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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 (Gösseringer *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; Gösseringer *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

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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 *pfk*, *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 (Ery^R) gene was introduced into a unique *Acc*I site located in the *ccpA* gene, resulting in strain NZ9870. Protein extracts of strain NZ9870 no longer contained a protein of 37 kDa that cross-reacted with the antiserum raised against the *B. megaterium* CcpA (data not shown). To 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%)

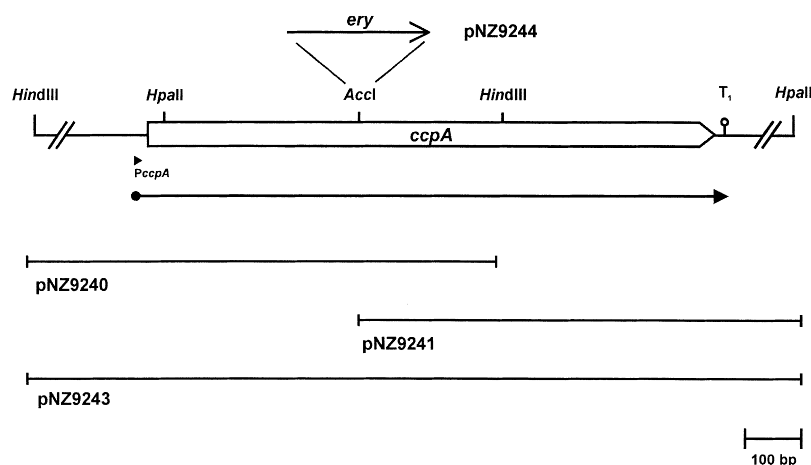

Fig. 1. Transcriptional organization of the *L. lactis ccpA* gene. The *ccpA* gene is shown with its gene product and the mapped promoter. The arrows denote the promoter and transcripts mapped by primer extension and Northern analysis respectively. Relevant restriction sites are indicated.

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

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

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

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 a *ccpA*-specific probe could be identified (Fig. 3). This suggests that transcription terminates at a rho-independent terminator structure, with a ΔG value of −12.6 kcal mol^{-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 μg) 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

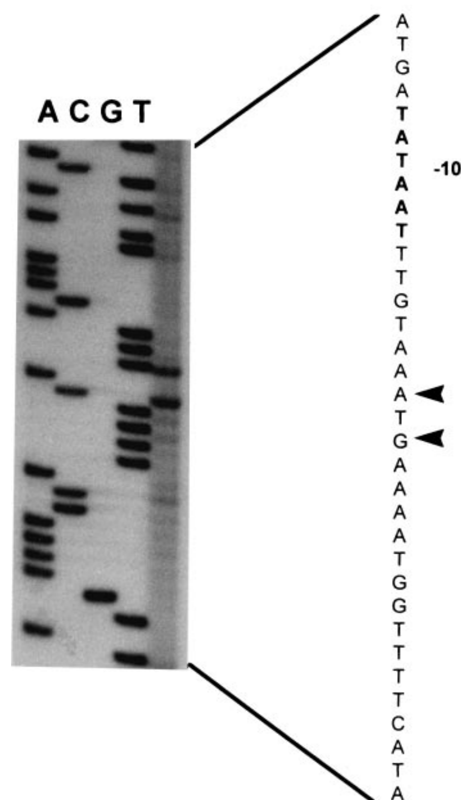

Fig. 2. Primer extension analysis of the *ccpA* promoter. The transcription start sites are indicated by arrows. The putative −10 region in the complementary strand is represented in bold. RNA was isolated from *L. lactis* strain NZ9800 grown on glucose.

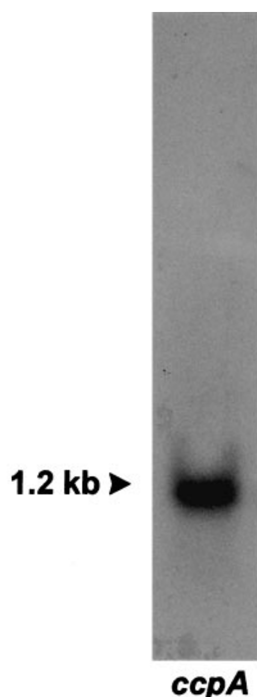

Fig. 3. Northern analysis of the *L. lactis ccpA* gene. After electrophoresis the RNA was transferred to nylon membranes and probed with a *ccpA* specific probe. The size of the *ccpA* transcript is indicated with an arrow. RNA was isolated from *L. lactis* strain NZ9800 grown on glucose.

