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ORIGINAL PAPER

Identification and functional characterisation of cellobiose and lactose transport systems in *Lactococcus lactis* IL1403

Magdalena Kowalczyk · Muriel Cocaign-Bousquet · Pascal Loubiere · Jacek Bardowski

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Abstract Physiological, biochemical and macroarray analyses of *Lactococcus lactis* IL1403 and its *ccpA* and *bglR* single and double mutants engaged in lactose and *β*-glucosides catabolism were performed. The kinetic analysis indicated the presence of different transport systems for salicin and cellobiose. The control of salicin catabolism was found to be mediated by the transcriptional regulator BglR and the CcpA protein. The transcriptional analysis by macroarray technology of genes from the PEP:PTS regions showed that several genes, like *ybhE*, *celB*, *ptcB* and *ptcA*, were expressed at higher levels both in wild type cells exposed to cellobiose and in the *ccpA* mutant. We also demonstrated that in *L. lactis* IL1403 cultured on medium with cellobiose and lactose as carbon sources, after the first phase of cellobiose consumption and then co-metabolism of the two sugars, when cellobiose is exhausted the strain uses lactose as the only carbon source. These data could indicate that lactose and cellobiose are transported by a unique system—a PTS carrier induced by the presence of cellobiose, and negatively controlled by the CcpA regulator.

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M. Kowalczyk · J. Bardowski (✉)
Institute of Biochemistry and Biophysics,
Polish Academy of Sciences, Pawinskiego 5a,
02-106 Warsaw, Poland
e-mail: jacek@ibb.waw.pl

M. Cocaign-Bousquet · P. Loubiere
Laboratoire Biotechnologie et Bioprocédés,
UMR CNRS 5504, UMR INRA 792, INSA,
135 Avenue de Rangueil,
31077 Toulouse Cedex 4, France

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Abbreviations
LAB Lactic acid bacteria
CDM Chemically defined medium

Introduction

Lactococcus lactis is a lactic acid bacterium (LAB) that is used as a dairy starter in the production of fermented milk products, particularly cheeses. From an industrial point of view the main role of lactococci is the conversion of sugar into lactic acid. Efficient internalisation and catabolism of lactose are important for growth of lactococci in milk as well as for generation of dairy products with low lactose concentration. On the other hand, LAB used in vegetable fermentations are exposed to various carbon sources, including *β*-glucosides like cellobiose, salicin, esculin or arbutin.

L. lactis converts rapidly metabolised sugars to lactate, while poorly metabolised substrates are converted to mixed-acid products, formate, acetate and ethanol (Cocaign-Bousquet et al. 1996; Garriques et al. 1997). Moreover, these observations clearly show that the central metabolic pathways, i. e., glycolysis and pyruvate oxidation pathways, are strongly regulated by several factors, one of which is the speed of sugar consumption. In addition to regulation at the level of particular steps in the metabolic pathway (Cocaign-Bousquet et al. 2002), another efficient way of controlling the central carbon flux is regulation at the level of substrate transport. In Gram-positive bacteria, the presence of

glucose, a rapidly metabolised sugar, hampers the expression of catabolite operons (Titgemeyer and Hillen 2002). This "carbon catabolite repression" is mediated by the regulatory catabolite control protein A (CcpA) (Kowalczyk and Bardowski 2007). The CcpA protein interacts with Hpr–Ser–P and early glycolytic intermediates (Deutscher et al. 1995; Saier et al. 1996). This complex binds to catabolite responsive elements, *cre* sites, located in the vicinity of the regulated genes (Aung-Hilbrich et al. 2002; Jones et al. 1997; Kowalczyk et al. 2007; Ramseier et al. 1995).

Since *L. lactis* IL1403 has been intensively studied in many laboratories and its genome has been sequenced (Bolotin et al. 2001), it can be considered as a model for genetic and physiological studies of LAB. It is a lactose-negative, plasmid-free strain. The absence of plasmidic genes coding for the lactose PEP:PTS carrier system and for enzymes of the tagatose 6-phosphate pathway necessary for lactose uptake and catabolism prohibits this strain from growth on lactose. Nevertheless, the *L. lactis* IL1403 strain was found to be able to cleave lactose, provided that cellobiose is also present in the medium (Aleksandrzak et al. 2005). It was previously shown that the *lacG* mutant (P-β-galactosidase negative) of *L. lactis* MG5267 a derivative of lactose negative *L. lactis* MG1363 strain containing a single chromosomal copy of the *lac* operon could still ferment lactose slowly (Simons et al. 1993). This ability was due to a cryptic P-β-glycohydrolase activity of a new protein cross-reacting with anti-LacG antibodies. Synthesis of this protein and its enzymatic activity were induced in both MG5267 strain and its LacG-deficient mutant on cellobiose. As the level of P-β-glycohydrolase activity is the same for LacG-deficient strain grown on lactose and for the Lac- MG1363 grown on cellobiose it was proposed that P-β-glycohydrolase activity on lactose is conferred by a P-β-glucosidase. These results prompted us to speculate that lactose could be transported by the PEP:PTS system dedicated to cellobiose.

Another way of inducing the β-galactosidase positive phenotype in the *L. lactis* IL1403 strain is by disruption of the *ccpA* gene (Aleksandrzak et al. 2000, 2005). In contrast to the wild type strain, the *ccpA* mutant exhibits a β-galactosidase-positive phenotype without cellobiose in the growth medium. The CcpA protein was shown to be involved in the regulation of β-glucosides and lactose catabolism (Aleksandrzak et al. 2000, 2005).

The regulatory system involved in the metabolism of β-glucosides relies also on the *bglR* gene (Bardowski et al. 1995). The BglR protein acts as a transcriptional antiterminator, which prevents the RNA polymerase from early transcription arrest of the β-glucosides operon. The *bglR* mutant is not able to grow on β-glucosides like salicin, arbutin or esculin, but is still able to grow on cellobiose (Bardowski et al. 1994).

