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ClaR – a novel key regulator of cellobiose and lactose metabolism in *Lactococcus lactis* IL1403.

TAMARA ALEKSANDRZAK-PIEKARCZYK*, LIDIA STASIAK-RÓŻAŃSKA†, JAROSŁAW CIEŚLA AND JACEK BARDOWSKI

Institute of Biochemistry and Biophysics, Polish Academy of Sciences (IBB PAS), Pawińskiego 5a, 02-106 Warsaw, Poland

†Present address: Department of Biotechnology, Microbiology and Food Evaluation, Faculty of Food Sciences, Warsaw University of Life Sciences, Nowoursynowska 159c, 02-776 Warsaw, Poland

*Corresponding author. Mailing address: IBB PAS, Pawińskiego 5a, 02-106 Warsaw, Poland, Phone: (4822) 592 1213, Fax: (4822) 658 4636, E-mail: tamara@ibb.waw.pl

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Abstract

In a number of previous studies, our group has discovered an alternative pathway for lactose utilization in *Lactococcus lactis* that, in addition to a sugar-hydrolyzing enzyme with both P-β-glucosidase and P-β-galactosidase activity (BglS), engages chromosomally-encoded components of cellobiose-specific PTS ($PTS^{Cel\text{-}Lac}$), including PtcA, PtcB and CelB. In this report, we show that this system undergoes regulation via ClaR, a novel activator protein from the RpiR family of transcriptional regulators. Although RpiR proteins are widely distributed among lactic acid bacteria, their roles have yet to be confirmed by functional assays. Here we show that ClaR activity depends on intracellular cellobiose-6-phosphate availability, while other sugars such as glucose or galactose have no influence on it. We also show that ClaR is crucial for activation of the *bglS* and *celB* expression in the presence of cellobiose, with some limited effects on *ptcA* and *ptcB* activation. Among 190 of carbon sources tested, the deletion of *claR* reduces *L. lactis* growth only in lactose- and/or cellobiose-containing media, suggesting a narrow specificity of this regulator within the context of sugar metabolism.

Introduction

Lactic acid bacteria (LAB), including *Lactococcus lactis*, are the focus of intensive research within the field of carbohydrate catabolism and its regulation, important during industrial fermentation processes (Mayo et al. 2010). The most prevalent pathway for carbohydrate uptake in LAB is the phosphoenolpyruvate-phosphotransferase system (PEP-PTS), which links sugar transport with phosphorylation of incoming sugars. Phosphoenolpyruvate serves as the first phosphoryl donor to Enzyme I, which utilizes a complex phosphoryltransfer cascade to phosphorylate sugars entering bacteria via the transmembrane protein - EIIC (Lorca et al. 2010). Among a wide array of carbohydrates that can be internalized via PTS, in LAB these systems are best known for efficient transport of milk (lactose) and plant (β-glucosides) sugars, which are highly important for industrial fermentation processes involving LAB species.

Due to the economic importance of lactose fermentation, the metabolism of this sugar is studied extensively in *L. lactis*. These studies mostly focus on two pathways: (i) plasmid-localized lactose-specific PTS (PTS^{Lac}) and (ii) the chromosomally encoded lactose permease-*β*-galactosidase system (Aleksandrzak-Piekarczyk 2013). The latter system transports lactose in an unphosphorylated form that enables its subsequent cleavage by *β*-galactosidase. Recently, we reported on the discovery of a third, alternative lactose uptake system that we found to be operative in *L. lactis* IL1403 (Aleksandrzak-Piekarczyk et al. 2011). This novel lactose utilization pathway engages chromosomally-encoded components of a cellobiose-specific PTS ($PTS^{Cel\text{-}Lac}$). The proteins of $PTS^{Cel\text{-}Lac}$ are encoded in two distinct regions that encompass *ptcA*, *ptcB* (one region) and *celB* (second region), which code for EIIA, EIIB and EIIC, respectively (Aleksandrzak-Piekarczyk et al. 2011). The latter region also contains the *bglS* gene, which

encodes a P-sugar hydrolyzing enzyme with both P-β-glucosidase and P-β-galactosidase activity. This BglS enzyme has been shown to promote the cleavage of cellobiose and lactose internalized via PTS$^{Cel\text{-}Lac}$ in *L. lactis* IL1403 (Aleksandrzak-Piekarczyk et al. 2005). Therefore, PtcAB, CelB and BglS form a complete system specific for cellobiose and lactose uptake and hydrolysis in *L. lactis* IL1403. We surmise that the existence of PTS$^{Cel\text{-}Lac}$ is not limited to *L. lactis* IL1403 and may occur commonly among other lactococcal strains (Aleksandrzak-Piekarczyk et al. 2011). This was confirmed by the later study of Solopova et al. (Solopova et al. 2012) in *L. lactis* MG1363. In this bacterium the alternative lactose metabolism pathway, despite being differently induced (by lactose or cellobiose), relies on the same PTS$^{Cel\text{-}Lac}$ components used by *L. lactis* IL1403.

The efficiency of transport and subsequent metabolism of incoming sugars is tightly controlled by several regulatory proteins, which form the regulatory network necessary for sensing environmental conditions and setting catabolic capacities of the cell. Guédon et al. (Guédon et al. 2002) distinguish two groups of regulators - general and secondary regulators. The main example of such a general regulator is catabolite control protein A (CcpA) (Hueck and Hillen 1995). CcpA is a well-conserved protein operating in many low-GC Gram-positive bacteria and, together with its corepressor Ser-P-HPr, it binds to 14-nucleotide *cis*-acting DNA target sites known as *cre* (catabolite responsive elements), promoting carbon catabolite activation (CCA) or repression (CCR) (Weickert and Chambliss 1990). In *L. lactis* strains, CcpA has been shown to repress different genes associated with uptake of β-glucosides, fructose, galactose or lactose, while activating the glycolytic *las* operon (Aleksandrzak-Piekarczyk et al. 2011; Aleksandrzak-Piekarczyk et al. 2005; Barrière et al. 2005; Luesink et al. 1998; Monedero et al. 2001).

In addition to global regulators, carbon catabolism might also be controlled by specific and local secondary regulators belonging to different protein families such as LacI, LysR, AraC, GntR, DeoR, RpiR or BglG, which are widely distributed among LAB. Regulators from some of these families have been shown to control genes encoding utilization of α-galactosides, β-glucosides, fructose, lactose, maltose, sorbose, sucrose and xylose in lactococci (Bardowski et al. 1994; Boucher et al. 2003; Mayo et al. 2010).

The YebF protein (now designated ClaR – cellobiose-lactose regulatory protein) from *L. lactis* IL1403, belongs to the RpiR family of regulators. In bacterial genomes, there are many genes that encode potential regulators belonging to the RpiR family (http://www.genome.jp/kegg/ssdb/). However, none of these genes have thus far been shown to play a role in carbohydrate catabolism among LAB. We previously suggested the probable involvement of ClaR in the activation of *bglS*, *celB*, *ptcA* and *ptcB*, genes, which encode components of the novel and the only pathway for lactose utilization in *L. lactis* IL1403 (Aleksandrzak-Piekarczyk et al. 2011; Aleksandrzak-Piekarczyk et al. 2005). It has been demonstrated that the expression of these genes is tightly regulated by the general catabolite repression system, whereas *bglS* and *celB* also require the presence of cellobiose to be fully induced (Aleksandrzak-Piekarczyk et al. 2011).

