

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

2 **IL1403.**

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15

16 **Abstract**

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

30

31 Introduction

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

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

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

64 The efficiency of transport and subsequent metabolism of incoming sugars is tightly
65 controlled by several regulatory proteins, which form the regulatory network necessary for
66 sensing environmental conditions and setting catabolic capacities of the cell. Guédon et al.
67 (Guédon et al. 2002) distinguish two groups of regulators - general and secondary regulators.
68 The main example of such a general regulator is catabolite control protein A (CcpA) (Hueck
69 and Hillen 1995). CcpA is a well-conserved protein operating in many low-GC Gram-positive
70 bacteria and, together with its corepressor Ser-P-HPr, it binds to 14-nucleotide *cis*-acting DNA
71 target sites known as *cre* (catabolite responsive elements), promoting carbon catabolite
72 activation (CCA) or repression (CCR) (Weickert and Chambliss 1990). In *L. lactis* strains,
73 CcpA has been shown to repress different genes associated with uptake of β -glucosides,
74 fructose, galactose or lactose, while activating the glycolytic *las* operon (Aleksandrak-
75 Piekarczyk et al. 2011; Aleksandrak-Piekarczyk et al. 2005; Barrière et al. 2005; Luesink et al.
76 1998; Monedero et al. 2001).

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

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

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

98 MATERIALS AND METHODS

99 **Bacterial strains, media, and plasmids.** Bacterial strains and plasmids used in this
100 study are listed in Table 1. *E. coli* cells were grown in Luria-Bertani (LB) medium (Wood
101 1983) at 37°C, while *L. lactis* was cultivated in M17 glucose medium (G-M17) (Terzaghi and
102 Sandine 1975) or in chemically defined medium (CDM) (Sissler et al. 1999) supplemented
103 with 1% glucose (G-CDM), 1% cellobiose (C-CDM), 1% lactose (L-CDM), 1% galactose
104 (Gal-CDM), 1% arbutin (A-CDM), 1% galactose with 1% cellobiose (GalC-CDM) or 1%
105 lactose together with an **inducing** (0.01%) concentration of cellobiose (LC-CDM). When
106 required, ampicillin (Amp; 100 µg ml⁻¹ for *E. coli*) or erythromycin (Em; 100 µg ml⁻¹ for *E.*
107 *coli* and 5 µg ml⁻¹ for *L. lactis*) were added to the medium. Solidified media contained 1.5%
108 agar and, when necessary, IPTG (isopropyl β-D-thiogalactopyranoside; 1 mM for *E. coli*) and
109 X-gal (5-bromo-4-chloro-3-indodolyl-β-D-galactopyranoside; 50 µg ml⁻¹ for *E. coli*).

110 **Construction of the *claR* deletion mutants and complementing plasmid.** The
111 mutant was created by double crossover between pGhost9 harboring DNA fragments
112 overlapping the *claR* gene **and the chromosomal region containing these DNA fragments**. The
113 overlapping DNA fragments were amplified using the appropriate forward and reverse primer
114 pairs (Table 1). The *claR* upstream region was amplified with the primers *claRPstI/claRERpr*
115 (Table1) using ExTaq polymerase and was cloned as a **PstI/EcoRI** fragment into the
116 corresponding sites in pGhost9. The *claR* downstream region was amplified using the primers
117 *claRERrv/claRSalI* (Table 1) and was cloned as an **EcoRI/SalI** fragment into the vector
118 carrying the upstream fragment, resulting in the pGhost9 Δ *claR* plasmid. The deletion plasmid
119 was introduced into *L. lactis* IL1403 and IL1403 Δ *ccpA*. Homologous recombination was
120 enforced by 10⁻³ dilution in fresh G-M17-Em medium of the overnight culture of the

121 lactococcal strains harboring pGhost9 Δ *claR*. Diluted cultures were incubated for 2.5 h in G-
122 M17 at non-permissive temperature (38°C). Integrants containing pGhost9 Δ *claR* in the
123 chromosome were selected at 38°C on G-M17 agar plates containing erythromycin. Excision
124 from the chromosome and removing of the integration vector from *L. lactis* was performed by
125 growth of integrants in the absence of antibiotic for at least 100 generations at the permissive
126 temperature of 28°C. The genetic structure of the resulting *claR* deletion strains, *L. lactis*
127 IL1403 Δ *claR*, was confirmed by colony PCR, determination of the strain sensitivity to Em and
128 sequencing of the DNA region containing the deleted *claR* gene.