This study undertakes the comparison of growth profiles of different strains, the wild type IL1403 and three mutants, the *ccpA* and *bglR* single mutants and the *ccpA bglR* double mutant, on different carbon sources. It is complemented by transcriptional analysis of genes from the PEP:PTS regions considered, on the basis of genomic sequence (Bolotin et al. 2001), to play a role in β-glucosides (including cellobiose) transport in *L. lactis*. These results led us to propose a way of lactose and β-glucosides transport in *L. lactis* IL1403 taking into account the specificities and the regulations of the carriers.

Materials and methods

Strains and growth conditions

The lactococcal strains used were the wild type *L. lactis* subsp. *lactis* IL1403 strain and its derivatives: the *ccpA* mutant, IBB 550 (Aleksandrzak et al. 2000), the *bglR* mutant, IL 3990 (Bardowski et al. 1994) and the *ccpA bglR* double mutant, IBB 1500 (this work). The double mutant was obtained in a way similar to that used to construct the *bglR* single mutant (Bardowski et al. 1994), involving electroporation of the IBB 550 strain with plasmid pIL1140 containing the *bglR* gene truncated with the extra HindIII site. The recombinants with chromosomal *bglR* gene replaced by the truncated copy were tested for their Em^s phenotype and identified by HindIII digestion of the PCR-amplified *bglR*-containing DNA fragment. The bacteria were grown in a chemically defined medium (CDM) described by Otto et al. (1983) and modified by Poolman and Konings (1988). The CDM broth was supplemented with cellobiose, lactose, salicin or glucose.

Culture conditions

Fermentations were carried out in anaerobic conditions, under N_2, in 2 l fermentors (Setric Genie Industriel Toulouse, France) at 30°C and at agitation speed of 250 rpm. The pH was kept constant at pH 6.6 by automatic addition of 10 M KOH. The precultures used as the inoculum were grown in anaerobic conditions, in CDM with 1% glucose at 30°C, harvested during exponential phase and washed with KH_2PO_4 (18 g l^{-1}) K_2HPO_4 (15 g l^{-1}) buffer. The bioreactors were inoculated to an initial $A_{580} = 0.2$.

Fermentation analysis

Bacterial growth was controlled by absorbance at 580 nm and calibrated against cell dry weight measurements. One

absorbance unit equals 0.3 g of cell dry weight per liter (data not shown).

Sugar substrates and fermentation end-products, lactate, formate, acetate and ethanol, were determined by high-pressure liquid chromatography in samples taken during the entire fermentation process. The HPLC was performed using an Aminex HPX-87 H column (Biorad) at 36°C with 10 mM H_2SO_4 at a flow rate of 0.5 ml min^{-1}. Quantitative analysis was made by refractometry and UV absorbance at 210 nm.

RNA isolation and transcript labeling

A volume of culture (corresponding to 6 mg dry weight of cells) was harvested during exponential growth phase as previously described (Even et al. 2001). Cells were disrupted (three cycles of 1 min on vortex at maximal speed with 3 min cooling periods) with glass beads (0.6 g), 50 µl 10% SDS, 500 µl phenol (pH 4.7), and 170 µl macaloid (2% in TE pH 8) used as RNase inhibitor. After centrifugation (4°C, 30 min at 13,000 rpm), the aqueous phase was used for RNA purification with RNEasy Midi kit (Qiagen). Following precipitation with ethanol the RNA was quantified and labeled (Fontaine et al. 2001).

Primer design and PCR amplification

Genes *ybhE*, *celB*, *bglS*, *rheB*, *yebF*, *ptcB*, *ptcA*, *ptcC*, *bglA*, *bglR*, *ptbA*, *bglH*, *yleE*, *yidB*, *yedE*, *yedF* from *L. lactis* IL1403 were amplified by PCR in a 100-µl reaction volume, with 1 U of Taq polymerase (Polgen), 200 µM deoxynucleoside triphosphates and 1 µM specific primers (Table 1). The PCR mixture was incubated for 45 cycles at 94°C for 30 s, 56°C for 30 s, and 70°C for 1 min, with a final cycle of 70°C for 7 min. The PCR products were purified by electrophoresis on 1.5% agarose gels and isolated from gels using the JETsorb gel extraction kit (Genomed).

Preparation of macroarrays

Approximately 20 ng of purified PCR product were blotted with a robotic spotter (Eurogridder, Eurogentec, Belgium) on a positively charged nylon membrane as described previously (Fontaine et al. 2001).

Hybridization, array detection and analysis

All hybridisation and detection steps with DDAO-phosphate as the alkaline phosphatase substrate were performed as described by Fontaine et al. (2001). The membranes were scanned at different time intervals with a phosphofluoroimager (Storm 860, Molecular Dynamics) at 50 µm pixel resolution. Images were stored electronically and analysed with ImageQuant version 5.1 analysis software (Molecular Dynamics). Since the amount of total RNA was always the same, the alkaline phosphatase activity corresponded to the abundance of the specific messenger in the total RNA population. Standard deviation was estimated to be 24%.

Results

Growth and kinetic characteristics of the *L. lactis* IL1403 strain and its *ccpA* and *bglR* mutants

The wild type *L. lactis* IL1403 strain and its *ccpA* and *bglR* single mutants as well as the *ccpA bglR* double mutant were cultivated in fermentors, in anaerobic conditions in the CDM broth supplemented with different carbon sources, lactose, cellobiose, glucose and salicin. Since the wild type IL1403 strain and the *bglR* mutant are lactose-negative, they were not cultivated in fermentors in the CDM with lactose. The growth profiles obtained are presented in Fig. 1. It was observed that the *ccpA* mutant was able to grow on lactose as the only carbon source (Fig. 1a), confirming that the *ccpA* gene mutation renders the IL1403 cells lactose-positive. All tested strains grew equally well on cellobiose (Fig. 1b), demonstrating that neither the *ccpA* nor the *bglR* mutation affect the cellobiose catabolism. In the presence of both sugars, cellobiose and lactose (Fig. 2), all strains demonstrated diauxic growth, indicating their ability to grow on both carbon sources. Since in the wild type *L. lactis* IL1403 strain cellobiose was previously assumed to induce lactose catabolism (Aleksandrzak et al. 2000, 2005), the CDM supplemented with glucose and lactose was used to confirm the inducible role of cellobiose (Fig. 1c). It was found that IL1403 could grow only on glucose, whereas the *ccpA* mutant grew on both sugars glucose and lactose (Fig. 1c). These observations indicated that glucose could not induce lactose catabolism in the wild type strain. Since the *ccpA* mutant was able to grow on lactose as the only carbon source, its growth in CDM with glucose and lactose was not the effect of glucose induction.