In this report, we show that in *L. lactis* IL1403 genes encoding the $PTS^{Cel\text{-}Lac}$ components in the presence of cellobiose are regulated via a novel activator ClaR. Moreover, global phenotypic analysis via phenotype microarray suggests that ClaR is a specific regulator indispensable for cellobiose and lactose metabolism.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* cells were grown in Luria-Bertani (LB) medium (Wood 1983) at 37°C, while *L. lactis* was cultivated in M17 glucose medium (G-M17) (Terzaghi and Sandine 1975) or in chemically defined medium (CDM) (Sissler et al. 1999) supplemented with 1% glucose (G-CDM), 1% cellobiose (C-CDM), 1% lactose (L-CDM), 1% galactose (Gal-CDM), 1% arbutin (A-CDM), 1% galactose with 1% cellobiose (GalC-CDM) or 1% lactose together with an inducing (0.01%) concentration of cellobiose (LC-CDM). When required, ampicillin (Amp; 100 µg ml^{-1} for *E. coli*) or erythromycin (Em; 100 µg ml^{-1} for *E. coli* and 5 µg ml^{-1} for *L. lactis*) were added to the medium. Solidified media contained 1.5% agar and, when necessary, IPTG (isopropyl *β*-D-thiogalactopyranoside; 1 mM for *E. coli*) and X-gal (5-bromo-4-chloro-3-indodyl-*β*-D-galactopyranoside; 50 µg ml^{-1} for *E. coli*).

Construction of the *claR* deletion mutants and complementing plasmid. The mutant was created by double crossover between pGhost9 harboring DNA fragments overlapping the *claR* gene and the chromosomal region containing these DNA fragments. The overlapping DNA fragments were amplified using the appropriate forward and reverse primer pairs (Table 1). The *claR* upstream region was amplified with the primers *claR*PstI/*claR*ERpr (Table1) using ExTaq polymerase and was cloned as a *Pst*I/*Eco*RI fragment into the corresponding sites in pGhost9. The *claR* downstream region was amplified using the primers *claR*ERrv/*claR*SalI (Table 1) and was cloned as an *Eco*RI/*Sal*I fragment into the vector carrying the upstream fragment, resulting in the pGhost9Δ*claR* plasmid. The deletion plasmid was introduced into *L. lactis* IL1403 and IL1403Δ*ccpA*. Homologous recombination was enforced by 10^{-3} dilution in fresh G-M17-Em medium of the overnight culture of the

lactococcal strains harboring pGhost9Δ*claR*. Diluted cultures were incubated for 2.5 h in G-M17 at non-permissive temperature (38°C). Integrants containing pGhost9Δ*claR* in the chromosome were selected at 38°C on G-M17 agar plates containing erythromycin. Excision from the chromosome and removing of the integration vector from *L. lactis* was performed by growth of integrants in the absence of antibiotic for at least 100 generations at the permissive temperature of 28°C. The genetic structure of the resulting *claR* deletion strains, *L. lactis* IL1403Δ*claR*, was confirmed by colony PCR, determination of the strain sensitivity to Em and sequencing of the DNA region containing the deleted *claR* gene.

In order to complement the *claR* deletion, the *claR* gene with its putative promoter region was amplified using *claR*Smaf and *claR*PstR primers (Table 1), cloned into pGEM-T and transferred into *E. coli* TG1. The resultant plasmid DNA was isolated, digested with *Pst*I and *Sma*I, ligated to pIL253 digested with the same restriction enzymes and transferred into *L. lactis* IL1403Δ*claR* giving rise to the IL1403Δ*claR*pIL253*claR* strain.

Cloning and activity measurement of the putative intrinsic terminator following the *yebE* gene. A DNA sequence resembling a rho-independent terminator downstream of *yebE* was amplified using *yebE*terf and *yebE*terr primers (Table 1). Further cloning procedures into pGBT58 bearing the *xylE* gene that encodes catechol 2,3-dioxygenase (Table 1) and measurements of its expression determined by enzymatic assay were performed as described previously (Aleksandrzak-Piekarczyk et al. 2011). Its activity was determined in three independent experiments. As a reference point, measurements of catechol 2,3-dioxygenase activity were also performed in the wild-type pGBT58 vector, which contains no terminator located between the *xylE* gene and its promoter.

Cloning of putative *claR* promoter region upstream of the *luxAB* genes. Construct consisting of pGEM-T, pJIM2374 and a non-coding region (amplified with *yebE*terf and *yebE*terr), containing a putative promoter of *claR* controlling the *luxAB* genes, was prepared as described previously (Aleksandrzak-Piekarczyk et al. 2011). Since pGEM-T is non-replicative in Gram-positive bacteria and pJIM2374 does not contain the *rep* gene, the recombinant pJIM2374*claRp* plasmid was transferred into *L. lactis* LL302, which encodes the RepA protein in its chromosome. The existence of a functional promoter in the cloned region was concluded from luciferase activity measurements performed in C-CDM.

Relative quantification of the gene expression by real-time quantitative PCR (RT-qPCR). For RT-qPCR total RNA was isolated with the use of TRI Reagent (Sigma) according to the instructions of the manufacturer, from 25 ml of the IL1403 wild-type strain and its mutants (IL1403Δ*ccpA* and IL1403Δ*claR*) cultures harvested in mid-exponential phase (OD_{600}=0.6). Cells were grown in G-CDM, C-CDM, Gal-CDM, A-CDM or GalC-CDM. RNA was extracted from at least three independent cultures.

First-strand cDNA was synthesized from DNAse I (Sigma)-treated 2 µg RNA samples. The synthesis of cDNA was performed by the use of the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer instructions.

Real-time quantitative PCR assays were carried out on the 7500 Real Time PCR System (Applied Biosystems). Each reaction was carried out in reaction mixture containing: 1x concentrated commercial buffer supplied with polymerase (Metabion), 0.625 U Taq polymerase (Metabion), 4 mM $MgCl_2$, 30000x diluted SYBR Green (Sigma), 5% DMSO, 0.5 ng/µl acetylated BSA (Sigma), 0.8% glycerol (ROTH), 400 µM dNTP mixture (Metabion), forward and reverse specific primers (400 µM each), cDNA template, and water up to 25 µl of

final volume. Reactions were performed with an initial denaturation step (95ºC for 3 min) followed by 45 cycles of denaturation (95ºC for 15 s) and primer annealing-extension (60ºC for 1 min). Fluorescence was read during the annealing-extension step of each cycle. After cycling, melting-point temperature analysis was performed in the range of 60ºC to 95ºC with temperature increments of 0.33ºC. In each experiment background range was adjusted automatically and the threshold for C_t evaluation was adjusted manually. For each cDNA sample six reactions were carried out using three template amounts, each in duplicate. For each gene studied the amounts of cDNA were chosen individually (if possible the same for all genes) to obtain C_t values in range between 14 and 34 cycles. Quality of results were evaluated based on expected C_t differences among three cDNA amounts as well as product melting curves. Rare outlying results were omitted in calculations. The results were normalized by the use of *L. lactis* the reference *tuf* and *purM* genes, which code for elongation factor TU and phosphoribosylaminoimidazole synthetase, respectively. Specific primers for each gene (Table 1) were designed using Primer Express software (Applied Biosystems). Before use, the primers were verified for equal efficiency of PCR reaction. The resulting data were processed using files exported from the qPCR cycler program and imported into Excel sheets to facilitate calculation of expression ratios between target and reference genes. These ratios were calculated by ΔC_t method using geometric mean of reference genes C_ts in each experiment separately.