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 the L-lactate dehydrogenase activities in cells grown on glucose (Table 2). The introduction of plasmid pNZ9245 into strain NZ9870 (Δ*ccpA*) 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.

Strain	Carbon source	Pyruvate kinase μmol NADH mg^{-1} min^{-1}	Lactate dehydrogenase μmol NADH mg^{-1} min^{-1}
NZ9800 (wild type)	Glucose	3.20 ± 0.19	14.20 ± 0.41
	Galactose	1.67 ± 0.13	9.05 ± 0.72
NZ9800 + pNZ9245 (*L. lactis ccpA*)	Glucose	2.91 ± 0.42	13.55 ± 0.52
	Galactose	2.81 ± 0.21	13.31 ± 0.71
NZ9870 (Δ*ccpA*)	Glucose	0.79 ± 0.08	6.32 ± 0.55
NZ9870 + pNZ9245 (*L. lactis ccpA*)	Glucose	2.72 ± 0.13	11.48 ± 1.02
	Galactose	2.58 ± 0.48	11.57 ± 0.66
NZ9870 + pNZ9246 (*B. subtilis ccpA*)	Glucose	2.65 ± 0.13	10.74 ± 0.34

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

```
                                       -35                          -10       >
Pgal operon AAAATAGAAAGGGTTTCCAAAAGATAAGCCGGAATTGGAAACCCTTTCTTAAACCAAAGTGTTATACTCTAAATGTGAGCGATTTCAAAA
                                                                              ||||||||||||||
                  TGWNANCGNTNWCA                 consensus cre                TGWNANCGNTNWCA
                  ||||||||||| ||
Plas operon AAAAGAAACAATGAAAACGTTT-CATACAGTTTGTAAAGAGATTTTTTATAAATACGTGATATAATGAACTAGACAACAAATGAACATG
                                    -35                          -10         >
```

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 $-4.6\,\text{kcal mol}^{-1}$ and $-3.0\,\text{kcal mol}^{-1}$ 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.

(Table 2). Remarkably, the activities of pyruvate kinase and L-lactate dehydrogenase in strain NZ9870 containing pNZ9245 grown on galactose were higher than in the wild-type strain NZ9800 grown on galactose. To 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.

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

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

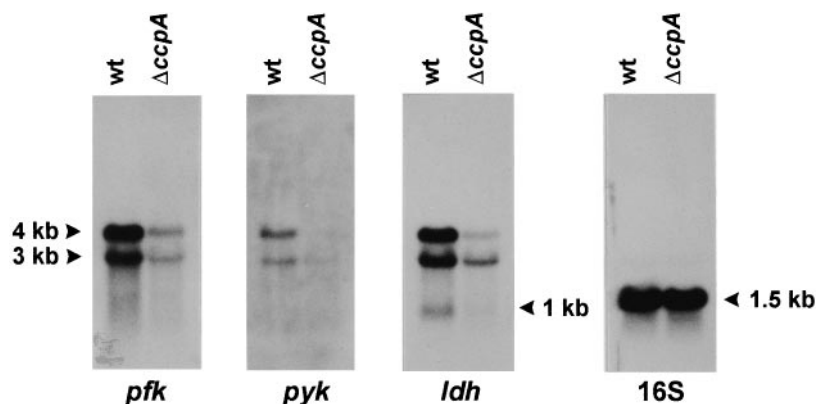

Fig. 6. Northern analysis of the expression of the *pfk*, *pyk* and *ldh* genes in the *L. lactis* strains NZ9800 and NZ9870 grown on glucose. The sizes of the different transcripts are indicated by arrows.

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 (Δ*ccpA*) 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.