129 In order to complement the *claR* deletion, the *claR* gene with its putative promoter
130 region was amplified using *claRSmaf* and *claRPstR* primers (Table 1), cloned into pGEM-T
131 and transferred into *E. coli* TG1. The resultant plasmid DNA was isolated, digested with *Pst*I
132 and *Sma*I, ligated to pIL253 digested with the same restriction enzymes and transferred into *L.*
133 *lactis* IL1403 Δ *claR* giving rise to the IL1403 Δ *claR*pIL253*claR* strain.

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

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

151 **Relative quantification of the gene expression by real-time quantitative PCR (RT-**
152 **qPCR).** For RT-qPCR total RNA was isolated with the use of TRI Reagent (Sigma) according
153 to the instructions of the manufacturer, from 25 ml of the IL1403 wild-type strain and its
154 mutants (IL1403 Δ *ccpA* and IL1403 Δ *claR*) cultures harvested in mid-exponential phase
155 (OD₆₀₀=0.6). Cells were grown in G-CDM, C-CDM, Gal-CDM, A-CDM or GalC-CDM. RNA
156 was extracted from at least three independent cultures.

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

160 Real-time quantitative PCR assays were carried out on the 7500 Real Time PCR
161 System (Applied Biosystems). Each reaction was carried out in reaction mixture containing: 1x
162 concentrated commercial buffer supplied with polymerase (Metabion), 0.625 U Taq
163 polymerase (Metabion), 4 mM MgCl₂, 30000x diluted SYBR Green (Sigma), 5% DMSO, 0.5
164 ng/ μ l acetylated BSA (Sigma), 0.8% glycerol (ROTH), 400 μ M dNTP mixture (Metabion),
165 forward and reverse specific primers (400 μ M each), cDNA template, and water up to 25 μ l of

166 final volume. Reactions were performed with an initial denaturation step (95°C for 3 min)
167 followed by 45 cycles of denaturation (95°C for 15 s) and primer annealing-extension (60°C for
168 1 min). Fluorescence was read during the annealing-extension step of each cycle. After cycling,
169 melting-point temperature analysis was performed in the range of 60°C to 95°C with
170 temperature increments of 0.33°C. In each experiment background range was adjusted
171 automatically and the threshold for C_t evaluation was adjusted manually. For each cDNA
172 sample six reactions were carried out using three template amounts, each in duplicate. For each
173 gene studied the amounts of cDNA were chosen individually (if possible the same for all
174 genes) to obtain C_t values in range between 14 and 34 cycles. Quality of results were evaluated
175 based on expected C_t differences among three cDNA amounts as well as product melting
176 curves. Rare outlying results were omitted in calculations. The results were normalized by the
177 use of *L. lactis* the reference *tuf* and *purM* genes, which code for elongation factor TU and
178 phosphoribosylaminoimidazole synthetase, respectively. Specific primers for each gene (Table
179 1) were designed using Primer Express software (Applied Biosystems). Before use, the primers
180 were verified for equal efficiency of PCR reaction. The resulting data were processed using
181 files exported from the qPCR cycler program and imported into Excel sheets to facilitate
182 calculation of expression ratios between target and reference genes. These ratios were
183 calculated by ΔC_t method using geometric mean of reference genes C_t s in each experiment
184 separately.

185 **Phenotypic testing of carbon source utilization.** The study of phenotypic changes
186 in mutant cells compared to parental strains was carried out through growth tests and analysis
187 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 OD₆₀₀
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

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

212 **Results**

213 **Structural characterization of DNA region encompassing the *claR* gene.** Figure 1
214 depicts the chromosomal region of the *claR* gene (formerly *yebF*) from *L. lactis* IL1403. ClaR
215 reveals high sequence similarity with transcriptional regulators of the RpiR family: it is a two-
216 domain protein, with a 67-residue N-terminal DNA binding helix-turn-helix (HTH) motif and a
217 123-residue C-terminal sugar isomerase (SIS) domain (Fig. 1A; <http://pfam.sanger.ac.uk/>).