Phenotypic testing of carbon source utilization. The study of phenotypic changes in mutant cells compared to parental strains was carried out through growth tests and analysis of fermentation patterns.

188 Growth tests were performed on a Microbiology Reader Analyser, Bioscreen C
189 (Labsystems) in 0.2 ml of CDM supplemented with the required sugars. Changes in the OD_{600}
190 of the bacterial cultures were recorded every 30 min of growth up to 100 h.
191 The fermentation patterns of 49 sugars were determined using the API 50CH test as
192 specified by the manufacturer (BioMérieux), and recorded after 12, 24 and 36 h of incubation
193 under aerobic or anaerobic conditions at 30°C.
194 Further metabolic profiles were measured globally by the Phenotype MicroArrays
195 system (Biolog, USA) according to manufacturer's instructions. *L. lactis* strains were streaked
196 on plates containing G-M17 agar. Colonies were scraped from the plates and titrated into IF-0a
197 inoculating fluid (Biolog) with growth supplements and Biolog redox tetrazolium dye until the
198 solution reached desired transmittance, according to standard protocols recommended by
199 Biolog for *Streptococcus* species. 100 µl aliquots were added to each well of carbon source
200 plates (PM1 and PM2). The plates were incubated at 30°C in an aerobic OmniLog incubator
201 plate reader, and the metabolic activity was measured kinetically by determining the
202 colorimetric reduction of a tetrazolium dye. Phenotype MicroArrays use Biolog's redox assays,
203 engaging cell respiration or fermentation as a universal reporter and provide precise
204 quantitation of phenotypes. If the phenotype is strongly "positive" in a well, the cells are
205 metabolically active, reducing a tetrazolium dye and forming a strong color. If their metabolic
206 activity is slowed or stopped less color or no color is formed. Data were collected
207 approximately every 10 min over a 72-h period. This was a sufficient time for color
208 development in the positive control wells, while the negative control wells remained colorless.
209 Data were analyzed with the Biolog Kinetic and Parametric software. The PM1 and PM2

Biolog assays assess the ability of a bacterium to utilize any of 190 carbon compounds used in the assay as the sole carbon source.

Results

Structural characterization of DNA region encompassing the *claR* gene. Figure 1 depicts the chromosomal region of the *claR* gene (formerly *yebF*) from *L. lactis* IL1403. ClaR reveals high sequence similarity with transcriptional regulators of the RpiR family: it is a two-domain protein, with a 67-residue N-terminal DNA binding helix-turn-helix (HTH) motif and a 123-residue C-terminal sugar isomerase (SIS) domain (Fig. 1A; http://pfam.sanger.ac.uk/).

Located upstream of *claR* is the *yebE* gene, which encodes a hypothetical protein. Within the intergenic region between these two genes a potential rho-independent terminator with the free energy value (dG) of -15 kcal/mol was localized (Fig. 1B and 1C). In order to verify its functionality, the terminator was cloned into the pGBT58 vector between a promoter and the *xylE* gene encoding a catechol 2,3-dioxygenase. In an *E. coli* strain harboring this construct levels of catechol 2,3-dioxygenase activity obtained reached 0.50±0.04 U, lower than the observed control value (pGBT58 without *yebE* terminator) of 1.25±0.16 U. Thus, the presence of the *yebE* terminator caused a 60% reduction in catechol oxygenase activity when compared to the strain harboring the wild-type pGBT58 vector.

According to an *in silico* analysis the *claR* gene is preceded by at least two putative promoters (Fig. 1B and 1C) suggesting multiple potential transcription start sites. None of the identified potential −10 and −35 promoter regions upstream of *claR* had a full agreement with promoter consensus sequences, defined as TATAAT and TTGACA respectively (Browning and Busby 2004). One of these potential promoters was localized upstream of the *yebE* rho-

232 independent terminator (Fig. 1B and 1C). In order to verify whether in the DNA region
233 upstream of *claR* a functional promoter(s) may exist, a DNA fragment encompassing the
234 abovementioned putative promoters was inserted in front of the *luxAB* reporter genes in the
235 promoter probe pJIM2374 vector and introduced into *L. lactis* LL302. Considerable luciferase
236 activity was detected when the *claR* upstream region was tested indicating functionality of at
237 least one promoter embedded there.

238 **Phenotypes of *claR* deletion mutants.** In our previous study, the lactose-fermenting
239 *ccpA* mutant was randomly mutagenized using pGhost9::IS*S1*. By the use of this procedure, on
240 X-Gal supplemented plates, we have isolated several *β*-galactosidase negative phenotype
241 mutants in the *ccpA* mutant background. Among them, the inactivation of *claR* (*yebF*) in the
242 *ccpA* mutant led to the loss of its lactose-fermentation ability (Aleksandrzak-Piekarczyk et al.
243 2005). Thus, in this study deletion of *claR* was performed both in the IL1403 wild-type strain
244 and IL1403Δ*ccpA* resulting in IL1403Δ*claR* and IL1403Δ*ccpA*Δ*claR* mutants, respectively.
245 Subsequently, IL1403Δ*claR* and IL1403Δ*ccpA*Δ*claR* growth was tested in CDM supplemented
246 with various sugars and compared with their respective parental strains. In C-CDM the
247 IL1403Δ*claR* mutant initially showed no growth ability, and a minor increase in optical density
248 of cells was observed after prolonged incubation (Fig. 2A). Deletion of *ccpA* in the
249 IL1403Δ*claR* strain led to partial growth restoration of the obtained IL1403Δ*ccpA*Δ*claR* strain
250 in C-CDM (Fig. 2A). Both IL1403Δ*claR* and IL1403Δ*ccpA*Δ*claR* displayed no growth in L-
251 CDM in comparison to their parental IL1403 and IL1403Δ*ccpA* strains (Fig. 2B). The
252 supplementation of this medium with an inducing concentration of cellobiose (LC-CDM) did
253 not restore mutants' growth (Fig. 2C). Deletion of the *claR* gene in the IL1403 and

IL1403Δ*ccpA* strains had no effect on mutants' growth in arbutin-, esculin-, galactose-, glucose- or salicin-supplemented medium (data not shown).

After a 24 hour incubation period, the API 50CH test confirmed the complete inability of IL1403Δ*claR* to ferment lactose and cellobiose. While the inability to assimilate lactose by both IL1403Δ*claR* and IL1403Δ*ccpA*Δ*claR* mutants lasted for 36 hours, efficient utilization of cellobiose was observed in IL1403Δ*claR* after the initial 24 hours (Fig. 2D). When assessed by API 50CH, no differences were found between the IL1403Δ*ccpA*Δ*claR* and its parental IL1403Δ*ccpA* within the context of cellobiose fermentation (Fig. 2D).

Global measurements of metabolic activity using the Phenotypic MicroArray approach indicated that deletion of *claR* led to complete abolishment of lactose utilization in IL1403Δ*claR*. In contrast, when cellobiose was available as a sole carbon source, this caused only a slight decrease of IL1403Δ*claR* metabolic activity compared to wild type (data not shown).

