The Lcn972-bacteriocin plasmid pBL1 impairs cellobiose metabolism in *Lactococcus lactis*

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Abstract

pBL1 is a *Lactococcus lactis* theta-replicating 10.9-kbp plasmid that encodes the synthetic machinery of the bacteriocin Lcn972. In this work, the transcriptomes of exponentially growing *L. lactis* with and without pBL1 were compared. A discrete response was observed with a total of ten genes showing significantly changed expression. Up-regulation of the lactococcal oligopeptide uptake system (*opp)* was observed, likely linked to a higher nitrogen demand required for Lcn972 biosynthesis. Strikingly, *celB* coding for the membrane porter IIC of the cellobiose-PTS and the upstream gene *llmg0186* were down-regulated. Growth profiles for *L. lactis* strains MG1363, MG1363/pBL1 and MG1363Δ*celB* grown in CDM-cellobiose confirmed slower growth of pBL1 and Δ*celB* while no differences were scored on glucose. The presence of pBL1 shifted the fermentation products towards a mixed acid profile and promoted substantial changes in intracellular pool sizes for glycolytic intermediates in cellobiose-growing cells as determined by HPLC and NMR. Overall, these data support the genetic evidence of a constriction in cellobiose uptake. Notably, several cell wall precursors accumulated, while other UDP-activated sugars pools were lower, which could reflect rerouting of precursors towards the production of structural or storage polysaccharides. Moreover, slow cellobiose-growing cells and those lacking *celB* were more tolerant to Lcn972 than cellobiose adapted cells. Thus, down-regulation of *celB* could help to build-up a response against the antimicrobial activity of Lcn972 enhancing self-immunity of the producer cells.

Keywords: bacteriocin, PTS, cellobiose, NMR, *Lactococcus*.

INTRODUCTION

Bacteriocins are ribosomally synthesized bacterial peptides with antimicrobial activity. Their production is a widespread trait in lactic acid bacteria (LAB). Bacteriocins form a rather structurally diverse group encompassing post-translationally modified lantibiotics (Class I), non-modified heat resistant peptides (Class II), heat labile proteins (Class III) and circular bacteriocins (Class IV) (10, 26 and references therein). Many LAB bacteriocins are inhibitory towards a wide panel of Gram positive bacteria including relevant pathogen and food spoilage bacteria. Thereby, a major effort has been made in the last decades to understand their ecological role in complex ecosystems (e.g. fermented foods and gastrointestinal tract) and the molecular basis of their inhibitory activity for rationalizing their use as natural preservatives in food or as promising anti-infectives (19, 23).

Although most LAB bacteriocins were shown to act as membrane permeabilizers when added at high concentrations, it is being recognized that bacteriocin killing is target mediated. Many bacteriocins make use of receptors or docking molecules present in the cell envelope of susceptible strains prior to pore formation. Nisin and many other structurally-related lantibiotics specifically bind to the cell wall precursor lipid II (5, 6). Moreover, several class II bacteriocins such as lactococcin A, sakacin A and enterocin P form a complex with the membrane component IIC of the mannose-PTS (phosphoenolpyruvate-depedent phosphotransferase system) transporter (16, 29).

Functional PTSs consist of two general proteins, enzyme I (*ptsI*) and the heat-stable phosphocarrier protein HPr (*ptsH*), and sugar-specific permeases or enzyme II complexes. The latter catalyze the translocation and concomitant phosphorylation of several different sugars and usually consist of three to four proteins or protein domains,

77 namely, IIA, IIB, IIC and, when present, IID. Phosphoryl relay proceeds sequentially
78 from PEP to EI, HPr, IIA, IIB and the incoming sugar, which is transported across the
79 membrane via the IIC porter (15, 39). Expression of PTSs coding genes is tightly
80 regulated and those of alternative sugars are often subjected to carbon catabolite
81 repression (14). Beyond the primary function of sugar transport, PTSs play roles in
82 various processes central to the physiology of the cell, including a wide number of
83 mechanisms for metabolic and transcriptional regulation (15). As described above, they
84 also play a role as receptors of some class II bacteriocins. In fact, resistance to class II
85 bacteriocins has been often linked to absence or repression of the genes coding for
86 mannose-PTS involved in glucose uptake (11, 16, 22, 41). Thereby, one immediate
87 consequence of class II bacteriocin resistance is impaired growth on glucose, while
88 utilization of other sugars is favored (28, 48).

89 Based on the proposed use of LAB bacteriocins as food preservatives and to
90 provide enough quantities for structure-function studies, several studies have focused on
91 improving bacteriocin production and/or reducing production costs by using byproducts
92 (12 and references therein). Engineering bacteriocin immunity has been proved to
93 increase nisin yields in *Lactococcus lactis* and in heterologous hosts (24, 46). Moreover,
94 bacteriocin clusters have been suggested as a food grade alternative to antibiotic
95 resistant markers (47). In spite of this, little is known about the impact that synthesis of
96 bacteriocins may have on the physiology of the producing strain. This issue is
97 particularly relevant when bacteriocin production is seen as a valuable technological
98 trait and needs to be transferred to industrial strains. Recently, Fallico *et al.* (18)
99 reported that conjugation of the plasmid pMRC01 carrying the lacticin 3147 bacteriocin
100 cassette imposed a metabolic burden on several *L. lactis* starter strains.