On the basis of growth measurements, the maximal specific growth rates of *L. lactis* IL1403 and its derivatives on CDM with the different sugar content were calculated (Table 2).

In order to precisely analyse the consumption of each sugar in mixed-substrate conditions and to observe the effect of cellobiose on the induction of lactose consumption,

Table 1 Oligonucleotide primers in PCR reactions

Gene	Homology	Oligonucleotide	Gene coordinates (bp)	Reference[a]
ybhE	hypothetical surface protein	5′- AGCGTGTCTTCACTTCT -3′	176338–177432	L176316
		5′- CCATAGCCTTCGTTGTC -3′		
celB	Cellobiose-specific PTS IIC component	5′- GCACCAGAGAAAGTCTGG -3′	177530–179014	L177520
		5′- GTACCACCTGATCCACC -3′		
bglS	β-glucosidase	5′- GGATGTCGCTGTTGATC -3′	179668–181104	L179659
		5′- CTCAAGCCTTACGGGTC -3′		
rheB	RNA helicase	5′- CATCACAACCACATGTCG -3′	415804–417144	L0340
		5′- GCACGTTTGTAACCTGG -3′		
yebF	hypothetical transcriptional regulator	5′- CACCTGAAGAAGTCCAAC -3′	417888–418634	L17893
		5′- CGGATACTGTTTGGACC -3′		
ptcB	Cellobiose-specific PTS IIB component	5′- GCACTTGCATGTGCAGCC -3′	418876–419202	L18872
		5′- CTCCGCGCATCATTCCA -3′		
ptcA	Cellobiose-specific PTS IIA component	5′- GACTACATGGGTGTTGTAATGG -3′	419287–419637	L19292
		5′- GGTAATCGCATTCATTAAGTGG -3′		
ptcC	Cellobiose-specific PTS IIC component	5′- TGGGTTCGAGATGCAGG -3′	420905–422074	L20847
		5′- CCACCTACAAGGTATCC -3′		
bglA	P-β-glucosidase	5′- GAGGAGCAGTTGCTGCAC -3′	422156–423592	L22116
		5′- GCGAACATGAACATCTGC -3′		
bglR	β-glucoside operon antiterminator	5′-GTTCTGAACAATAATGTAGTCATTGCTC -3′	1492982–1493791	L0154
		5′-CTTCGTACGAACTTGTCT -3′		
ptbA	β-glucoside-specific PTS IIABC component	5′-GTGCAGATGTAGATGCTG -3′	1490930–1492840	L90678
		5′-GGAGCACGAACCTCACC -3′		
bglH	β-glucosidase	5′-GGCAATTCTTGCAAGTCCTGA -3′	1489446–1490882	L89194
		5′-CTTGTATCGTTCCGTCTTCTTG -3′		
yleE	β-glucoside-specific PTS IIABC component	5′-CGTGTTGTGACAACTCCAG -3′	1146884–1147471	L146642
		5′-GTGATAACCACAGGTG -3′		
yidB	Cellobiose-specific PTS IIC component	5′-CACGAGCGATGACTGC -3′	831369–832787	L31294
		5′-CCACCAAATGAGCCGCCTA -3′		
yedE	hypothetical protein (homolog II C component)	5′-GGGTGATGGTTTTGC -3′	437338–437823	L37338
		5′-CGTGCATTGGCTGAACCA -3′		
yedF	β-glucoside-specific PTS IIABC component	5′-GGCACAAGCAGTCGCTGT -3′	437921–439525	L37906
		5′-CTTGGCAAAGCAACGG -3′		

[a] Bolotin et al. 2001

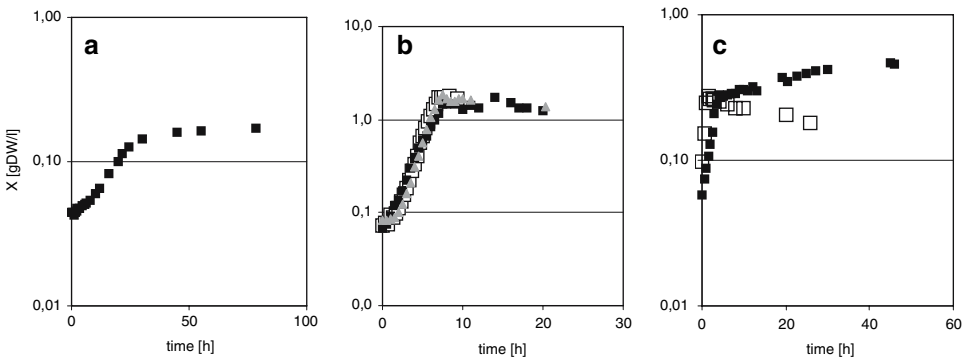

Fig. 1 Growth profiles (in g biomass l^{-1}) of (*open square*) the wild type *L. lactis* IL1403 strain and its single mutants (*filled square*) *ccpA* and (*filled triangle*) *bglR*, cultivated in a chemically defined medium (CDM) with different carbon sources. **a** 1% lactose, **b** 1% cellobiose, **c** 0.1% glucose + 1% lactose

two cellobiose concentrations were tested in the presence of lactose. The growth rates during exponential phase in the CDM with cellobiose were quite similar for the wild type IL1403 strain and the *bglR* mutant, while the *ccpA* single and *ccpA bglR* double mutants demonstrated lower maximal specific growth rates (Table 2).