No further pronounced differential phenotypes could be identified between strains tested on all other 47 sugars available on API 50CH or the 188 carbon sources available on Biolog PM1 and PM2 arrays.

Transformation of pIL253*claR* into IL1403Δ*claR*, which led to the creation of the IL1403Δ*claR*pIL253*claR* strain, fully complemented the effects of *claR* deletion, restoring the mutant's growth in cellobiose and/or lactose containing medium (data not shown).

ClaR is expressed constitutively at low level and independently from CcpA-mediated regulation. Apart from direct regulation of gene expression mediated by CcpA binding to *cre* sequences (Kowalczyk et al. 2007), indirect control has been observed previously (Ludwig et al. 2002). Although the *claR* upstream DNA region lacks a sequence

277 resembling the *cre* box, its expression could be still regulated by CcpA in an indirect manner.
278 To test whether this phenomenon also occurs in the case of regulation of *claR* and whether its
279 expression is influenced by the presence of different sugars, levels of *claR* expression were
280 estimated by real-time quantitative RT-PCR in response to arbutin, cellobiose, galactose and
281 glucose in IL1403 and IL1403Δ*ccpA*. In both of these strains and in presence of all sugars
282 tested, the *claR* gene was constitutively expressed at a low level reaching an approximate mean
283 relative gene expression level value of 0.05 (data not shown). This suggests that *claR*
284 expression is neither induced nor repressed by any of the sugars tested and does not undergo
285 CcpA-dependent regulation.

286 **Expression of *celB, bglS* and *ptcBA* genes is positively regulated by ClaR in the**
287 **presence of cellobiose.** Previously we showed that genes encoding PTS$^{Cel\text{-}Lac}$ components
288 (*celB* and *ptcBA*) and *bglS* are negatively regulated by CcpA in *L. lactis* IL1403
289 (Aleksandrzak-Piekarczyk et al. 2011). In order to estimate the influence of ClaR on the
290 expression of *bglS, celB* and *ptcBA* in response to the presence of different carbon sources,
291 RNAs were extracted from independent bacterial cultures of IL1403 and IL1403Δ*claR* grown
292 in C-CDM, G-CDM, Gal-CDM or GalC-CDM. Galactose in GalC-CDM was used to support
293 the growth of IL1403Δ*claR*, since this mutant is unable to utilize cellobiose as a carbon source.
294 Lactose was not included in the array of sugars due to *L. lactis* IL1403 poor growth parameters
295 (growth rate, lag phase and optical density of the final culture) in comparison with other sugars
296 used (such as cellobiose, galactose or glucose).

297 The striking difference between the levels of *bglS* and *celB* expression in IL1403 wild-
298 type and IL1403Δ*claR* was evident when the strains were grown in GalC-CDM. In this case,
299 levels of *celB* and *bglS* expression in the presence of ClaR (in IL1403) were significantly

higher than the values obtained in the absence of ClaR (in IL1403Δ*claR*) reaching ClaR activation ratio (calculated as a quotient of relative gene expression in IL1403 and IL1403Δ*claR*) of 8 and 36, respectively (Table 2). In the case of *ptcBA* ClaR activation ratios were close to 3, suggesting minor ClaR-dependent regulation (Table 2).

The lowest transcript levels were detected in IL1403 wild-type and IL1403Δ*claR* cells growing under repressive conditions (G-CDM) due to probable downregulation of *bglS, celB* and *ptcBA* expression by CcpA. Levels of *bglS* and *celB* expression were only slightly varied, whereas the *ptcBA* transcription was increased in both strains in medium supplemented with galactose (Table 2). Comparable expression levels between IL1403 wild-type and IL1403Δ*claR* strains in the presence of galactose or glucose (ClaR activation ratio ~1; Table 2) imply that these sugars do not elicit ClaR-dependent activation of *bglS, celB* and *ptcBA*.

Substantially higher transcription levels of all genes tested were detected when the wild-type strain was grown in a medium supplemented with cellobiose (C-CDM) as a sole carbon source (Table 2). The addition of galactose (GalC-CDM) caused a 2-4 fold transcription decrease of *bglS, celB* and *ptcAB* when compared to C-CDM. This may suggest that the presence of galactose leads to the repression of transcription in a similar manner to glucose.

Furthermore, to determine the influence of cellobiose on *celB, bglS, ptcB* and *ptcA* transcription in the presence (in IL1403) or absence (in IL1403Δ*claR*) of ClaR cellobiose activation ratios were calculated as a quotient of relative gene expression in the wild-type strain IL1403 or IL1403Δ*claR* grown in GalC-CDM and in Gal-CDM. These ratios obtained in IL1403 varied from 25 (*bglS*) and 7 (*celB*) to as low as 3-4-fold (*ptcBA*) implying that cellobiose has different levels of influence on the activation of associated genes (Table 2). These cellobiose activation ratios were similar to ClaR activation values calculated for GalC-

CDM suggesting that this cellobiose-dependent regulation may be due to ClaR action. This was confirmed by the observation that the cellobiose activation ratios obtained in the absence of ClaR were generally around a factor of 1, indicating a lack of genetic expression regulation in IL1403Δ*claR* even in the presence of cellobiose (Table 2).

Discussion

The *claR* gene product from *L. lactis* IL1403 belongs to the RpiR family of potential regulators, of which members are abundantly represented in many other related or distantly related bacteria from different genera (http://www.kegg.jp/kegg/ssdb/). The strongest ClaR orthologs are found only in other *Lactococcus* species (e.g. *L. lactis* subspecies *cremoris* MG1363 and SK11, and *L. lactis* subspecies *lactis* KF147). There are eight paralogs (encoded by *yidA, yecA, yljC, yfeA, yugA, gntR* and *yleF*) from the RpiR family, with ClaR being the prototypical example, encoded in the *L. lactis* IL1403 genome (Bolotin et al. 2001; Guédon E. et al. 2002). Thus far, no function has been assigned to any of the RpiR family genes in *L. lactis*.

However, the conservation of ClaR orthologs found in many different bacterial species suggests an important role in host organisms. Indeed, few members of the RpiR family have been shown to function as positive or negative transcriptional regulators of genes involved in the metabolism of different carbon sources. For example, in *B. subtilis* maltose metabolism is positively regulated by GlvR (Yamamoto et al. 2001). Until now, GlvR was the only representative from the family of RpiR regulators characterized within a group of Gram-positive bacteria. All other functionally characterized examples originate from Gram-negative bacteria, where RpiR family regulators appear to downregulate gene expression. These RpiR-

like repressors have been shown to regulate the metabolism of ribose and *N*-acetylmuramic acid in *E. coli*, glucose in *Pseudomonas putida* and inositol in *Caulobacter crescentus*, *Salmonella enterica* and *Sinorhizobium meliloti* (Boutte et al.; Daddaoua et al. 2009; Jaeger and Mayer 2008; Kohler et al. 2011; Kröger and Fuchs 2009; Sørensen and Hove-Jensen 1996). Members of the RpiR family contain a HTH motif at their N-terminus and a SIS domain on their C-terminal end. The SIS domain is a phospho-sugar-binding domain found in many proteins that regulate the expression of genes involved in synthesis of phospho-sugars (Bateman 1999; Teplyakov et al. 1998).