In this work, we have studied the impact of the plasmid pBL1 encoding the bacteriocin lactococcin 972 (Lcn972) on *L. lactis*. Lcn972 is an atypical 66-aa non-modified bacteriocin synthesized by *L. lactis* IPLA972. It targets exclusively the *Lactococcus* genus and thus far is the only non-lantibiotic that binds to lipid II, inhibiting cell wall biosynthesis, without disrupting membrane integrity (34, 36). The synthetic machinery is encoded by the 11 kbp-plasmid pBL1 (GenBank AF242367.1) and consists of a structural gene and two other open-reading-frames encoding a putative ABC transporter, presumably involved in self-immunity. Introduction of pBL1 in *L. lactis* rendered strains that were able to produce Lcn972 and that were immune to it, without showing other particular trait (34, 36). In this study, however, wide-genome transcriptomics revealed changes in the expression of genes directly related to oligopeptide and sugar uptake. Bearing in mind the role of sugar-PTSs in the mode of action of other bacteriocins and in bacterial metabolism, we have characterized in more detail the response of *L. lactis* to the presence of the bacteriocin coding plasmid pBL1.

MATERIALS AND METHODS

Bacteria, plasmids and culture conditions. Strains and plasmids used in this study are listed in Table 1. Lactococcal strains were routinely grown in M17 (Oxoid) with (0.5% wt/vol, ~27.5 mM) glucose at 30ºC (optimal growth temperature) or 37°C (when required for genetic manipulation). *E. coli* DH10B was used for intermediate cloning and grown on 2xYT (44) at 37ºC with shaking. For physiological characterization lactococcal cultures were grown statically and without pH control (initial pH 6.5), at 30°C, in Chemically Defined Medium (CDM) containing 1% (wt/vol) glucose (~55.5 mM) or cellobiose (~29.2 mM), in rubber-stoppered bottles, as described previously (8). Growth was started by addition of a pre-culture (inoculum) in early stationary phase to an initial optical density at 600 nm (OD_{600}) of approximately 0.05. Pre-cultures were grown in glucose-CDM, except for adapted cells in which the substrate was the same as for the culture; all other conditions were as above. When necessary, erythromycin was used at a final concentration of 5 μg ml^{-1} and ampicillin at 100 μg ml^{-1}. Growth was monitored by measuring the optical density at 600 nm. Growth rates (μ) were calculated through linear regressions of the plots of ln(OD_{600}) versus time during the exponential growth phase. Growth rates and other growth parameters were analyzed using the Instat software (GraphPad Software).

Transcriptome analysis. Genome-wide transcriptional experiments were performed using DNA microarrays containing 2457 annotated genes in the genome of *L. lactis* MG1363 and were essentially carried out following the methods for cell disruption, RNA isolation, RNA quality control, complementary DNA synthesis, indirect labeling, hybridization, and scanning as described (50). RNA from three biological replicates was extracted from exponentially growing (OD_{600} of 0.4) *L. lactis* MG1614 and MG1614.2

142 cultures in GM17 at 30 ºC. Data was processed as described previously (37). The DNA
143 microarray data is available at Gene Expression Omnibus (GEO) repository under
144 accession number GSE30625.
145
146 **Construction of *L. lactis* MG1363Δ*celB* and pBL1E.** Standard molecular cloning
147 techniques were followed as described elsewhere (44). Restriction enzymes were
148 purchased from Takara (Otsu, Shiga, Japan) and T4 ligase from Invitrogen (Barcelona,
149 Spain). Oligonucleotides were supplied by Sigma (Madrid, Spain) and shown in Table
150 2. PCRs were carried out using PuRe Taq Ready-to-go PCR Beads (GE Healthcare,
151 Buckinghamshire, UK). celB-1 and celB-4 primers were used to amplify a 2.4 kbp
152 chromosomal fragment containing *celB* plus 0.9 kbp flanking regions. PCR conditions
153 were: 95 ºC 5' (1x), 95 ºC 30'' - 60 ºC 30'' - 72 ºC 1'- (35x), 72 ºC 10' (1x). The
154 resulting amplicon was cloned in the E. *coli* plamid pCR2.1, generating pCR::celB4-1.
155 This plasmid was *Hinc*II-digested and religated, generating pCR::dcelB lacking a 1.0
156 kbp *Hinc*II internal fragment. The incomplete *celB* gene plus flanking regions were
157 released from pCR::dcelB as a 1.4 kpb *Xba*I-*Spe*I fragment and subsequently cloned in
158 the thermosensitive *L. lactis* plasmid pGhost9 digested with *Spe*I to obtain
159 pGhost::dcelB. *L. lactis* MG1363 was transformed with pGhost::dcelB and first and
160 second recombination events were followed essentially as previously described (33).
161 *celB* deletion was confirmed by PCR with appropriate primer pairs and DNA
162 sequencing.
163 To construct the recombinant plasmid pBL1E, the erythromycin resistance gene
164 *erm* was excised from pNG8048 (51) by *Sma*I-*Eco*RV restriction and ligated to the
165 unique *Eco*RV site present in pBL1_*orf4*. *L. lactis* MG1363/pBL1E transformants were
166 selected on GM17 with erythromycin 5 μg ml^{-1}.

Reverse transcriptase quantitative PCR. RNA was extracted using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare) and the RNA concentration was determined by absorbance at 260 nm. cDNA was generated with the iScript cDNA Synthesis Kit (Bio-Rad). PCR amplification was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Primers used for RT-qPCR are listed in Table 2. Amplification was carried out in 25 µL containing either 0.01 or 0.002 µg cDNA (according to the expected expression levels), 1x Power SYBR Green (Applied Biosystems) and each primer at a concentration of 0.56 µM. After incubation at 95 ºC for 10 min, amplification proceeded with 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Standard curves were generated by plotting the cycle threshold values (Ct) of reactions performed on serial dilutions of cDNA against the logarithm of cDNA concentrations. cDNA concentrations were correlated to quantify relative gene expression levels. The housekeeping gene *tuf*, encoding the elongation factor Tu, was used to normalize (43).

