increased activities were measured in glucose-grown cells compared with 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 explanations, such as indirect effects on the transcription or changes in the RNA stability, cannot be excluded. Because the intracellular concentration of early glycolytic intermediates such as 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 probably depends on the concentration of these metabolites.

Northern analysis of RNA isolated from the wild-type and the ccpA mutant strains grown on glucose showed that the observed regulation occurred at the transcriptional level, as a fourfold 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, as no promoter-like sequences have been 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; 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, as 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 CO₂ respectively. Further analysis is required to analyse whether the concentrations of other possible end-products, such as 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 probably 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*, as they are part of the pyruvate metabolism and catalyse the conversion of pyruvate to compounds that can easily be removed from the cell. Activation of the expression of these genes can be regarded as a mechanism for preventing the possible toxic accumulation of end-products of the glycolysis.

Several authors have suggested that the overall reduction in the growth rate observed in ccpA knock-out strains might be caused by the interference of CcpA with central metabolic pathways such as 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 *E. 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⁻¹, chloramphenicol 5 mg l⁻¹ and erythromycin 2.5 mg l⁻¹.

**DNA techniques and sequence analysis**

All manipulations with recombinant DNA were carried out according to standard procedures (Sambrook et al., 1989) and the specifications of the enzyme manufacturers (Gibco BRL, Life Technologies, US 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 10 mM Tris-HCl (pH 8.8), 50 mM NaCl, 2 mM MgCl₂, 10 μg gelatin, 200 μM each deoxy-nucleoside triphosphate, 1 U Taq polymerase (Gibco BRL), 10 pmol of each primer and 10–100 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 using a DNA thermocycler (Perkin-Elmer).

**Construction of plasmids**

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

A 0.9 kb AccI fragment from pUC19E containing an EryR gene was cloned into pNZ9243 digested with AccI, resulting in plasmid pNZ9244 carrying an interrupted ccpA gene. A NcoI site was introduced at the ATG start codon of the *L. lactis* ccpA gene. PCR was performed using primers CCPANCO and the complementary strand of bases 1352–1373, respectively, in order to amplify the *B. subtilis* ccpA gene without its promoter but with its ribosome binding site. The PCR product obtained containing the 5′ end of the ccpA gene was digested with *NcoI* and HindIII and cloned in *NcoI*–HindIII-digested pNZ9243. A 0.9 kb *HpaII*–Smal 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 analysed and found to contain no deviations. Oligonucleotides BSCCPA5 and BSCCPA3, 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 306–322 and the complementary strand of bases 1352–1373, respectively, in order to amplify the *B. subtilis* ccpA gene without its promoter but with its ribosome binding site. The PCR product obtained was cloned in BamHI and EcoRI-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 OD₆₀₀ 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 zirconium 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.
standard procedures (Sambrook et al., 1989). The RNA was on a 1% agarose gel containing formaldehyde according to Q, 1998 Blackwell Science Ltd, Molecular Microbiology, references 30: 789–798.

RNA analysis
RNA was isolated from L. lactis cultures as described previously (Kuipers et al., 1993). Northern analysis was performed with 20 \mu g of 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 dilution steps resulting in different RNA concentrations. Blots were probed with the following oligonucleotides: PECCPA (5'-GTGCCACATCATAATTGTGGTGTGGT-3' ; ccpA), GALR1 (5'-ACCAGACAACCTTCGTGTA-3' ; galK), LAS2 (5'-CTGCAAGATAAGGGCCATT-3' ; pck), LAS3 (5'-CATCATGGGATAACACCC-3' ; pyk), LAS4 (5'-GATCAGATGGTGAAG-3' ; idh) and 3.2 (5'-ATCTACCG-ATTCCCGGTAC-3'; 16S rRNA; Klijn et al., 1991). After autoradiography, bands were cut out, and total radioactivity was determined using a liquid scintillation counter (Beckman 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'-GTGCCACATCATAATTGTGGTGTGGT-3') complementary to nucleotides 189–215 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 \mu g of total RNA as described previously (Kuipers et al., 1993).

End-product determination
Cells were grown to an OD\textsubscript{600} of 1, concentrated by centrifugation and resuspended to a final OD\textsubscript{600} of 10 in 100 mM sodium phosphate buffer, pH 7.0, containing 50 mM glucose. After incubation for 1 h 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 high-performance liquid chromatography (HPLC) as described previously (Starrenburg and Hugenholtz, 1991). A Clostridium acetobutylicum regulator gene (regA) affecting amylose production in Bacillus subtilis. Microbiology 141: 989–996.

References

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