It is tempting to speculate that this type of regulation is operative in *L. lactis* IL1403. Theoretically, phosphorylated upon entry into the IL1403 cell via CelB cellobiose or lactose bind to the SIS domain of ClaR. Such association may result in a stimulation of the ClaR regulator and allows its binding to the respective DNA regions, resulting in ClaR-dependent regulation of gene expression. We postulate that through this mechanism the expression of *bglS, celB* and *ptcBA* could be activated in the presence of cellobiose or lactose. Evidence confirming this assumption includes strong observed decreases in *bglS*, *celB*, and to a lesser extent, *ptcBA* mRNA levels in response to ClaR absence upon the growth of a *claR* mutant in cellobiose-containing medium (Table 2). This effect is not observable when galactose or glucose is present as a sole carbon source, indicating that these sugars have no activating role toward ClaR.

Additionally, the hypothesis that ClaR plays a role in lactose and cellobiose metabolism is strongly confirmed by the growth deficiencies of *claR* mutants in cellobiose- and/or lactose-supplemented media (Fig. 2A-C). Deletion of *ccpA* led to the partial growth restoration of the IL1403Δ*ccpA*Δ*claR* mutant in cellobiose-supplemented media, but not in

lactose-supplemented media (Fig. 2A-C). Thus, it seems reasonable to conclude that both mutants are barely or not able to utilize these sugars due to a lack of observed *bglS* and *celB* transcriptional activation by ClaR. Indeed, in the absence of ClaR transcription of *bglS* and *celB* decrease 36 and 8-fold, respectively (Table 2). This may result in a severe decrease in BglS and CelB, which are indispensable for *L. lactis* IL1403 growth on cellobiose and lactose as shown previously (Aleksandrzak-Piekarczyk et al. 2011; Aleksandrzak-Piekarczyk et al. 2005).

When assayed globally (by Phenotype MicroArrays and API 50CH) no additional pronounced differences in utilization of tens of different carbon sources were detected between the *claR* mutants and their parental strains. This implies a narrow but significant specificity of ClaR. The contradictory phenomenon that IL1403Δ*claR* is barely able to grow on cellobiose (Fig.2A) while efficiently respiring on this sugar (when tested by phenotype MicroArrays) could be explained by the fate of cellobiose metabolism in IL1403. Previously, we showed that the EIIC CelB permease is the only protein dedicated to cellobiose and lactose uptake in *L. lactis* IL1403 (Aleksandrzak-Piekarczyk et al. 2011), while BglS is the only glycosidase crucial for lactose hydrolysis (Aleksandrzak-Piekarczyk et al. 2005). Although cellobiose is also a substrate for BglS, it was postulated that one or more additional enzyme(s) capable of hydrolysis of this sugar may exist in IL1403, and may be subject to CcpA-mediated negative regulation (Aleksandrzak-Piekarczyk et al. 2005). Moreover, results of the current study clearly indicate that the transcription of *bglS* tightly depends on its activation by ClaR, whereas transcription of *celB* is to a lesser extent dependent on the activation by ClaR (Table 2). Thus, we may assume that in IL1403Δ*claR* small amounts of CelB are still present and may transport traces of lactose or cellobiose. Internalized by IL1403Δ*claR* lactose does not serve as an energy

391 source due to a lack of BglS resulting from the absence of ClaR. On the other hand, in
392 IL1403Δ*claR* small amounts of cellobiose-derived metabolites, which are formed after
393 cellobiose uptake via CelB and activity of different than BglS hydrolases, are insufficient for
394 effective mutant cell division, resulting in the limited mutant's growth.

395 The results determined during this study by RT-qPCR indicate that *claR* is weakly
396 expressed under all conditions tested (in presence of different sugars [Table 2] and in presence
397 or absence of the CcpA protein [data not shown]), in which it reaches the relative gene
398 expression levels only slightly above zero. This is in agreement with the fact that many
399 transcriptional regulators exist endogenously in low amounts, and, in the case of ClaR, it may
400 be due to several reasons. First, both of the putative promoters found upstream of the *claR* gene
401 show incomplete correlation with the consensus sites for both -10 and -35 sequences, and thus
402 may drive a low-level transcription. Second, one these putative promoters yield transcript that
403 starts upstream of the rho-independent terminator sequence (Fig. 1B and 1C) that may
404 prematurely terminate *claR* transcription. We proved that it is a moderately strong terminator,
405 as it arrested the transcription of *claR* with about 60% efficiency. Thus, it might be assumed
406 that the generation of full-length transcript covering the *claR* gene is likely achieved by a low
407 amount of read-through transcription. However, we cannot exclude the possibility that the
408 efficiency of read-through termination could be strengthened by the influence of unknown
409 antitermination factors.

410 Despite the narrow specificity of ClaR within the context of sugar metabolism, it
411 seems that this regulator has an invaluable role in lactococcal cells. We demonstrated that its
412 activity is crucial for the assimilation of two sugars that are commonly found in ecosystems
413 inhabited by *L. lactis* strains - plant material (cellobiose), and milk (lactose). Having such a

regulator allows for a settlement of more diverse environments, and thus gives *L. lactis* an advantage over other microorganisms. The unprecedented role of the ClaR protein is made even more significant by the fact that the metabolism of lactose and *β*-glucosides is of great economic importance for biotechnological processes involving *L. lactis*, in regard to the production of both fermented milk and plant products. Obtaining detailed knowledge related to sugar metabolism and the regulation of associated gene expression in *L. lactis* may contribute to the improvement of mechanisms controlling significant cellular processes in these bacteria. In the case of industrial microorganisms such as *L. lactis*, modification of defined regulatory networks may drastically affect the properties of the bacteria and have implications on bioprocesses. Finally, we can assume that through our research, we can introduce changes in the metabolic potential of *Lactococcus* strains, which by themselves are not able to assimilate lactose. We can initiate this process through the inactivation of *ccpA* or activation of other genes by addition of cellobiose. In contrast to plasmid-located *lac*-operons, genes encoding $PTS^{Cel\text{-}Lac}$ components are located on the chromosome, which ensures their stability, a potentially important feature for industrial applications.

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REFERENCES

Aleksandrzak-Piekarczyk T (2013) Lactose and β-glucosides metabolism and its regulation in *Lactococcus lactis*: A review. In: Kongo JM (ed) Lactic acid bacteria - R & D for food, health and livestock purposes. InTechOpen. doi: 10.5772/50889

Aleksandrzak-Piekarczyk T, Kok J, Renault P, Bardowski J (2005) Alternative lactose catabolic pathway in *Lactococcus lactis* IL1403. Appl Environ Microbiol 71:6060–6069. doi: 10.1128/AEM.71.10.6060-6069.2005

Aleksandrzak-Piekarczyk T, Polak J, Jezierska B, Renault P, Bardowski J (2011) Genetic characterization of the CcpA-dependent, cellobiose-specific PTS system comprising CelB, PtcB and PtcA that transports lactose in *Lactococcus lactis* IL1403. Int J Food Microbiol 145:186–194. doi: 10.1016/j.ijfoodmicro.2010.12.011