Enzyme-linked immunoassay detection of Lcn972. The bacteriocin Lcn972 in culture supernatants was quantified by a non-competitive enzyme-linked immunoassay (NCI-ELISA) with rabbit polyclonal antibodies against Lcn972 (1). Primary and secondary antibodies were used at 1:1,000 and 1:40,000 dilutions, respectively. Pure Lcn972 (15 to 1 µg/ml) was used as standard.

Lcn972 susceptibility tests. Minimal Inhibitory Concentration (MIC) was assayed by the broth microdilution method in GM17 as described elsewhere (34). Dose-response curves were carried out in CDM in microtiter plates. Overnight cultures in CDM-

192 glucose or CDM-cellobiose were adjusted to OD_{600} of 0.1 and 100 µl were used to
193 inoculate wells containing Lcn972 from 9.6 to 0 µg/ml. Growth was followed in a
194 microtiter reader (BioRad) at 30 ºC until control cultures without Lcn972 reached an
195 OD_{600} of 0.7-0.8 that was taken as 100% growth. For challenging tests, exponentially
196 growing cultures in CDM-cellobiose at OD_{600} of 0.2 were treated with Lcn972 at 0.1
197 µM (5x the MIC). Sodium phosphate buffer 50 mM, pH 6.8, was used as control.
198 Cultures were incubated for 1 h at 30 ºC and appropriated ten-fold dilutions were plated
199 on GM17 agar plates for counting. Survival (%) was defined as cfu/ml of treated
200 cultures divided by cfu/ml in the control samples.
201
202 **Quantification of fermentation products during growth.** Samples (2 ml) were taken
203 at different growth stages, centrifuged (13200 × *g*, 5 min, 4ºC), filtered (0.22 µm), and
204 the supernatant solutions were stored at -20ºC until analysis by high performance liquid
205 chromatography (HPLC). Substrate and fermentation end products (lactate, acetate,
206 ethanol, formate, acetoin, 2,3-butanediol, pyruvate) were quantified in an HPLC
207 apparatus equipped with a refractive index detector (Shodex RI-101, Showa Denko K.
208 K., Japan) using an HPX-87H anion exchange column (Bio-Rad Laboratories Inc.,
209 California, USA) at 60ºC, with 5 mM H_2SO_4 as the elution fluid and a flow rate of
210 0.5 ml min^{-1}. Cellobiose was similarly quantified in a ICSep ION-300 column preceded
211 by ICSep ICE-GC-801 precolumn (Transgenomic, San Jose, CA), at 65 ºC in 8.5 mM
212 H_2SO_4 at 0.4 ml min^{-1} using a refractive index detector RI2414 (Waters, Milford, MA).
213 For the yield calculation, two time-points, one immediately after inoculation and
214 the other at the time of growth arrest (t30, non-adapted cells), were considered. ATP
215 production was calculated from the fermentation products, assuming that all ATP was
216 synthesized by substrate-level phosphorylation. The average specific consumption rates

of cellobiose were estimated from a first-order derivative of a polynomial fit of the observed substrate consumption time series.

Determination of intracellular metabolites during growth. Ethanol extracts for analysis by ^{31}P-NMR and quantification of phosphorylated metabolites in MG1363/pBL1 and control strain at mid-exponential growth phase were prepared as described elsewhere (42). The dried extracts were dissolved in 2 ml of aqueous solution containing 5 mM EDTA and 12.5% (vol/vol) $^{2}H_2O$ (final pH approximately 6.5). Assignment of resonances and quantification of phosphorylated metabolites was based on previous studies (38, 42) or by spiking the NMR-sample extracts with the suspected, pure compounds. Intracellular metabolite concentrations were calculated using a value of 2.9 µl (mg of protein)$^{-1}$ for the intracellular volume of *L. lactis* (40). The reported values for intracellular phosphorylated compounds are averages of two independent growth experiments and the accuracy was around 15%.

RESULTS

Transcriptional analysis of *L. lactis* with the bacteriocin Lcn972 plasmid pBL1.

To get a deeper insight into the impact of the presence of the Lcn972 encoding plasmid pBL1 may exert in *L. lactis,* a genome wide transcriptional analysis was carried out with *L. lactis* MG1614.2, carrying the bacteriocin plasmid pBL1, and compared to the parental strain *L. lactis* MG1614 when growing under standard laboratory conditions in GM17. A relative discrete transcriptional response was observed with a total of 10 genes showing significantly ($p<0.001$) changed expression over a factor of two (Table 3). Besides those coding for proteins of unknown function, up-regulation of the lactococcal oligopeptide uptake system was observed. *oppA* and *oppB* were clearly overexpressed but other members of the system (*oppF, oppC, oppD,* and the endopeptidase *pepO*) were also up-regulated although just below the established cut-off levels (see public array data GSE30625). Overexpression of genes coding for proteins involved in DNA rearrangement/mobilization was also noted. This could be due to cross-hybridization with transposases present in the composite plasmid pBL1. Indeed, pBL1_*orf9* shares 86% and 99% identity at the nucleotide level with *llmg0674_tnp1297* and *llmg0717_tnp946*, respectively, according to BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The same could have accounted for the hypothetical acetyl transferase gene *llmg0676* to which pBL1_*orf1* shows 99% identity.