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

227 According to an *in silico* analysis the *claR* gene is preceded by at least two putative
228 promoters (Fig. 1B and 1C) suggesting multiple potential transcription start sites. None of the
229 identified potential -10 and -35 promoter regions upstream of *claR* had a full agreement with
230 promoter consensus sequences, defined as TATAAT and TTGACA respectively (Browning
231 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::ISS1. 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 (Aleksandrak-Piekarczyk et al.
243 2005). Thus, in this study deletion of *claR* was performed both in the IL1403 wild-type strain
244 and IL1403 Δ *ccpA* resulting in IL1403 Δ *claR* and IL1403 Δ *ccpA* Δ *claR* mutants, respectively.
245 Subsequently, IL1403 Δ *claR* and IL1403 Δ *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 IL1403 Δ *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 IL1403 Δ *claR* strain led to partial growth restoration of the obtained IL1403 Δ *ccpA* Δ *claR* strain
250 in C-CDM (Fig. 2A). Both IL1403 Δ *claR* and IL1403 Δ *ccpA* Δ *claR* displayed no growth in L-
251 CDM in comparison to their parental IL1403 and IL1403 Δ *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

254 IL1403 Δ *ccpA* strains had no effect on mutants' growth in arbutin-, esculin-, galactose-,
255 glucose- or salicin-supplemented medium (data not shown).

256 After a 24 hour incubation period, the API 50CH test confirmed the complete inability
257 of IL1403 Δ *claR* to ferment lactose and cellobiose. While the inability to assimilate lactose by
258 both IL1403 Δ *claR* and IL1403 Δ *ccpA* Δ *claR* mutants lasted for 36 hours, efficient utilization of
259 cellobiose was observed in IL1403 Δ *claR* after the initial 24 hours (Fig. 2D). When assessed by
260 API 50CH, no differences were found between the IL1403 Δ *ccpA* Δ *claR* and its parental
261 IL1403 Δ *ccpA* within the context of cellobiose fermentation (Fig. 2D).

262 Global measurements of metabolic activity using the Phenotypic MicroArray approach
263 indicated that deletion of *claR* led to complete abolishment of lactose utilization in
264 IL1403 Δ *claR*. In contrast, when cellobiose was available as a sole carbon source, this caused
265 only a slight decrease of IL1403 Δ *claR* metabolic activity compared to wild type (data not
266 shown).

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

270 Transformation of pIL253*claR* into IL1403 Δ *claR*, which led to the creation of the
271 IL1403 Δ *claR*pIL253*claR* strain, fully complemented the effects of *claR* deletion, restoring the
272 mutant's growth in cellobiose and/or lactose containing medium (data not shown).

273 **ClaR is expressed constitutively at low level and independently from CcpA-**
274 **mediated regulation.** Apart from direct regulation of gene expression mediated by CcpA
275 binding to *cre* sequences (Kowalczyk et al. 2007), indirect control has been observed
276 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 IL1403 Δ *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-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 IL1403 Δ *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 IL1403 Δ *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 IL1403 Δ *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

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

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

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

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

323 CDM suggesting that this cellobiose-dependent regulation may be due to ClaR action. This was
324 confirmed by the observation that the cellobiose activation ratios obtained in the absence of
325 ClaR were generally around a factor of 1, indicating a lack of genetic expression regulation in
326 IL1403 Δ *claR* even in the presence of cellobiose (Table 2).

327 Discussion

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

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

345 like repressors have been shown to regulate the metabolism of ribose and *N*-acetylmuramic
346 acid in *E. coli*, glucose in *Pseudomonas putida* and inositol in *Caulobacter crescentus*,
347 *Salmonella enterica* and *Sinorhizobium meliloti* (Boutte et al.; Daddaoua et al. 2009; Jaeger
348 and Mayer 2008; Kohler et al. 2011; Kröger and Fuchs 2009; Sørensen and Hove-Jensen
349 1996). Members of the RpiR family contain a HTH motif at their N-terminus and a SIS domain
350 on their C-terminal end. The SIS domain is a phospho-sugar-binding domain found in many
351 proteins that regulate the expression of genes involved in synthesis of phospho-sugars
352 (Bateman 1999; Teplyakov et al. 1998).