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

Barrière C, Veiga-da-Cunha M, Pons N, Guédon E, van Hijum SAFT, Kok J, Kuipers OP, Ehrlich DS, Renault P (2005) Fructose utilization in *Lactococcus lactis* as a model for low-GC Gram-positive bacteria: Its regulator, signal, and DNA-binding site. J Bacteriol 187:3752–3761. doi: 10.1128/JB.187.11.3752-3761.2005

Bateman A (1999) The SIS domain: a phosphosugar-binding domain. Trends Biochem Sci 24:94–95. doi: 10.1016/S0968-0004(99)01357-2

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. doi: 10.1101/gr.169701

Boucher I, Vadeboncoeur C, Moineau S (2003) Characterization of genes involved in the metabolism of α-galactosides by *Lactococcus raffinolactis*. Appl Environ Microbiol 69:4049–4056. doi: 10.1128/AEM.69.7.4049-4056.2003

Boutte CC, Srinivasan BS, Flannick JA (2008) Genetic and computational identification of a conserved bacterial metabolic module. PLOS Genetics. 8:e1000310. doi: 10.1371/journal.pgen.1000310

Browning DF, Busby SJW (2004) The regulation of bacterial transcription initiation. Nat Rev Micro 2:57–65. doi: 10.1038/nrmicro787

Chopin A, Chopin M-C, Moillo-Batt A, Langella P (1984) Two plasmid-determined restriction and modification systems in *Streptococcus lactis*. Plasmid 11:260–263. doi: 10.1016/0147-619X(84)90033-7

Daddaoua A, Krell T, Ramos J-L (2009) Regulation of glucose metabolism in *Pseudomonas*: the phosphorylative branch and Entner-Doudoroff enzymes are regulated by a repressor

containing a sugar isomerase domain. J. Biol. Chem. 284:21360–21368. doi: 10.1074/jbc.M109.014555

Delorme C, Ehrlich SD, Renault P (1999) Regulation of expression of the *Lactococcus lactis* histidine operon. J Bacteriol 181:2026–2037.

Guédon E., Jamet E., Renault P (2002) Gene regulation in *Lactococcus lactis*: the gap between predicted and characterized regulators. Antonie Van Leeuwenhoek 82:93–112. doi: 10.1007/978-94-017-2029-8_6

Hueck CJ, Hillen W (1995) Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the Gram-positive bacteria? Mol Microbiol 15:395–401. doi: 10.1111/j.1365-2958.1995.tb02252.x

Jaeger T, Mayer C (2008) The transcriptional factors MurR and catabolite activator protein regulate N-acetylmuramic acid catabolism in *Escherichia coli*. J Bacteriol 190:6598–6608. doi: 10.1128/JB.00642-08

Jagura-Burdzy G, Khanim F, Smith CA, Thomas CM (1992) Crosstalk between plasmid vegetative replication and conjugative transfer: repression of the *trfA* operon by *trbA* of broad host range plasmid RK2. Nucleic Acids Res 20:3939–3944. doi: 10.1093/nar/20.15.3939

Kohler PRA, Choong E-L, Rossbach S (2011) The RpiR-like repressor IolR regulates inositol catabolism in *Sinorhizobium meliloti*. J Bacteriol 193:5155–5163. doi: 10.1128/JB.05371-11

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

Kröger C, Fuchs TM (2009) Characterization of the myo-inositol utilization island of *Salmonella enterica* serovar Typhimurium. J Bacteriol 191:545–554. doi: 10.1128/JB.01253-08

Leenhouts K, Bolhuis A, Venema G, Kok J (1998) Construction of a food-grade multiple-copy integration system for *Lactococcus lactis*. Appl Microbiol Biotechnol 49:417–423.

Lorca G, Reddy L, Nguyen A, Sun EI, Tseng J, Yen M-R, Saier MH (2010) Lactic acid bacteria: comparative genomic analyses of transport systems. In:. Mozzi F, Raya RR, Vignolo GM (eds) Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Oxford, UK, pp 73–87. doi: 10.1002/9780813820866.ch1

Ludwig H, Rebhan N, Blencke H-M, Merzbacher M, Stülke J (2002) Control of the glycolytic *gapA* operon by the catabolite control protein A in *Bacillus subtilis*: a novel mechanism of CcpA-mediated regulation. Mol Microbiol 45:543–553. doi: 10.1046/j.1365-2958.2002.03034.x

Luesink EJ, Van Herpen REMA, 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 30:789–798. doi: 10.1046/j.1365-2958.1998.01111.x

Maguin E, Prévost H, Ehrlich SD, Gruss A (1996) Efficient insertional mutagenesis in lactococci and other Gram-positive bacteria. J Bacteriol 178:931–935.

Mayo B, Aleksandrzak-Piekarczyk T, Fernández M, Kowalczyk M, Álvarez-Martín P, Bardowski J (2010) Updates in the metabolism of lactic acid bacteria. In:. Mozzi F, Raya RR, Vignolo GM (eds) Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Oxford, UK, pp 3-33. doi: 10.1002/9780813820866.ch1

Monedero V, Kuipers OP, Jamet E, Deutscher J (2001) Regulatory functions of serine-46-phosphorylated HPr in *Lactococcus lactis*. J Bacteriol 183:3391–3398. doi: 10.1128/JB.183.11.3391-3398.2001

Simon D, Chopin A (1988) Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie 70:559–566. doi: 10.1016/0300-9084(88)90093-4

Sissler M, Delorme C, Bond J, Ehrlich SD, Renault P, Francklyn C (1999) An aminoacyl-tRNA synthetase paralog with a catalytic role in histidine biosynthesis. Proc Natl Acad Sci 96:8985–8990. doi: 10.1073/pnas.96.16.8985

Solopova A, Bachmann H, Teusink B, Kok J, Neves AR, Kuipers OP (2012) A specific mutation in the promoter region of the silent *cel* cluster accounts for the appearance of lactose-utilizing *Lactococcus lactis* MG1363. Appl Environ Microbiol 78:5612–5621. doi: 10.1128/AEM.00455-12

Sørensen KI, Hove-Jensen B (1996) Ribose catabolism of *Escherichia coli*: characterization of the *rpiB* gene encoding ribose phosphate isomerase B and of the *rpiR* gene, which is involved in regulation of *rpiB* expression. J Bacteriol 178:1003–1011.

Teplyakov A, Obmolova G, Badet-Denisot M-A, Badet B, Polikarpov I (1998) Involvement of the C terminus in intramolecular nitrogen channeling in glucosamine 6-phosphate synthase: evidence from a 1.6 å crystal structure of the isomerase domain. Structure 6:1047–1055. doi: 10.1016/S0969-2126(98)00105-1

Terzaghi BE, Sandine WE (1975) Improved medium for lactic streptococci and their bacteriophages. Appl Microbiol 29:807–813.

Weickert MJ, Chambliss GH (1990) Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*. Proc Natl Acad Sci 87:6238–6242. doi: 10.1073/pnas.87.16.6238

Wood EJ (1983) Molecular cloning. A laboratory manual by T Maniatis, E F Fritsch and J Sambrook. Cold Spring Harbor Laboratory, New York. Biochem Educ 11:82–82. doi: 10.1016/0307-4412(83)90068-7

Yamamoto H, Serizawa M, Thompson J, Sekiguchi J (2001) Regulation of the *glv* operon in *Bacillus subtilis*: YfiA (GlvR) is a positive regulator of the operon that is repressed through CcpA and *cre*. J Bacteriol 183:5110–5121. doi: 10.1128/JB.183.17.5110-5121.2001

547 **TABLES AND FIGURES**

548 **TABLE 1**. Bacterial strains, plasmids and primers.