The cellobiose-specific PTS permease (IIC^{Cel} domain) *celB* and the upstream *llmg0186* were the only genes down-regulated in the presence of pBL1 (Table 3). *llmg0186* codes for a conserved hypothetical protein, also identified in *L. lactis* IL1403 (*ybhE*), which might be functionally related to *celB* as both genes seem to form a single transcriptional unit (3). Absence of specific PTS permeases has been phenotypically linked to resistance to several class II baceriocins. Moreover, PTSs are main players in

bacterial metabolism mediating sugar uptake, the main energy source in *Lactococcus*. Thus, we proceeded to further investigate *celB* repression in the presence of pBL1.

Expression of cellobiose-related genes in the presence of pBL1.

It has been recently shown that point mutations, which might be selected within very closely related *L. lactis* strains, may have a pronounced effect on gene expression and, subsequently, on particular phenotypes. This has been exemplified by comparative transcriptomics of *L. lactis* MG1363 and its derivative *L. lactis* NZ9000, generated by the insertion of the two component system *nisKR* that allows nisin inducible gene expression. Specific point mutations in the latter strain accounted for altered expression of several genes involved in carbohydrate metabolism that translated into different growth rates on specific sugars (31). The reference strain we used for the transcriptional analysis was *L. lactis* MG1614, an antibiotic resistant derivative of *L. lactis* MG1363 which is poorly characterized and whose genome sequence is unknown (20). To avoid possible interferences and unequivocally confirm by RT-qPCR that repression of *celB* is a direct consequence of the presence of pBL1, we transformed this plasmid into *L. lactis* MG1363 (Table 1). As shown later (see table 5), *L. lactis* MG1363/pBL1 transformants were able to synthesize Lcn972 and were resistant to it. Furthermore, we generated a *celB* knockout mutant MG1363Δ*celB* to evaluate the consequences of *celB* repression.

Initially, an insight into the *celB* operon structure was carried out by RT-PCR. The genomic context of *celB* in MG1363 is similar to that described in *L. lactis* IL1403 (3). *celB* is flanked upstream by the conserved gene *llmg0186* and downstream by two small *orfs* followed by *bglS* potentially encoding a phospho-β-glucosidase involved in cellobiose hydrolysis. The presence of mRNA encompassing *llmg0186* and *celB* in *L. lactis* MG1363 cells growing on cellobiose was confirmed (data not shown). This result

284 is in agreement with the co-downregulation of both genes noted in the transcriptomic
285 analysis (Table 3). On the contrary, we failed to detect a putative *celB-bglS* mRNA by
286 RT-PCR.

287 The expression of cellobiose-related genes, i.e. *celB*, *ptcC*, and *bglS*, in the
288 presence of pBL1 was determined by RT-qPCR (Table 4) using total RNA isolated
289 from glucose- and cellobiose-growing cultures in CDM. *ptcC* was also included as a
290 cellobiose/glucose IIC porter (8, 30). In the presence of cellobiose, the three analysed
291 genes were induced in MG1363, but *celB* showed the highest fold-induction which
292 supports its role as a main cellobiose uptake system in *L. lactis* (Table 4). Interestingly,
293 loss of CelB (MG1363Δ*celB*) resulted in a higher expression ratio of *ptcC*, possibly as a
294 way to counteract the lack of *celB* and facilitate cellobiose uptake. On the contrary,
295 induction of *celB* and *bglS* was inhibited 5-fold and *ptcC* 3-fold by the presence of
296 pBL1 confirming the hypothesis raised from the transcriptomic results that pBL1
297 downregulates *celB*.

298

299 **pBL1 impairs growth on cellobiose**

300 Based on the differential expression of cellobiose-related genes in the presence of
301 pBL1, we proceeded to determine possible effects in the catabolism of relevant sugars.
302 As *celB* encodes the cellobiose PTS porter, cellobiose was an obvious choice to use as
303 carbon source in physiological studies. Glucose was used as control sugar.

304 Slim differences in growth, although not statistically significant ($P>0.05$), were
305 observed for glucose-grown cells subcultured in fresh glucose-CDM as the growth rates
306 of MG1363/pBL1 and MG1363Δ*celB* were slightly reduced when compared to that of
307 MG1363 (Fig. 1A). Similarly, pBL1 did not affect the homolactic behavior of MG1363.
308 In contrast, subculturing glucose-grown cells in CDM supplemented with cellobiose

309 (i.e. non-adapted cells) resulted in major and very significant (P<0.01) differences in the
310 growth profiles. Cultures of MG1363 and derivatives were characterized by long lag
311 phases, and exponential growth started only 18-19 h after inoculation (Fig. 1B). This
312 was not surprising as this behavior was already reported for *L. lactis* MG1363 (31). The
313 growth rate and the maximal biomass produced were substantially lower for strains
314 MG1363/pBL1 and MG1363Δ*celB* than for the control strain. These remarkable
315 differences led us to examine in greater detail the kinetics of substrate consumption and
316 fermentation product formation in non-adapted cells growing on cellobiose (Fig. 2). The
317 three strains showed a mixed acid fermentation profile, but the product distribution was
318 markedly different in the parent strain as compared with the strain carrying pBL1 or
319 MG1363Δ*celB*. In the manipulated strains, formate, acetate and ethanol, ratio 2:1:1,
320 were the major products, while lactate accounted for less than 20% of the substrate
321 consumed. In contrast, lactate was the main product from cellobiose metabolism in *L.*
322 *lactis* MG1363, and curiously its yield increased dramatically along growth from 0.4 at
323 mid-exponential (T24) to 1.3 in late stationary phase (T42). At the time of growth arrest
324 (T30), MG1363 had consumed about 50% of the initial substrate, whereas
325 MG1363/pBL1 and MG1363Δ*celB* used only 30% of the cellobiose in the medium. The
326 biomass yield was, however, similar in MG1363 and the Δ*celB* mutant (about 17 g mol^{-1}
327 of substrate) and slightly lower in the presence of pBL1 (about 16.2 g mol^{-1} of
328 substrate), correlating well with the OD_{600} values determined (Fig. 1B). In view of these
329 data, it is reasonable to speculate that the observed differences in growth rate and
330 maximal biomass in MG1363 as compared to MG1363/pBL1 and MG1363Δ*celB* are
331 directly associated with cellobiose transport. In line, the average cellobiose
332 consumption rate during exponential growth was higher in MG1363 (3.6 mmol g^{-1} h^{-1})

than in the presence of pBL1 (2.1 mmol g^{-1} h^{-1}) or the absence of *celB* (2.3 mmol g^{-1} h^{-1}).