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

364 Additionally, the hypothesis that ClaR plays a role in lactose and cellobiose
365 metabolism is strongly confirmed by the growth deficiencies of *claR* mutants in cellobiose-
366 and/or lactose-supplemented media (Fig. 2A-C). Deletion of *ccpA* led to the partial growth
367 restoration of the IL1403 Δ *ccpA* Δ *claR* mutant in cellobiose-supplemented media, but not in

368 lactose-supplemented media (Fig. 2A-C). Thus, it seems reasonable to conclude that both
369 mutants are barely or not able to utilize these sugars due to a lack of observed *bglS* and *celB*
370 transcriptional activation by ClaR. Indeed, in the absence of ClaR transcription of *bglS* and
371 *celB* decrease 36 and 8-fold, respectively (Table 2). This may result in a severe decrease in
372 BglS and CelB, which are indispensable for *L. lactis* IL1403 growth on cellobiose and lactose
373 as shown previously (Aleksandrak-Piekarczyk et al. 2011; Aleksandrak-Piekarczyk et al.
374 2005).

375 When assayed globally (by Phenotype MicroArrays and API 50CH) no additional
376 pronounced differences in utilization of tens of different carbon sources were detected between
377 the *claR* mutants and their parental strains. This implies a narrow but significant specificity of
378 ClaR. The contradictory phenomenon that IL1403 Δ *claR* is barely able to grow on cellobiose
379 (Fig.2A) while efficiently respiring on this sugar (when tested by phenotype MicroArrays)
380 could be explained by the fate of cellobiose metabolism in IL1403. Previously, we showed that
381 the EIIC CelB permease is the only protein dedicated to cellobiose and lactose uptake in *L.*
382 *lactis* IL1403 (Aleksandrak-Piekarczyk et al. 2011), while BglS is the only glycosidase crucial
383 for lactose hydrolysis (Aleksandrak-Piekarczyk et al. 2005). Although cellobiose is also a
384 substrate for BglS, it was postulated that one or more additional enzyme(s) capable of
385 hydrolysis of this sugar may exist in IL1403, and may be subject to CcpA-mediated negative
386 regulation (Aleksandrak-Piekarczyk et al. 2005). Moreover, results of the current study clearly
387 indicate that the transcription of *bglS* tightly depends on its activation by ClaR, whereas
388 transcription of *celB* is to a lesser extent dependent on the activation by ClaR (Table 2). Thus,
389 we may assume that in IL1403 Δ *claR* small amounts of CelB are still present and may transport
390 traces of lactose or cellobiose. Internalized by IL1403 Δ *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 IL1403 Δ *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

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

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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		
<i>L. lactis</i> IL1403	Lac ⁻ , Cel ⁺ , plasmid-free wild-type, host strain	INRA (Chopin et al. 1984)
LL302	<i>L. lactis</i> MG1363-derivative, RepA ⁺	(Leenhouts et al. 1998)
IL1403Δ <i>claR</i>	Lac ⁻ , Cel ⁻ , Δ <i>claR</i> , Em ^s , plasmid-free, IL1403-derivative	This study
IL1403Δ <i>ccpA</i>	Lac ⁺ , CcpA ⁻ (ISS1), Em ^s , plasmid-free, IL1403-derivative	(Aleksandrak-Piekarczyk et al. 2005)
IL1403Δ <i>ccpA</i> Δ <i>claR</i>	Lac ⁻ , Cel ⁻ , CcpA ⁻ (ISS1), Δ <i>claR</i> , Em ^s , plasmid-free, IL1403-derivative	This study
IL1403Δ <i>claRp</i> IL253 <i>claR</i>	Lac ⁻ , Cel ⁺ , Em ^r , IL1403Δ <i>claR</i> -derivative carrying pIL253 <i>claR</i>	This study
<i>E. coli</i>		
TG1	Δ(<i>hsdMS-mcrB</i>)5 Δ(<i>lac-proAB</i>) <i>supE thi-1</i> F' <i>(traD36 proAB⁺ lacI^qZΔM15)</i>	Laboratory collection
Plasmids		
pGEM-T	Amp ^r , M13ori, linear T-overhang vector	Promega
pGhost9	Em ^r , <i>repA</i> (Ts)	INRA (Maguin et al. 1996)
pGBT58	Km ^r , 10.35 kb, pSC101 replicon, carrying <i>xylE</i> under <i>trfAp</i>	(Jagura-Burdzy et al. 1992)
pIL253	Em ^r , high-copy number lactococcal vector	(Simon and Chopin 1988)
pJIM2374	Em ^r , integrative vector carrying <i>luxAB</i> genes	(Delorme et al. 1999)
Recombinant plasmids		
pJIM2374 <i>claRp</i>	Em ^r , integrative vector carrying <i>luxAB</i> under the control of the putative <i>claR</i> promoter (<i>claRp</i>)	This study
pGBT58 <i>yebEt</i>	Km ^r , pGBT58 derivative, carrying the <i>yebE</i> terminator (<i>yebEt</i>) between <i>trfAp</i> and <i>xylA</i>	This study
pGhost9Δ <i>claR</i>	Em ^r , pGhost9 derivative, carrying the <i>claR</i> upstream and downstream DNA regions	This study
pIL253 <i>claR</i>	Em ^r , pIL253 vector carrying the <i>claR</i> gene under the control of its promoter	This study
Primers ^d		
For cloning of <i>yebE</i> promoter and terminator or deletion and complementation of the <i>claR</i> gene		
<i>claRp</i> StI/ <i>claRER</i> pr	AACTGCAGGGGTGTTACATTCCAGC/GGAATTCGCTCCGCCACAAATTC	
<i>claRER</i> rv/ <i>claRS</i> all	GGAATTCGCAACTCCCAGTACTAGC/ACGCGTTCGACGCAGGCGTTGTTCT	