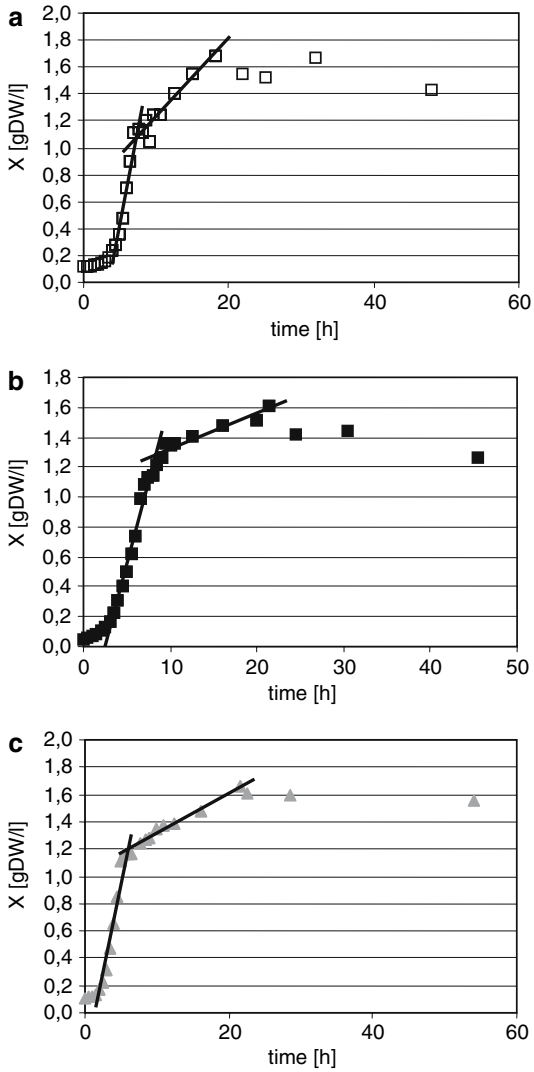

Fig. 2 Diauxic growth profiles (in g biomass l^{-1}) of **a** the wild type *L. lactis* IL1403 strain and its single mutants, **b** *ccpA* and **c** *bglR*, cultivated in a chemically defined medium (CDM) with 0.5% cellobiose + 1% lactose

Growth rates of all four strains cultivated on cellobiose and lactose were similar to those on cellobiose alone, except for the wild type strain grown on lactose in the presence of 0.1% cellobiose. In the latter case, the growth rate was lower compared to that on 1% cellobiose alone. In the medium with both cellobiose and lactose, all strains grew very slowly on lactose with growth rates lower than 0.1 h^{-1}. A similar growth rate, less than 0.1 h^{-1}, was also observed for the *ccpA* mutant growing on lactose as the only carbon source. For the wild type strain, the growth rate on glucose was higher than that on cellobiose, while for the *ccpA* mutant these growth rates were very similar. While the *bglR* mutant was unable to grow on salicin, the wild type strain, the *ccpA* mutant and the *ccpA bglR* double mutant grew on this β-glucoside, although at different growth rates of 0.08, 0.28 and 0.25 h^{-1}, respectively.

To characterise changes in substrate and product concentrations during fermentation, culture supernatants were analysed with HPLC. These experiments showed that both sugars, cellobiose and lactose, are metabolised by each of the three strains tested, the wild type IL1403 and its *ccpA* and *bglR* single mutants (Fig. 3). Three phases can be distinguished in sugar consumption in each of these strains. In the first phase, only cellobiose is fermented. In the second phase, both sugars are co-consumed. In the last phase, lactose alone continues to be consumed after cellobiose exhaustion. Regardless of the strain used and the consumed sugar, more than 80% of carbon sources were recovered as lactate, while carbon recovery was within the range of 91–96%. Other products such as formate, acetate and ethanol were only found in minor quantities.

Next, for each cellobiose and lactose, the maximal specific rates, q_{max}, of substrate consumption were calculated and found to be similar for all the tested strains (Table 3).

The specific rates of lactose consumption for the *ccpA* mutant strain grown on lactose alone and on lactose

Table 2 Maximal specific growth rates (in h^{-1}) of *L. lactis* IL1403 and its derivatives in chemically defined medium (CDM) with various carbon sources

Strain	IL 1403 Wild type	IBB 550 *ccpA*	IL 3990 *bglR*	IBB 1500 *ccpA bglR*
Carbon source				
Cel 1%	0.59 ± 0.04	0.46 ± 0.02	0.63 ± 0.05	0.48 ± 0.02
Lac 1%	–	0.05 ± 0.01	–	ND
Cel 1%	0.60 ± 0.04	0.49 ± 0.02	0.63 ± 0.03	ND
Lac 1%	0.04 ± 0.01	ND	ND	ND
Cel 0.1%	0.49 ± 0.02	0.45 ± 0.02	0.59 ± 0.03	0.49 ± 0.02
Lac 1%	0.04 ± 0.01	0.08 ± 0.02	0.04 ± 0.01	0.06 ± 0.01
Glu 0.1%	0.94 ± 0.06	0.41 ± 0.02	ND	ND
Lac 1%	–	0.02 ± 0.01	ND	ND
Sal 1%	0.08 ± 0.02	0.28 ± 0.03	–	0.25 ± 0.03

Cel Cellobiose, *Lac* Lactose, *Glu* Glucose, *Sal* Salicin, *ND* Not determined, – no growth