This view was further supported by the growth profiles obtained for adapted cells, i.e. early stationary cellobiose-grown pre-cultures subcultured in CDM-cellobiose (Fig. 1C). Adaptation abolished the long lag-phases (maximal growth after 1h at 30ºC) and resulted in improved growth rate (20-35% increase) for all strains. Nevertheless, both the maximal biomass produced and the growth rate were still considerably higher in strain MG1363 than MG1363/pBL1 or MG1363Δ*celB*. Adaptation also promoted the formation of lactate both in the presence or absence of pBL1 in MG1363 (Fig. 1C).

pBL1 changes the intracellular dynamics of phosphorylated metabolites

Prompted by the notable effect of pBL1 on growth properties and end product profiles of MG1363 we asked whether intracellular metabolite levels would also be affected by the plasmid during growth on cellobiose. Glucose was used as a control condition. As expected, pBL1 had no effect on the pool sizes of glycolytic intermediates (Fig. 3A) or sugar-nucleotides (Fig. 3C) during growth on glucose as determined by ^{31}P-NMR in cell extracts. Contrastingly, pBL1 promoted substantial changes in intracellular pool sizes in cellobiose-growing cells. Fructose 1,6-bisphosphate (FBP), the major mid-exponential glycolytic intermediate in glucose-growing cells and in cellobiose-growing MG1363, was reduced by about two times (Fig. 3A). A similar effect was observed for other upper glycolytic metabolites (DHAP and G6P). The concentration of cellobiose 6-phosphate, the product of the transport step, was also 2-fold lower in the presence of pBL1. On the other hand, the lower glycolytic metabolites 3-PGA, 2-PGA and PEP, showed increased concentrations.

Of note was the drastic effect of pBL1 on the concentration of the cell wall cytoplasmic precursors, UDP-N-acetylmuramoyl-pentapeptide (UDP-MurNAc-pPep) and UDP-N-acetyl muramic acid (UDP-MurNAc) (Fig. 3D). The latter only accumulated in cellobiose-growing MG1363/pBL1, while UDP-MurNAc-pPep increased by about 14-fold in this strain. In contrast, the other peptidoglycan cytoplasmic precursor, UDP-N-acteylglucosamine (UDP-GlcNAc) was slightly lower, as were all other UDP-activated sugars detected and 5-phosphorylribose 1-pyrophosphate.

Contribution of impaired growth on cellobiose to production of and resistance to Lcn972

According to the physiological data, the presence of pBL1 correlated well with defective growth of *L. lactis* on cellobiose in a similar fashion as a non-functional *celB*. Since the main phenotype that could be attributed to pBL1 is the production of the bacteriocin Lcn972 (35, 45), we attempted to establish if there was a link between Lcn972 synthesis or immunity and impaired growth on cellobiose.

We determined Lcn972 production in supernatants from *L. lactis* MG1363/pBL1 grown on glucose (i.e. low *celB* expression) or cellobiose (i.e. high *celB* expression) and in the Δ*celB* background where *celB* is not present. In this case, we had to make use of pBL1E in which the erythromycin resistance marker was cloned in the unique *Eco*RV site of pBL1 disrupting pBL1_*orf4*, because we were unable to recover any *L. lactis* Δ*celB*/pBL1 transformants by Lcn972 selection. Supernatant samples were taken at the transition towards the stationary phase (OD_{600} of 2.8 and 1.6 in glucose and cellobiose cultures, respectively). As shown in Table 5, the mutation in pBL1E did not affect production of Lcn972 as similar yields were detected compared to the wildtype plasmid

382 pBL1 (Table 5). No large differences in Lcn972 production were observed when the
383 strains were growing in glucose. On the contrary, in cellobiose-growing cultures, yields
384 were somewhat higher in the Δ*celB* background, sustaining the idea that cells lacking
385 this gene may support higher Lcn972 production levels.