TGACC

yebEfor/claRrev GCTTTTGTGGTCTGATT/CGGATACTGTTTGGACC

claRSmaf/claRPstR CCCGGGCTTGCTTTGATTCTCAG/CTGCAGGCAACCTTTAGGCGAC

yebEterf/yebEterr GTGGTACCCTGTGATTGTCAGTAGCG/CATCCCATGGCTAGCGTTTCAAGCTCG

For cDNA synthesis and RT-qPCR amplification

LIBglSaF/LIBglSaR GCATGGAATCCAGTTGACGG/GCAATTCTCAAGCCTTCAGGG

LICcpAaF/LICcpAaR AAAAGACGCGCCAGAAGGTC/TGGAATCGATATCATCAACCCC

LICelBaF/LICelBaR GGAGTCAATGACCTCGCTGG/GGTTTCCAAGCGGCAAGTC

LIPtcAaF/LIPtcAaR TTATCATGAGTGGAGGAAATGCC/TTTTTCGCCTTGAGCTAAACG

LIPtcBbF/LIPtcBbR ACAGCGGATATTGATAACATGCTTG/ATCTCCGCGCATCATTCC

LIPurMaF/LIPurMaR ATTGCGTAGCCATGTGCGTC/CTGTTTCTCCACCAATCAGCG

LITufaF/LITufaR CGTGACCTCTTGAGCGAATACG/GAGTGGTTTGTGAGTGTGCGG

LIYbhEaF/LIYbhEaR CAGCAACATTTGGTCCTTGGC/TGCTTGGCTCATCGCTTTAAG

549 ^a Strains obtained in this study are deposited in the publicly accessible IBB PAS laboratory

550 culture collection.

551 ^b Amp, ampicillin; Em, erythromycin; Km, kanamycin; r, resistance; s, sensitivity.

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

553 ^d All primers were designed on the basis of the *L. lactis* IL1403 genome nucleotide sequence

554 (Bolotin et al. 2001), which is available from NCBI (<http://www.ncbi.nlm.nih.gov/genome>)

555 with accession no. AE005176. To certain primers restriction sites were added for digestion

556 with EcoRI, KpnI, NcoI, PstI and SalI.