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

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 CO_2 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 $MgCl_2$, 10 μg gelatin, 200 μM each deoxynucleoside 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 *Hin*dIII DNA fragment from a phage containing a gene encoding a protein that cross-reacted with the CcpA antibodies into *Hin*dIII-digested pUC19 (Yanisch-Perron *et al*., 1985). A 0.5 kb *Hpa*II–*Hin*dIII fragment from plasmid pNZ9240 was used as a probe to clone a 1.7 kb *Hpa*II fragment from the chromosomal DNA from strain NZ9800 into *Acc*I-digested pUC19, yielding pNZ9242. After the orientation of both inserts was determined, a 1.4 kb *Acc*I–*Kpn*I fragment from pNZ9242 was cloned into *Acc*I–*Kpn*I-digested pNZ9240. The resulting plasmid was designated pNZ9243 and contains the intact *ccpA* gene.

A 0.9 kb *Acc*I fragment from pUC19E containing an Ery^R gene was cloned into pNZ9243 digested with *Acc*I, resulting in plasmid pNZ9244 carrying an interrupted *ccpA* gene. A *Nco*I site was introduced at the ATG start codon of the *L. lactis ccpA* gene. PCR was performed using primers CCPANCO (5′-GATAGCCAACCATGGTAGAATC-3′) containing the *Nco*I site (underlined) and the anti-parallel primer CCPAR5 (5′-CGGTTGATTAACAGAAGTT-3′) using chromosomal DNA from strain NZ9800 as template. The obtained PCR product containing the 5′ end of the *ccpA* gene was digested with *Nco*I and *Hin*dIII and cloned in *Nco*I–*Hin*dIII-digested pNZ8030 (de Ruyter *et al*., 1996). The resulting plasmid was digested with *Xho*I and made blunt using Klenow DNA polymerase. Afterwards, the plasmid was digested with *Hpa*II, and a 1.7 kb *Hpa*II–*Sma*I fragment from plasmid pNZ9243 containing the 3′ end of the *ccpA* gene was cloned in these sites. The resulting plasmid pNZ9245 carried the entire *ccpA* gene translationally fused to the *nisA* promoter. The nucleotide sequence of the DNA obtained by PCR was analysed and found to contain no deviations. Oligonucleotides BSCCPA5 (5′-CAGTGGATCCAGTAAAAGGAGTGG-3′) and BSCCPA3 (5′-CGCAGAATTCACCATAAAGGTGAAGC-3′), based on the sequence data published under accession number M85182, were used to amplify the *B. subtilis* IG33 *ccpA* gene. The oligonucleotides were based on bases 306–322 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 *Bam*HI and *Eco*RI-digested pNZ8020 (de Ruyter *et al*., 1996), resulting in plasmid pNZ9246. Its nucleotide sequence was determined and found to be identical to the published sequence.

Western blot analysis

Cells were grown to an OD_{600} of 1 and concentrated by centrifugation. Cell pellets were resuspended in 1 ml of a sodium phosphate buffer (0.1 M, pH 7). The resulting suspension was subjected to mechanical disruption in the presence of 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

(Küster *et al.*, 1996). These antibodies were detected using goat anti-rabbit peroxidase conjugate (Gibco BRL) as described by the manufacturer.

Enzyme assays

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

RNA analysis

RNA was isolated from *L. lactis* cultures as described previously (Kuipers *et al.*, 1993). Northern analysis was performed with 20 μg 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′-GTGCCACATCATAAATTGTTGTTGTTG-3′; *ccpA*), GALR1 (5′-ACCGACAACTTCTTCGTA-3′; *galK*), LAS2 (5′-CTGCACGAATAGCCGCATTC-3′; *pfk*), LAS3 (5′-CATCATTGGGATAACACCC-3′; *pyk*), LAS4 (5′-GCATCAGAGTAGTCTGCAGAG-3′; *ldh*) and 3.2 (5′-ATCTACGCATTTCACCGCTAC-3′; 16S rRNA; Klijn *et al.*, 1991). 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′-GTGCCACATCATAAATTGTTGTTGTTG-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 μg of total RNA as described previously (Kuipers *et al.*, 1993).

End-product determination

Cells were grown to an OD_{600} of 1, concentrated by centrifugation and resuspended to a final OD_{600} of 10 in 100 mM 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).

Nucleotide sequence accession number

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

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