386 Next, we hypothesized that CelB may act as a putative receptor to facilitate
387 Lcn972 antimicrobial activity and, consequently, producer cells would have a tendency
388 to suppress gene expression. According to MIC values in GM17, *L. lactis* MG1363 and
389 Δ*celB* were equally susceptible to Lcn972 (MIC=0.15 μg/ml). Moreover, dose-response
390 curves to increasing Lcn972 concentrations were essentially identical for *L. lactis*
391 MG1363 and Δ*celB* regardless whether cultures were grown on glucose or cellobiose
392 (Fig. 4A). In this light, in contrast to the mannose PTS which is targeted by several class
393 II bacteriocins and determines bacteriocin activity, CelB is unlikely to be an essential
394 receptor for Lcn972. Interestingly, both strains wildtype and Δ*celB* were somewhat
395 more susceptible to Lcn972 when growing on cellobiose than on glucose (Fig.4A).
396 Thereby, we asked whether repression of *celB* and the subsequent metabolic changes
397 could make lactococcal cells able to cope better with the presence of Lcn972, a scenario
398 that Lcn972-producing cells have to face. To test this, early exponentially cellobiose
399 growing (OD_{600}=0.2) non-adapted *L. lactis* MG1363 and Δ*celB* cells were challenged
400 with Lcn972 and survival was scored (Fig. 4B). The highest percentage of surviving
401 cells was found for *L. lactis* Δ*celB* compared to the wildtype *L. lactis* MG1363.
402 Moreover, resistance to Lcn972 decreased further when *L. lactis* MG1363 had been
403 previously subcultured in CDM-cellobiose for 30 generations (adapted cells) prior to the
404 treatment. In this case, only 0.8% of the cells survived (Fig. 4B). These results sustain
405 the hypothesis that downregulation of *celB* somehow triggers a defence mechanism
406 against the antimicrobial activity of Lcn972. Furthermore, it was also confirmed by RT-

qPCR that *celB* RNA levels in *L. lactis* MG1363 cellobiose adapted cells were 6.4 times higher than in non-adapted cells when growing on glucose (data not shown). This suggests that adaptation to cellobiose in *L. lactis* MG1363 may imply increased basal expression of *celB* in the presence of glucose.

DISCUSSION

The plasmid pBL1 had been previously shown to encode for the production of and immunity to the bacteriocin Lcn972 (35, 45). This plasmid could be transferred into the susceptible *L. lactis* MG1614 conferring to the new transformants the ability to produce Lcn972 while any other obvious phenotype remained elusive under standard laboratory conditions (35). However, the genome-wide transcriptional analysis carried out in this work revealed unexpected changes in gene expression that suggested that bacteriocin synthesis is not gratuitous for producing cells and may impose a metabolic burden.

Although we have not further investigated, it seems reasonable to speculate that the overexpression of genes involved in oligopeptide transport in the presence of pBL1 responds to a higher nitrogen demand needed for Lcn972 biosynthesis. *L. lactis* is auxotrophic for multiple amino acids and depends on its proteolytic system and peptide uptake for growth. Moreover, the *opp* operon is highly repressed by the presence of peptides in the growth medium via the pleiotropic transcriptional repressor CodY that senses the intracellular pool of branched-chain amino acids (BCAAs), co-repressors of CodY (13, 17 and references therein). The intracellular amino acid content in Lcn972-producing cultures would be supposedly lower relieving *opp* from CodY repression.

Another response to the presence of pBL1 deduced from the microarray data was repression of *llmg0186* and *celB* involved in cellobiose metabolism. This PTS has been more deeply characterized in *L. lactis* IL1403, but our results showed that the structural organization is similar and both genes form an operon also in *L. lactis* MG1363. The specific downregulation of *celB* by pBL1 was further confirmed by transforming this plasmid into *L. lactis* MG1363 and demonstrating by RT-qPCR that *celB* RNA levels were lower in the presence of pBL1. In this way, the possibility of strain to strain variation based on non-identified mutations prompted by the plasmid was discarded.

438 Moreover, although to a lesser extent, induction of the other cellobiose transporter gene
439 *ptcC* was also inhibited by the presence of pBL1 supporting the notion that cellobiose
440 metabolism was specifically targeted by pBL1. It is worth mentioning that *celB* RNA
441 levels were almost identical between cells growing on glucose regardless of the
442 presence of pBL1 (expression ratio 1.2 in MG1363 *vs* pBL1). This observation is not in
443 agreement with the initial transcriptomic analysis carried out with cultures grown on
444 glucose in the complex medium M17. In *L. lactis* IL1403, induction of *celB* requires the
445 presence of cellobiose (3). Residual dextrins present in the formulation of M17 may be
446 enough to induce *celB* expression in the control cells and, thereby, making more evident
447 the repression posed by pBL1.

448 The genetic evidence that cellobiose uptake was hindered in Lcn972-producing
449 cells was further demonstrated by the subsequent physiological studies. All the results
450 are in agreement with a constrained sugar uptake based on the lower growth rate in
451 CDM-cellobiose, the more pronounced mixed acid fermentation profile and the lower
452 substrate consumption rate observed in *L. lactis* MG1363/pBL1. Moreover, these
453 variables paralleled those defined for the *celB*-defective strain. This view was further
454 supported by the differences found in the pool of internal metabolites. Overall, the pool
455 sizes of glycolytic metabolites reflect a constriction in cellobiose utilization, which most
456 likely arises from the transport step limitation, and are in accordance to previous studies
457 on mutants lacking particular sugar PTSs (8).

458 The lower pool sizes of UDP-activated sugars and/or aminosugars might reflect
459 rerouting carbon flux to the production of structural or storage polysaccharides in the
460 presence of pBL1. Moreover, since the lipid carrier C-55 is used both for exocellular
461 polysaccharide biosynthesis and peptidoglycan biosynthesis, a higher demand for the
462 former would lead to the accumulation of cell wall precursors as previously described

463 (42). Lcn972 itself could also contribute as it is a cell wall active bacteriocin that binds
464 to lipid II precluding its incorporation into preexisting peptidoglycan (34). Recently, it
465 has been shown that *L. lactis* MG1363 is able to synthesize a cell wall polysaccharide
466 pellicle that acts as a protective barrier (9). Curiously, dense cell suspensions of
467 MG1363/pBL1 were considerably slimier than those of MG1363 (data not shown).
468 Whether pBL1 induces the synthesis of this protective pellicle is currently under
469 investigation.