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

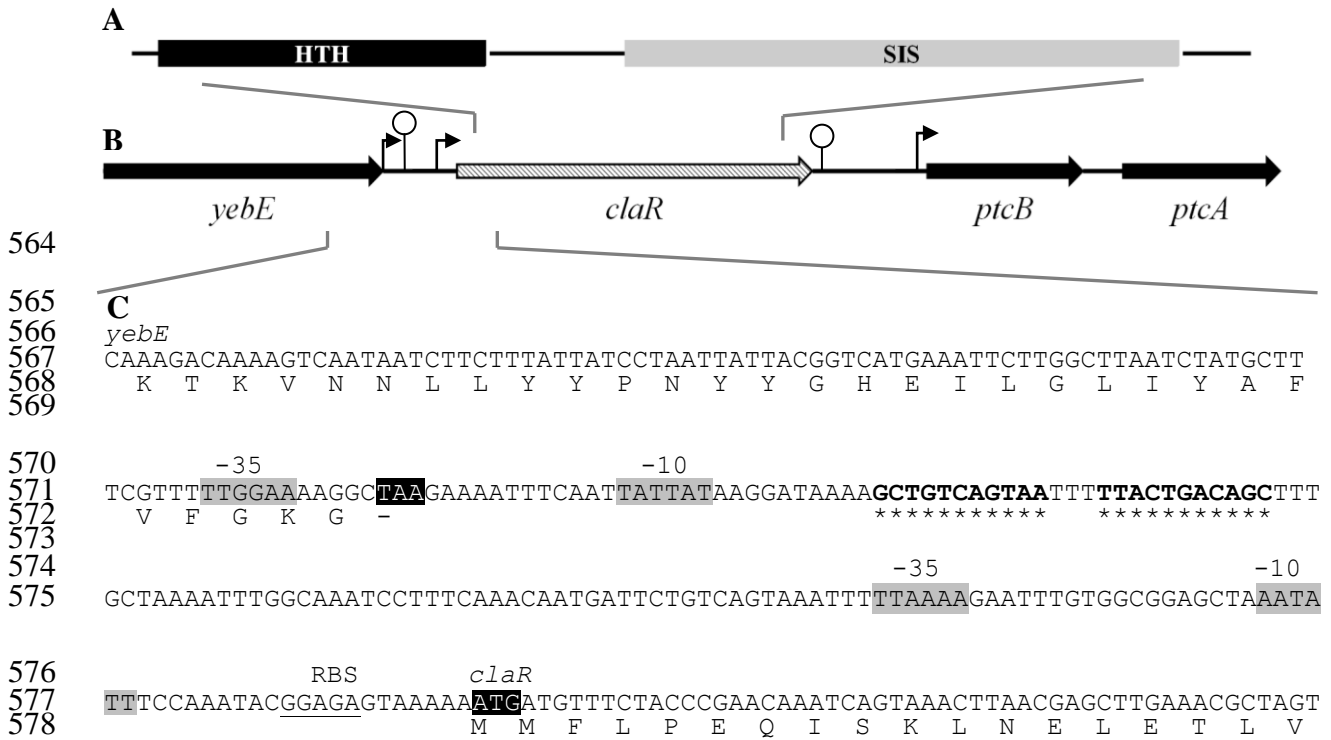
gene	Relative gene expression level							ClaR activation ratio ^a on			Cellobiose activation ratio ^b in	
	galactose		glucose		cellobiose/galactose		cellobiose	glucose	galactose	galactose/ cellobiose	IL1403	IL1403 Δ <i>claR</i>
	IL1403	IL1403 Δ <i>claR</i>	IL1403	IL1403 Δ <i>claR</i>	IL1403	IL1403 Δ <i>claR</i>	IL1403					
<i>bglS</i>	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
<i>celB</i>	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
<i>ptcA</i>	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
<i>ptcB</i>	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
<i>claR</i>	0.046±0.008	-	0.061±0.010	-	0.049±0.012	-	0.037±0.009	-	-	-	1.1	-

559 ^a ClaR activation ratio was calculated as a quotient of relative gene expression in IL1403 and IL1403 Δ *claR*.

560 ^b Cellobiose activation ratio was calculated as a quotient of relative gene expression in the wild-type strain IL1403 or IL1403 Δ *claR*
 561 grown in GalC-CDM and in Gal-CDM.

562 - not determined.

563



580 **Fig. 1.** The organization of ClaR domains (A), the *claR* chromosomal region (B) and

581 nucleotide sequence of the *yebE-claR* intergenic region (C). The ClaR domains (A) and the

582 genes of the *claR* chromosomal region (B) are drawn to scale. Stem-loop structures denote rho-

583 independent terminators. Gray-shaded sequences (C) highlight the potential -10/-35 promoter