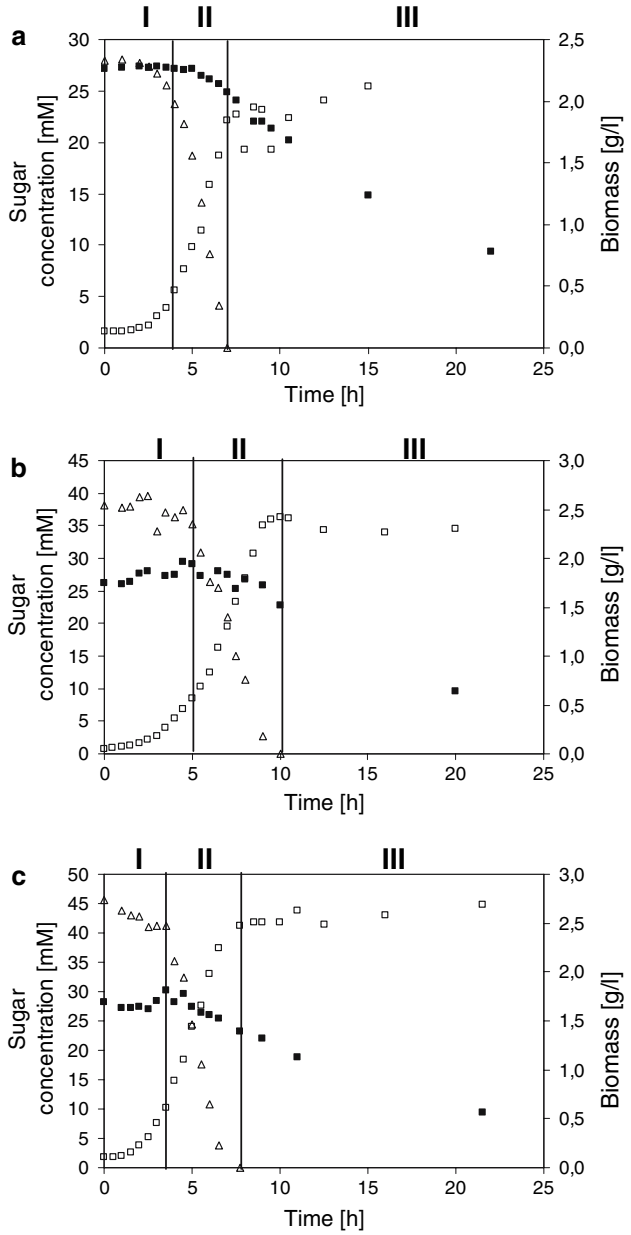

Fig. 3 Fermentation profiles of *L. lactis* IL1403 and its single mutants *ccpA* and *bglR* cultivated in a chemically defined medium (CDM) with cellobiose and lactose. **a** IL1403 on 1% cellobiose + 1% lactose, **b** IBB 550 (*ccpA* mutant) on 1.5% cellobiose + 1% lactose, **c** IL3990 (*bglR* mutant) on 1.5% cellobiose + 1% lactose. (*open triangle*) cellobiose, (*filled square*) lactose, (*open square*) biomass (g DW l^{-1})

together with cellobiose, were close to 0.5 mmol g^{-1} h^{-1}. The maximal specific rates of cellobiose consumption were to some extent dependent on the cellobiose concentration, reaching the value of 8 mmol g^{-1} h^{-1} in the CDM with 1% cellobiose, with lower values of about 5.5 mmol g^{-1} h^{-1} on 0.1% cellobiose.

Transcriptional analysis of the genes from PEP:PTS regions

The abundance of transcripts corresponding to genes of the PEP:PTS regions (Fig. 4) was estimated using DNA macroarrays in exponentially growing cells. On cellobiose, four genes, *ybhE*, *celB*, *ptcB* and *ptcA* were expressed at higher levels than on glucose exceeding the relative transcript abundance of 1.5.

The relative transcript abundance in IL1403 and its *ccpA* and *bglR* single mutants cultivated on cellobiose and glucose was also determined (Fig. 5a, b). During growth on cellobiose, the transcript abundance of *ybhE*, *celB*, *rheB*, *ptcB*, *ptcA*, *yedE* and *yedF* increased in the *ccpA* mutant compared with the wild type, while the *bglR* mutation caused a decrease in expression levels of nearly all genes (Fig. 5). Genes with increased transcription on cellobiose were also overexpressed in the *ccpA* mutant on glucose.

A general conclusion that can be drawn from this experiment is that during growth on cellobiose the expression of the cellobiose-specific PTS systems, encoded by *celB*, *ptcB* and *ptcA* genes and located in two separate DNA regions, is increased. These results also showed that cellobiose transport is repressed by the CcpA protein.

Discussion

As demonstrated in this work, the *L. lactis* IL1403 and all of its tested mutants catabolise glucose, lactose or β-glucoside substrates by the homofermentative pathway. While sugar catabolic pathways of *L. lactis* are well known, the carrier systems used to internalise β-glucosides are not fully established. In this work, the growth on cellobiose of the *bglR* mutant was shown to be unchanged compared to the wild type strain. However, it was found that the *bglR* mutant, but not the wild type, was unable to grow on salicin. This result, in agreement with previous observations (Bardowski et al. 1994), clearly indicates the presence of different transport systems for salicin and cellobiose. Interestingly, the double mutant, just like the *ccpA* mutant, grows even faster than the wild type strain on salicin at 0.25–0.28 h^{-1} compared to 0.08 h^{-1}, respectively, suggesting a regulation of salicin catabolism by CcpA.

It was previously observed that cellobiose can induce a lactose-positive phenotype in the lactose-negative *L. lactis* IL 1403 strain (Aleksandrzak et al. 2000, 2005). Reversion of Lac^- phenotype to a partial Lac^+ phenotype was also observed for other strains of *L. lactis* (Sinha 1991). This Lac^+ phenotype in revertants was accompanied by a slow lactose metabolism and was proposed to be mediated through unidentified chromosomal gene(s). *L. lactis* IL

Table 3 Maximal specific rates of cellobiose and lactose consumption (q_{max}) and lactate formation (r_{max}) during growth of *L. lactis* IL1403 and its derivatives on chemically defined medium (CDM) with cellobiose and lactose as carbon sources

Strain	Phenotype	Carbon source in CDM	$q_{max}\left(\frac{mmol}{h\,gDW}\right)$		$r_{max}\left(\frac{mmol}{h\,gDW}\right)$
			Cellobiose	Lactose	Lactate
IL 1403	wild type	cel 1–1.5%	8.73		25.94
		lac		0.53	1.92
		cel 0.1%	5.36		10.80
		lac		1.06	1.75
IBB 550 x	*ccpA*	cel 1–1.5%	7.53		20.98
		lac		0.64	1.75
		lac		0.49	2.45
IL 3990	*bglR*	cel 1–1.5%	8.07		30.87
		lac		0.53	1.37
		cel 0.1%	5.67		19.33
		lac		0.59	1.85
IL 1500	*ccpA bglR*	cel 1%	6.44		19.50
		cel 0.1%	6.53		20.31
		lac		1.09	4.56

Rates are expressed in mmol × (gram dry weight)$^{-1}$ h^{-1}, lactose was used in 1% concentration

q_{max} maximal specific rates of cellobiose and lactose consumption

r_{max} maximal specific rates of lactate formation

1403 is a Lac$^-$ strain, and this phenotype results from the lack of any lactose-specific PTS genes usually located on the "lactose plasmid". Furthermore, disruption of *lacS* coding for the only chromosomal lactose permease had no effect on IL1403 growth in lactose-CDM broth with cellobiose added at the inducible concentration (Aleksandrzak-Piekarczyk et al. 2005). These results suggest that lactose is transported in this strain by other chromosomally encoded system(s). Taking into account an inducible role of cellobiose, the good candidates for this function could be several β-glucoside-specific PTS systems present in IL1403 genome.