470 For some bacteriocins, it has been shown that engineering bacteriocin immunity
471 leads to higher bacteriocin production yields (25, 27) showing that self-toxicity poses a
472 burden to increase bacteriocin productivity. Moreover, downregulation of key enzymes
473 involved in sugar metabolism has been shown to be involved in tolerance to antibiotics
474 (4). On this background, the observed metabolic response of *L. lactis* to the presence of
475 pBL1 suggests that pBL1 carrying cells seem to mount a response to counteract the
476 antimicrobial activity of Lcn972, even in the presence of the putative immune system,
477 and signalling might occur via downregulation of *celB*. In favour of this hypothesis is: i)
478 the fact that slightly higher Lcn972 yields were obtained in a Δ*celB* background, ii) that
479 susceptibility of *L. lactis* to Lcn972 increases when growing on cellobiose and, iii) that
480 *L. lactis* Δ*celB* is more tolerant than the wildtype. Moreover, *celB* is also downregulated
481 in *L. lactis* strains resistant to the bacteriocin Lcn972 (our own unpublished results)
482 supporting its role in self-protection against Lcn972. On the other hand, CelB itself
483 might also play a role as a docking molecule to facilitate access of Lcn972 to its target
484 lipid II. However, contrary to the mannose PTSs which are targeted by class II
485 bacteriocins (16, 22, 29, 41), CelB seems to be dispensable because *L. lactis* Δ*celB* are
486 still susceptible to Lcn972.

Beyond the significance of tuning *celB* expression as a trigger to increase tolerance of *L. lactis* MG1363 to Lcn972, our results have also added some hints on cellobiose metabolism in this strain which support future research on this particular PTS. First, *celB* seems to be the main cellobiose transporter in this strain as it is highly induced in cellobiose growing cultures and at levels more than 70-times higher than *ptcC.* Moreover, co-regulation of *bglS,* demonstrated by both induction by cellobiose and similar inhibition rate posed by pBL1, supports its role as the putative phospho-β-glucosidase responsible of cleavage of cellobiose-6-phosphate as described in IL1403 (2). Our data are also in agreement with the recent report showing that rapid growth of *L. lactis* MG1363 on cellobiose is preceded by the induction of cellobiose-specific genes (31). In this regard, our results showed that adaptation seems to rely on the higher basal expression levels of the operon *llmg0186-celB*, and once the cells were grown on cellobiose, the initial lag phase is no longer observed. The underlying molecular mechanism remains to be clarified.

Many references in the literature regarding altered carbon metabolism have been linked to class IIa bacteriocin resistance (7, 28, 48, 49). However, some other practical consequences in the field of bacteriocin production stems from the results of this work. In the case of the bacteriocin Lcn972, choice of inexpensive or renewable sources rich on dextrins or cellobiose as substrates should be avoided as lower cell biomass will be reached. Furthermore, *celB* has been shown to be involved in lactose uptake by *L. lactis* lacking a lactose-specific PTS (3), meaning that Lcn972 production by the recombinant *L. lactis* MG1363/pBL1 would be seriously compromised in milk or dairy products where lactose is the main available carbohydrate.

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689 **Figure legends**

690 **Figure 1.** Growth profiles of *L. lactis* MG1363 and its isogenic strains MG1363/pBL1
691 and MG1363Δ*celB* on glucose or cellobiose. Cultures were grown in CDM
692 supplemented with 1% (wt/vol) sugar substrate at 30ºC, in rubber-stoppered bottles
693 without pH control (initial pH 6.5). (A) Pre-culture and culture grown on glucose. (B)
694 Pre-culture grown on glucose and culture on cellobiose (non-adapted cells). (C) Pre-
695 culture and culture grown on cellobiose (adapted cells). Growth rate (μ), maximal
696 OD_{600}, and the percentage of lactate (% Lct) at OD_{600}=0.7-0.9 formed from the substrate
697 consumed are also shown for each condition tested. Growth curves are from a
698 representative experiment. Growth was repeated at least twice and the values are
699 averages for each condition. Symbols: (squared), MG1363; (circles) MG1363/pBL1;
700 (triangles) MG1363Δ*celB*.

701

702 **Figure 2.** Substrate consumption and end-product profiles during the fermentation of
703 cellobiose (1% wt/vol) by non-adapted *L. lactis* cells. Strains (A) MG1363, (B)
704 MG1363/pBL1, and (C) MG1363Δ*celB* were grown overnight in CDM supplemented
705 with glucose (1% wt/vol) and subcultured in fresh medium containing cellobiose (1%
706 wt/vol) as in Fig. 1B. Supernatants obtained at different growth stages were analyzed by
707 HPLC as described in Materials & Methods. The values are averages of at least two
708 independent experiments, and the average accuracy was ±5%. Symbols: (closed
709 diamonds), cellobiose; (white), lactate; (light grey), formate; (mid grey), acetate; (dark
710 grey), ethanol; (black), acetoin + 2,3-butanediol.

711

712 **Figure 3.** Effect of pBL1 on intracellular phosphorylated metabolites during growth on
713 glucose or cellobiose. Phosphorylated metabolites were measured by ^{31}P-NMR in

ethanol extracts of adapted MG1363 and MG1363/pBL1 cells (same sugar substrate in pre-cultures and cultures) grown to mid-exponential phase in CDM supplemented with 1% (wt/vol) glucose (A, C) or cellobiose (B, D). Gycolytic metabolites (A, B) and UDP-activated metabolites (C, D) are depicted. The average accuracy was ±15%. Symbols: (dark grey), MG1363; (white), MG1363/pBL1. DHAP: dihydroxyacetone phosphate; G6P: glucose-6-phosphate; FBP: fructose 1,6-biphosphate; 3PGA: 3-phosphoglycerate; 2PGA: 2- phosphoglycerate; PEP: phosphoenolpyruvate; Cel6P: cellobiose-6-phosphate; Gal: galactose; Glc:glucose; GlcNAc: N-acetyl-glucosamine; MurNAc: N-acetyl-muramic acid; pPep: pentapeptide; PRPP: 5-phosphorylribose 1-pyrophosphate.