Strain[a], plasmid or primers pair	Relevant genotypic or phenotypic properties[b]	Source and/or reference[c]
Strains		
L. lactis		
IL1403	Lac$^-$, Cel$^+$, plasmid-free wild-type, host strain	INRA (Chopin et al. 1984)
LL302	*L. lactis* MG1363-derivative, RepA$^+$	(Leenhouts et al. 1998)
IL1403Δ*claR*	Lac$^-$, Cel$^-$, Δ*claR*, Em^s, plasmid-free, IL1403-derivative	This study
IL1403Δ*ccpA*	Lac$^+$, CcpA$^-$ (IS*S1*), Em^s, plasmid-free, IL1403-derivative	(Aleksandrzak-Piekarczyk et al. 2005)
IL1403Δ*ccpA*Δ*claR*	Lac$^-$, Cel$^-$, CcpA$^-$ (IS*S1*), Δ*claR*, Em^s, plasmid-free, IL1403-derivative	This study
IL1403Δ*claR*pIL253*claR*	Lac$^-$, Cel$^+$, Em^r, IL1403Δ*claR*-derivative carrying pIL253*claR*	This study
E. coli		
TG1	Δ(*hsdMS-mcrB*)5 Δ(*lac-proAB*) *supE thi-1* F'(*traD36 proAB*$^+$ *lacI*^{q}*ZΔM15*)	Laboratory collection
Plasmids		
pGEM-T	Amp^r, M13*ori*, linear T-overhang vector	Promega
pGhost9	Em^r, *repA* (Ts)	INRA (Maguin et al. 1996)
pGBT58	Km^r, 10.35 kb, pSC101 replicon, carrying *xylE* under *trfAp*	(Jagura-Burdzy et al. 1992)
pIL253	Em^r, high-copy number lactococcal vector	(Simon and Chopin 1988)
pJIM2374	Em^r, integrative vector carrying *luxAB* genes	(Delorme et al. 1999)
Recombinant plasmids		
pJIM2374*claRp*	Em^r, integrative vector carrying *luxAB* under the control of the putative *claR* promoter (*claRp*)	This study
pGBT58*yebEt*	Km^r, pGBT58 derivative, carrying the *yebE* terminator (*yebEt*) between *trfAp* and *xylA*	This study
pGhost9Δ*claR*	Em^r, pGhost9 derivative, carrying the *claR* upstream and downstream DNA regions	This study
pIL253*claR*	Em^r, pIL253 vector carrying the *claR* gene under the control of its promoter	This study
Primers[d]		
For cloning of *yebE* promoter and terminator or deletion and complementation of the *claR* gene		
*claR*PstI/*claR*ERpr	AACTGCAGGGGTGTTACATTCCAGC/GGAATTCGCTCCGCCACAAATTC	
*claR*ERrv/*claR*SalI	GGAATTCGCAACTCCCAGTACTAGC/ACGCGTCGACGCAGGCGTTGTTCT	

	TGACC
yebEfor/*claR*rev	GCTTTTGTTGGTCTGATT/CGGATACTGTTTGGACC
*claR*Smaf/*claR*PstR	CCCGGGCTTGCTTTGATTCTCAG/CTGCAGGCAACCTTTAGGCGAC
*yebE*terf/*yebE*terr	GTGGTACCCTGTGATTGTCACTAGCG/CATCCCATGGCTAGCGTTTCAAG CTCG
For cDNA synthesis and RT-qPCR amplification	
LlBglSaF/LlBglSaR	GCATGGAATCCAGTTGACGG/GCAATTCTCAAGCCTTCAGGG
LlCcpAaF/LlCcpAaR	AAAAGACGCGCCAGAAGGTC/TGGAATCGATATCATCAACCCC
LlCelBaF/LlCelBaR	GGAGTCAATGACCTCGCTGG/GGTTTCCAAGCGGCAAGTC
LlPtcAaF/LlPtcAaR	TTATCATGAGTGGAGGAAATGCC/TTTTTCGCCTTGAGCTAAACG
LlPtcBbF/LlPtcBbR	ACAGCGGATATTGATAACATGCTTG/ATCTCCGCGCATCATTCC
LlPurMaF/LlPurMaR	ATTGCGTAGCCATGTGCGTC/CTGTTTCTCCACCAATCAGCG
LlTufaF/LlTufaR	CGTGACCTCTTGAGCGAATACG/GAGTGGTTTGTCAGTGTCGCG
LlYbhEaF/LlYbhEaR	CAGCAACATTTGGTCCTTGGC/TGCTTGGCTCATCGCTTTAAG

[a] Strains obtained in this study are deposited in the publicly accessible IBB PAS laboratory culture collection.

[b] Amp, ampillicin; Em, erythromycin; Km, kanamycin; r, resistance; s, sensitivity.

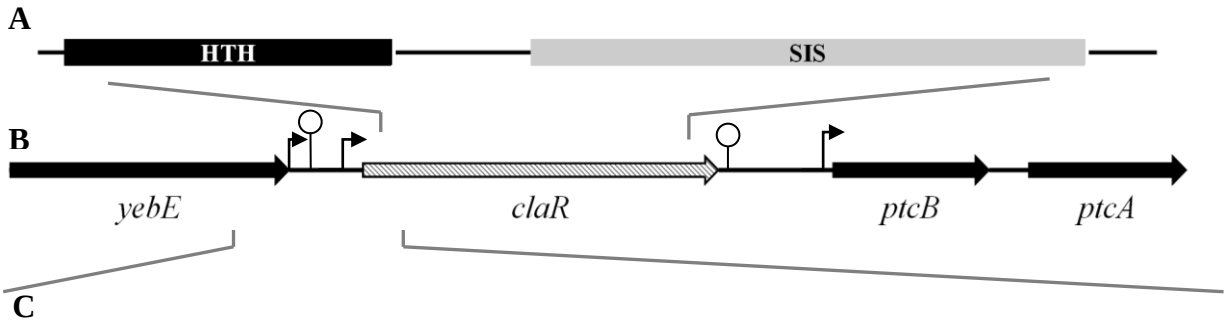
[c] INRA. Institut National de la Recherche Agronomique (Jouy-en-Josas. France).

[d] All primers were designed on the basis of the *L. lactis* IL1403 genome nucleotide sequence (Bolotin et al. 2001), which is available from NCBI (http://www.ncbi.nlm.nih.gov/genome) with accession no. AE005176. To certain primers restriction sites were added for digestion with *Eco*RI, *Kpn*I, *Nco*I, *Pst*I and *Sal*I.