In the present work, it has been demonstrated that the IL1403 strain, grown in a lactose and cellobiose containing medium, is not only able to cleave lactose but also to metabolise it to lactic acid, confirming the inducible role of cellobiose in lactose consumption. Kinetic analysis of lactose and cellobiose consumption showed that after the first phase of co-metabolism of the two sugars, cellobiose is exhausted and the strain uses lactose as the only carbon source. This observation led us to conclude that under these conditions an unidentified transport system enables lactose to be taken up inside the *L. lactis* IL1403 cells. Moreover, this cellobiose-inducible lactose-positive phenotype was observed in the IL1403 wild type strain and its *ccpA* and *bglR* mutants.

Results from cellobiose indicate that this sugar is taken up by a carrier system different from that involved in the uptake of other β-glucosides, like salicin. The analysis of transcripts of genes putatively involved in cellobiose transport showed an important increase in the expression of *celB*, *ptcB* and *ptcA* in the wild type strain, grown on cellobiose in comparison to glucose. This observation suggests that these three genes are involved in cellobiose metabolism unlike other genes, *ptcC* and *yidB*, also annotated as cellobiose PTS genes, but being constantly expressed on cellobiose.

Analysis of the conditions enabling lactose uptake in the studied strains (IL1403 and its *ccpA* and *bglR* mutants) brought to light several important characteristics of lactose transport: (1) lactose metabolism was induced when expression of *celB*, *ptcB* and *ptcA* genes putatively involved in cellobiose transport was increased; this effect was observed for strains grown on cellobiose but not on other substrates like glucose or salicin; (2) the *ccpA* mutation, which results in an increased expression of all of the three genes, allowed the *ccpA* mutant to grow on lactose alone without cellobiose added to the medium; (3) for cellobiose, as well as for lactose, the maximal specific rates of consumption were very similar and low in all tested strains. Finally, it appeared that the regulation of lactose transport is identical to that of cellobiose. Our previous results showed that the only activity of the enzyme hydrolysing cellobiose as well as lactose was P-β-galactosidase activity (Aleksandrzak-Piekarczyk et al. 2005). This information suggest that the transport of sugar is probably concomitant with its phosphorylation which is characteristic for PEP:PTS transporters. According to the fact that we analysed all out of six β-glucoside-specific PEP:PTS systems present in *L. lactis* IL1403 genome we assumed that cellobiose and lactose are transported by the unique PEP:PTS system (*celB*, *ptcB* and *ptcA*). This hypothesis can be assured by the fact that the permease-β-galactosidase system plays a minor role in lactose internalisation in the *L. lactis* IL1403 and its *ccpA* mutant and the LacS function is limited to transport of X-Gal (Aleksandrzak-Piekarczyk et al. 2005). In addition disruption of *bglS* (encoding a P-β-glucosidase) directly proceeded by the *celB* leads to complete inability to grow in