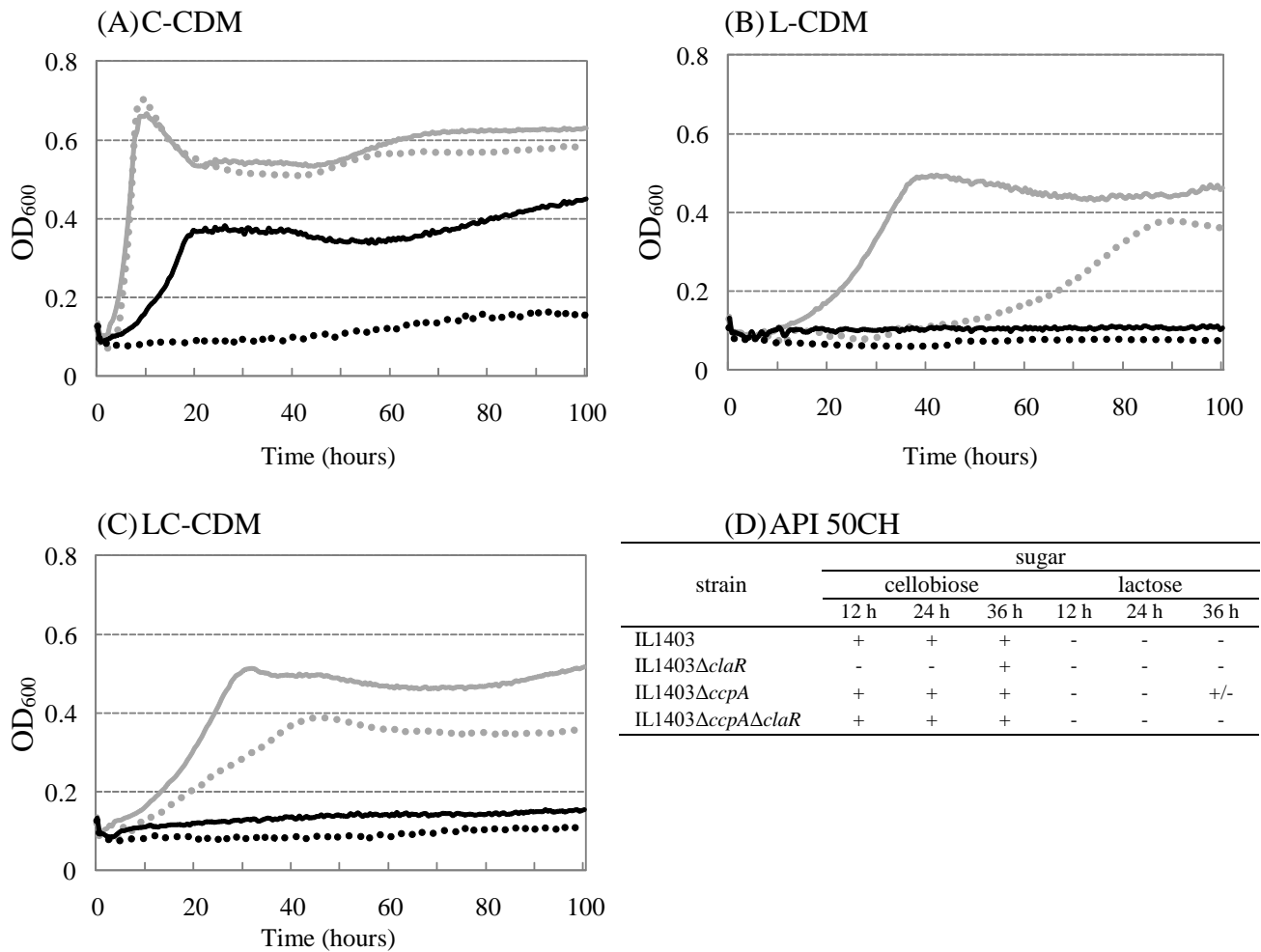
584 regions upstream of *claR*. The putative ribosome binding site (RBS) is underlined, and the start

585 and stop codons of *claR* and *yebE*, respectively, are shown as black shading. Stars following

586 *yebE* indicate a rho-independent terminator, the functionality of which was measured in this

587 work.

588



590 **Fig.2.** Growth of the *L. lactis* IL1403 wild-type strain (grey dots) and its derivatives:
 591 IL1403 Δ *ccpA* (grey line), IL1403 Δ *claR* (black dots) and IL1403 Δ *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: IL1403 Δ *ccpA*, IL1403 Δ *claR* and
 596 IL1403 Δ *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)