TABLE 2. ClaR and cellobiose activation ratios and the relative gene expression levels in IL1403 wild-type and IL1403Δ*claR* measured by RT-qPCR in response to different sugars.

gene	Relative gene expression level							ClaR activation ratio[a] on			Cellobiose activation ratio[b] in	
	galactose		glucose		cellobiose/galactose		cellobiose					
	IL1403	IL1403Δ*claR*	IL1403	IL1403Δ*claR*	IL1403	IL1403Δ*claR*	IL1403	glucose	galactose	galactose/ cellobiose	IL1403	IL1403 Δ*claR*
bglS	0.069±0.019	0.070±0.018	0.009±0.002	0.008±0.002	1.743±0.485	0.048±0.013	4.206±0.971	1.1	1.0	36.1	25.2	0.7
celB	0.066±0.016	0.070±0.013	0.010±0.002	0.005±0.001	0.487±0.128	0.058±0.014	2.040±0.438	1.8	1.0	8.4	7.3	0.8
ptcA	1.532±0.165	0.952±0.220	0.030±0.007	0.029±0.004	5.907±1.011	1.515±0.105	17.215±0.490	1.0	1.6	3.9	3.9	1.6
ptcB	7.536±1.509	8.391±1.927	0.177±0.05	0.204±0.057	24.006±6.003	9.773±2.118	36.166±6.054	0.9	0.9	2.5	3.2	1.2
claR	0.046±0.008	-	0.061±0.010	-	0.049±0.012	-	0.037±0.009	-	-	-	1.1	-

[a] ClaR activation ratio was calculated as a quotient of relative gene expression in IL1403 and IL1403Δ*claR*.
[b] Cellobiose activation ratio was calculated as a quotient of relative gene expression in the wild-type strain IL1403 or IL1403Δ*claR* grown in GalC-CDM and in Gal-CDM.
- not determined.



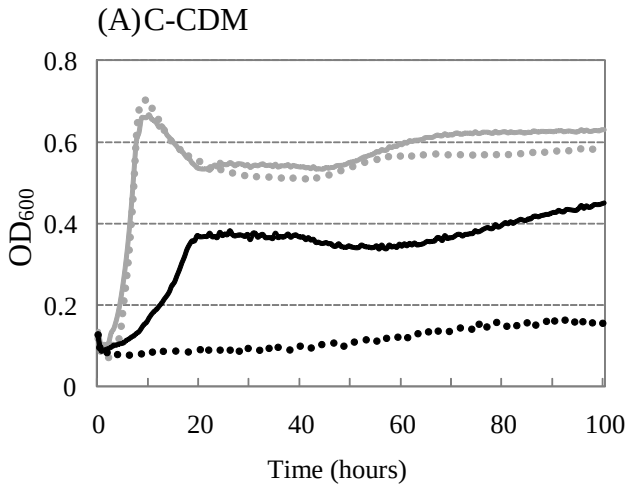
C

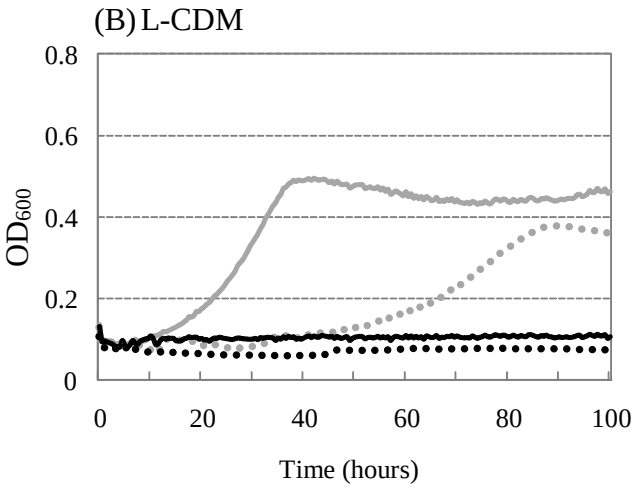
yebE

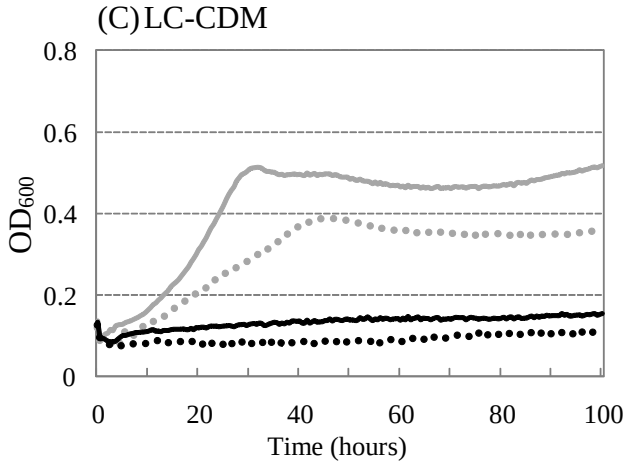
```
CAAAGACAAAAGTCAATAATCTTCTTTATTATCCTAATTATTACGGTCATGAAATTCTTGGCTTAATCTATGCTT
  K  T  K  V  N  N  L  L  Y  Y  P  N  Y  Y  G  H  E  I  L  G  L  I  Y  A  F

       -35                          -10
TCGTTTTTGGAAAAGGCTAAGAAAATTTCAATTATTATAAGGATAAAAGCTGTCAGTAATTTTTACTGACAGCTTT
  V  F  G  K  G  -                                  **********   **********

                                                  -35                  -10
GCTAAAATTTGGCAAATCCTTTCAAACAATGATTCTGTCAGTAAATTTTTAAAAGAATTTGTGGCGGAGCTAAATA

             RBS       claR
TTTCCAAATACGGAGAGTAAAAAATGATGTTTCTACCCGAACAAATCAGTAAACTTAACGAGCTTGAAACGCTAGT
                       M  M  F  L  P  E  Q  I  S  K  L  N  E  L  E  T  L  V
```

Fig. 1. The organization of ClaR domains (A), the *claR* chromosomal region (B) and nucleotide sequence of the *yebE-claR* intergenic region (C). The ClaR domains (A) and the genes of the *claR* chromosomal region (B) are drawn to scale. Stem-loop structures denote rho-independent terminators. Gray-shaded sequences (C) highlight the potential −10/−35 promoter regions upstream of *claR*. The putative ribosome binding site (RBS) is underlined, and the start and stop codons of *claR* and *yebE*, respectively, are shown as black shading. Stars following *yebE* indicate a rho-independent terminator, the functionality of which was measured in this work.

589







(D) API 50CH

strain	sugar					
	cellobiose			lactose		
	12 h	24 h	36 h	12 h	24 h	36 h
IL1403	+	+	+	-	-	-
IL1403Δ*claR*	-	-	+	-	-	-
IL1403Δ*ccpA*	+	+	+	-	-	+/-
IL1403Δ*ccpA*Δ*claR*	+	+	+	-	-	-

590 **Fig.2.** Growth of the *L. lactis* IL1403 wild-type strain (grey dots) and its derivatives:
591 IL1403Δ*ccpA* (grey line), IL1403Δ*claR* (black dots) and IL1403Δ*ccpA*Δ*claR* (black line) in
592 CDM containing cellobiose (A), lactose (B), or lactose + cellobiose (C). Sugar concentrations
593 were 1% except for 0.01% cellobiose in lactose + cellobiose supplemented CDM. X axis =
594 time (in hours), and the Y axis = optical density at 600 nm. Sugar fermentation patterns of the
595 *L. lactis* IL1403 wild-type strain and its derivatives: IL1403Δ*ccpA*, IL1403Δ*claR* and
596 IL1403Δ*ccpA*Δ*claR* determined by the API 50CH test (D). The results are shown for 12, 24
597 and 36 h of incubation; +, good fermentation; +/-, weak fermentation; -, no fermentation (D)

598