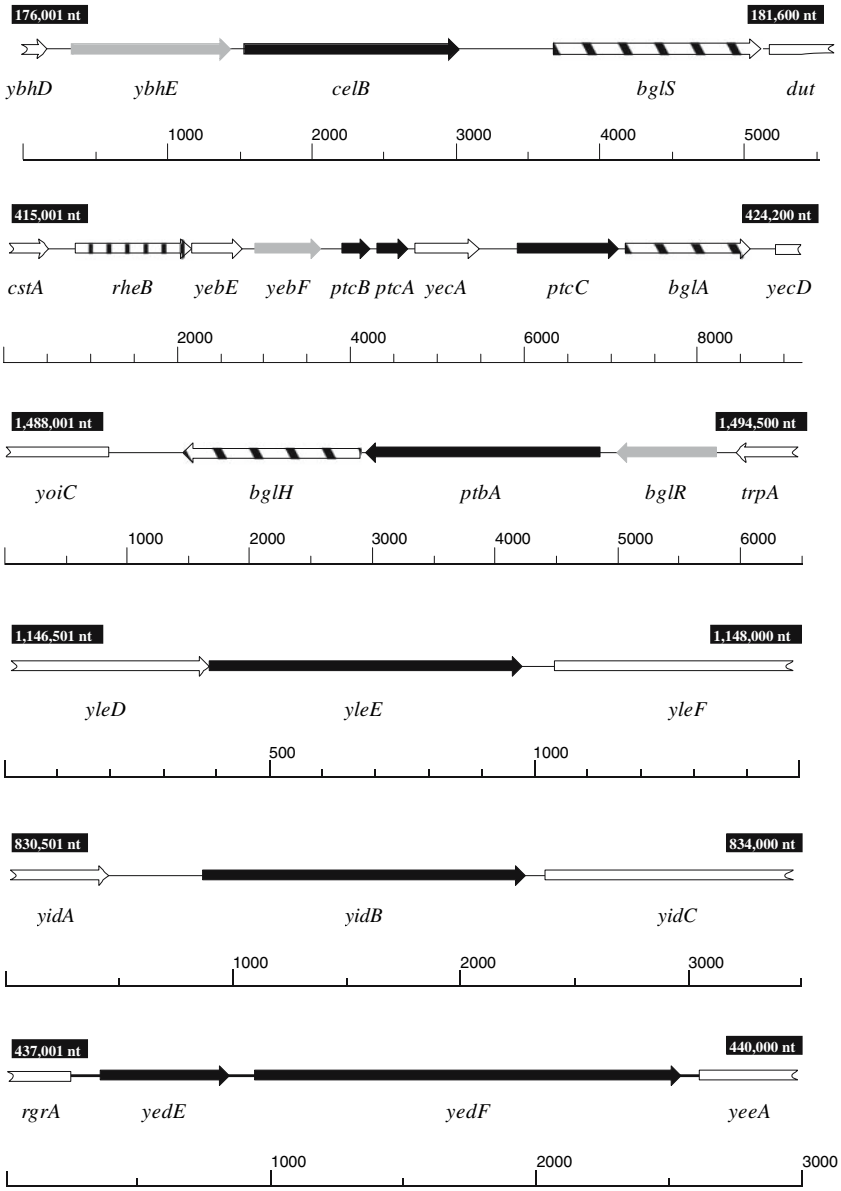

Fig. 4 Genetic organisation of the studied PEP:PTS regions. Region I of 5,600 bp, region II of 9,200 bp, region III of 6,500 bp, region IV of 1,500 bp, region V of 3500 bps, region VI of 3,000 bp. Sequences derived from the *L. lactis* IL1403 genome (AE005176). Genes encoding proteins homologous to the A, B and C components of the PEP:PTS system are denoted in *black* and those homologous to hypothetical regulators in *grey*

all lactose-containing media both in IL1403 and the *ccpA* mutant (Aleksandrzak-Piekarczyk et al. 2005). Very recently, Aleksandrzak-Piekarczyk et al. found that disruption of *celB* fully abolished the growth on cellobiose, lactose and lactose with cellobiose of both the IL1403 wild-type strain and its *ccpA* mutant. In addition a severe impairment or lack of growth was observed for *ptcB* and *ptcA* mutants grown in CDM with all of these substrates (Aleksandrzak-Piekarczyk, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, personal communication).

It is worth to mention that cellobiose and lactose are almost alike in molecular composition differing only in the orientation of the C4 hydroxyl group. Transport of such similar compounds by one carrier is quite common (Curtis and Epstein 1975) and has been also proposed in the case of cellobiose and lactose for *Listeria monocytogenes* (Dalet et al. 2003). Moreover, regardless of the strain, the specific consumption rate of cellobiose depends on cellobiose concentration, the consumption rate being lower for low substrate concentration. This observation could indicate low affinity of the cellobiose carrier for its cognate substrate (in the range of mM) or a lower expression of the respective genes as cellobiose induces this PEP:PTS causing a lower amount of carriers to be present. Therefore, a constant value of the specific cellobiose consumption rate observed for each strain for a given cellobiose concentration is another argument in favour of the presence of only one transport system for cellobiose.

Another interesting observation was that the *ccpA* mutation provoked a significant decrease of the growth rate of the *ccpA* mutant on glucose and cellobiose, compared to

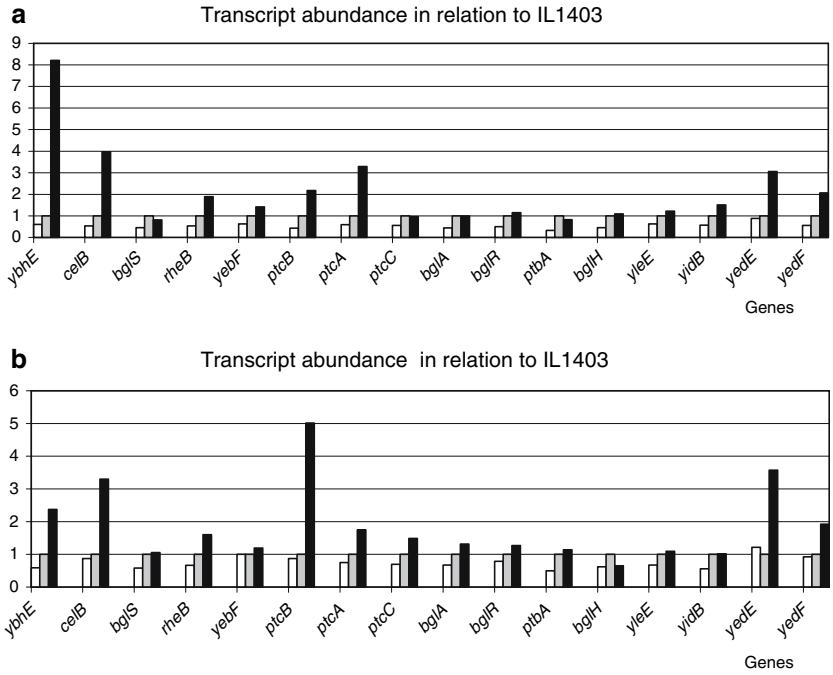

Fig. 5 The ratios of transcript abundance between the single mutants (*open square*) *bglR* and (*filled square*) *ccpA*, and (*shaded square*) the wild type *L. lactis* IL1403, in chemically defined medium (CDM) with **a** cellobiose or **b** glucose as carbon sources. All ratios represent averages of two independent determinations. Only ratios ≥1.5 or ≤0.7 are significant, taking into account the precision of the method (24%)

the wild type. The decreased growth rate resulting from the *ccpA* mutation was also reported for *Bacillus megaterium*, *Bacillus subtilis* and *L. lactis* MG1363 (Hueck et al. 1995; Wray et al. 1994; Luesink et al. 1998). However, the expression of *celB*, *ptcB* and *ptcA*, genes identified as cellobiose carriers, was significantly increased in the *ccpA* mutant compared to the wild type, on both glucose and cellobiose. The *ybhE* gene located in the regions of *celB* gene, showed a similar increase of the expression level, indicating a coordinated regulation of these different loci. Taking all of these results into account, a negative control of cellobiose PTS genes by CcpA was postulated. Furthermore, a direct control rather than an indirect one was assumed since *cre* sites upstream of the *ybhE–celB* and *ptcB–ptcA* loci were identified (Guédon et al. 2002; Kowalczyk and Bardowski, 2007). Since the growth rate on glucose and on cellobiose decreased in the *ccpA* mutant compared to the wild type despite the increase of expression of cellobiose PTS genes, a pleiotrophic negative effect of CcpA on the growth abilities was postulated. The separation of the growth effect from catabolite repression exerted by CcpA was established for the first time in *B. megaterium* (Küster et al. 1999).

In conclusion, it is proposed that the main cellobiose and lactose carrier is a PTS system (comprising the *celB*, *ptcB* and *ptcA* genes) inducible by the presence of cellobiose, negatively controlled by CcpA and differing from that operating for other *β*-glucosides (salicin) as it is not controlled by the BglR antiterminator.

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References

Aleksandrzak T, Kowalczyk M, Kok J, Bardowski J (2000) Regulation of carbon catabolism in *Lactococcus lactis*. In: Bielecki S, Tramper J, Polak J (eds) Food biotechnology, vol 17. Elsevier Science BV, Amsterdam, The Netherlands, pp 61–66

Aleksandrzak-Piekarczyk T, Kok J, Renault P, Bardowski J (2005) Alternative lactose catabolic pathway in *Lactococcus lactis*. Appl Environ Microbiol 71(10):6060–6069

Aung-Hilbrich LM, Seidel G, Wagner A, Hillen W (2002) Quantification of the influence of HprSer46P on CcpA-*cre* interaction. J Mol Biol 319:77–85

Bardowski J, Ehrlich SD, Chopin A (1994) BglR protein, which belongs to the BglG family of transcriptional antiterminators, is involved in *β*-glucoside utilization in *Lactococcus lactis*. J Bacteriol 176:5681–5685

Bardowski J, Ehrlich SD, Chopin A (1995) A protein, belonging to a family of RNA-binding transcriptional antiterminators, controls *β*-glucoside assimilation in *Lactococcus lactis*. Dev Biol Stand Basel Karger 85:555–558

Bolotin A, Wincker P, Mauger S, Jaillon O, Malarme K, Weissenbach J, Ehrlich SD, Sorokin A (2001) The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp *lactis* IL1403. Genome Res 11:731–753

Cocaign-Bousquet M, Garriques C, Loubiere P, Lindley ND (1996) Physiology of pyruvate metabolism in *Lactococcus lactis*. Antonie van Leeuwenhoek 70:253–267

Cocaign-Bousquet M, Even S, Loubiere P, Lindley ND (2002) Anaerobic sugar catabolism in *Lactococcus lactis*: genetic regulation and enzyme control over pathway flux. Appl Microbiol Biotechnol 60:24–32

Curtis SJ, Epstein W (1975) Phosphorylation of D-glucose in *Escherichia coli* mutants defective in glucose phosphotransferase, mannose phosphotransferase, and glucokinase. J Bacteriol 122:1189–1199

Dalet K, Arous S, Cenatiempo Y, Hechard Y (2003) Characterization of a unique sigma54-dependent PTS operon of the lactose family in *Listeria monocytogenes*. Biochimie 85(7):633–638

Deutscher J, Küster E, Bergstedt U, Charrier V, Hillen W (1995) Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria. Mol Microbiol 15:1049–1053

Even S, Lindley ND, Cocaign-Bousquet M (2001) Molecular physiology of sugar catabolism in *Lactococcus lactis* IL1403. J Bacteriol 183:3817–3824

Fontaine L, Even S, Soucaille P, Lindley ND, Cocaign-Bousquet M (2001) Transcript quantification based on chemical labeling of RNA associated with fluorescent detection. Anal Biochem 298:246–252

Garriques C, Loubiere P, Lindley ND, Cocaign-Bousquet M (1997) Control of the shift from homolactic acid to mixed-acid fermentation in *Lactococcus lactis*: predominant role of the NADH/NAD^+ ratio. J Bacteriol 179:5282–5287

Hueck CJ, Kraus A, Schmiedel D, Hillen W (1995) Cloning, expression and functional analyses of the catabolite control protein CcpA from *Bacillus subtilis*. Mol Microbiol 16(5):855–864

Jones BE, Dossonnet V, Küster E, Hillen W, Deutscher J, Klevit RE (1997) Binding of the catabolite repressor protein CcpA to its DNA target is regulated by phosphorylation of its corepressor HPr. J Biol Chem 272:26530–26535

Kowalczyk M, Bardowski J (2007) Regulation of sugar catabolism in *Lactococcus lactis*. Crit Rev Microbiol 33:1–13

Kowalczyk M, Borcz B, Plochocka D, Bardowski J (2007) In vitro DNA binding of purified CcpA protein from *Lactococcus lactis* IL1403. Acta Biochim Pol 54(1):71–78

Küster E, Hilbich T, Dahl MK, Hillen W (1999) Mutations in catabolite control protein CcpA separating growth effects from catabolite repression. J Bacteriol 181(13):4125–4128

Luesink EJ, van Herpen RE, Grossiord BP, Kuipers OP, de Vos WM (1998) 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. Mol Microbiol 30(4):789–798

Otto R, ten Brink T, Veldkamp H, Konings WN (1983) The relation between growth rate and electrochemical proton gradient of *Streptococcus cremoris*. FEMS Microbiol Lett 16:69–74

Poolman B, Konings WN (1988) Relation of growth of *Streptococcus lactis* and *Streptococcus cremoris* to amino acid transport. J Bacteriol 170:700–707

Ramseier TM, Reizer J, Küster E, Hillen W, Saier MH Jr (1995) *In vitro* binding of the CcpA protein of *Bacillus megaterium* to *cis*-acting catabolite responsive elements (CREs) of Gram-positive bacteria. FEMS Microbiol Lett 129:207–214

Saier MH Jr, Chauvaux S, Cook GM, Deutscher J, Paulsen IT, Reizer J, Ye JJ (1996) Catabolite repression and inducer control in gram-positive bacteria. Microbiology 142:217–230

Simons G, Nijhuis M, de Vos WM (1993) Integration and gene replacement in the *Lactococcus lactis lac* operon: induction of a cryptic phospho-beta-glucosidase in LacG-deficient strains. J Bacteriol 175(16):5168–5175

Sinha RP (1991) Genetic characterization of partial lactose-fermenting revertants from lactose-negative mutants of lactococci. Can J Microbiol 37:281–286

Titgemeyer F, Hillen W (2002) Global control of sugar metabolism: a Gram-positive solution. Antonie van Leeuwenhoek 82:59–71

Wray LV Jr, Pettengill FK, Fisher SH (1994) Catabolite repression of the *Bacillus subtilis hut* operon requires a cis-acting site located downstream of the transcription initiation site. J Bacteriol 176(7):1894–1902