Figure 4. Susceptibility of *L. lactis* strains to Lcn972. (A) Dose response curves of *L. lactis* MG1363 (squares) and *L. lactis* MG1363Δ*celB* (triangles) to increasing Lcn972 concentrations growing in CDM-glucose (closed symbols) and CDM-cellobiose (open symbols) at 30 ºC in a microtiter plate. Growth in the absence of Lcn972 was taken as 100% as monitored by OD_{600}. Results are mean values of triplicate wells and standard deviations never exceeded 10% of the given value. (B) Survival of *L. lactis* MG1363Δ*celB* and *L. lactis* MG1363 non-adapted and adapted to cellobiose. Exponentially growing cells on cellobiose (OD_{600}=0.2) were treated with 0.1 µM Lcn972 for 1 h at 30 ºC before plating appropriate dilutions on GM17. Survival (%) was defined as cfu/ml of treated cultures divided by cfu/ml of control cultures.

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant phenotype or genotype[a]	Reference
L. lactis		
MG1363	Plasmid-free derivative of NCDO712	20
MG1363/pBL1	MG1363 carrying pBL1, *lcn972*$^{+}$, *lcn972*^{R}	This work
MG1363/pBL1E	MG1363 carrying pBL1E, *lcn972*$^{+}$, *lcn972*^{R},Em^{R}	This work
MG1363Δ*celB*	MG1363; chromosomal deletion of *celB*	This work
MG1614	Str^{R}, Rif^{R} derivative of MG1363	20
MG1614.2	MG1614 carrying pBL1	35
MG1363Δ*celB*/pBL1E	MG1363Δ*celB* carrying pBL1E, Em^{R}, *lcn972*$^{+}$, *lcn972*^{R}	This work
E. coli		
DH10B	Plasmid-free	21
Plasmids		
pBL1	Lcn972 coding plasmid, 10.9 kbp	35, 45
pBL1E	*erm* from pNG8048 cloned in the unique *Eco*RV of pBL1_*orf4*	This work
pCR2.1	Cloning of PCR products, Ap^{R}	Invitrogen
pCR::celB4-1	*celB* and flanking regions cloned in pCR2.1	This work
pCR::dcelB	pCR::celB4-1 with a 1,019 bp deletion in *celB*	This work
pGhost9	Thermosensitive, Em^{R}	33
pGhost::dcelB	Incomplete *celB* cloned in pGhost9	This work

[a] Str, streptomycin; Rif, rifampicin; Em, erythromycin; Ap, ampicillin

Table 2. Oligonucleotides used in this study.

Primer	Sequence[a] 5'-3'	Description
celB-qF1	ATTTGGCCCGTGCTTACG	qRT-PCR *celB*
celB-qR1	TTTGGCAAACCTGCAAATAGG	
QptcC-F	CGTGTTCGGTATTGCTTACG	qRT-PCR *ptcC*
QptcC-R	TGTTAAACCAGCGGGTACTC	
qBglS-F	TACACCGCAGTATGCTAAGG	qRT-PCR *bglS*
qBglS-R	TTGGCCGACTTCAAGAGTTC	
Tuf-F	GGTAGTTGTCGAAGAATGGAGTGTGA	qRT-PCR internal control
Tuf-R	TAAACCAGGTTCAATCACTCCACACA	
celB-1	AACTCtAGATGGCCTTTGTA (*Xba*I)	Cloning and disruption of *celB*
celB-4	gAagAtctAAGACAGCCGCTCC (*Bgl*II)	

[a] Changes introduced to generate restriction sites (underlined and shown in brackets) are indicated in lower cases.

Table 3. Significant changes in gene expression induced by the presence of the Lcn972-coding plasmid pBL1 in *L. lactis* MG1614 cultures growing exponentially in GM17 broth.

Genes	Ratio[a]	Bayesian *p*[b]	Annotation[c]
Upregulated			
llmg0701_*oppA*	4.83	9.50E-08	Oligopeptide-binding protein OppA
llmg1012	4.73	4.00E-06	Putative ABC transporter substrate-binding protein
llmg0676	3.14	1.28E-05	Hypothetical acetyltransferase
llmg0699_*oppB*	2.85	2.31E-06	Peptide transport system permease protein OppB
llmg0642	2.41	5.22E-05	Hypothetical protein
llmg0711_*tnpR*	2.40	5.13E-04	DNA-invertase/resolvase
llmg2348	2.36	7.65E-05	Hypothetical protein
llmg0674_*tnp1297*	2.03	3.28E-06	Transposase for insertion sequence element IS1297
Downregulated			
llmg0186	-4.93	5.49E-10	Conserved hypothetical protein
llmg0187_*celB*	-4.59	4.53E-04	Cellobiose-specific PTS system IIC component

[a] Genes whose expression changes over twofold in the presence of pBL1 are shown. Negative values mean down-regulation.

[b] Determined by Cyber-T test (32)

[c] According to GenBank AM406671.

Table 4. Expression ratio of cellobiose-related genes as determined by RT-qPCR in exponentially growing *L. lactis* MG1363, MG1363/pBL1 and MG1363Δ*celB* in CDM-cellobiose relative to growth in CDM-glucose.

L. lactis strain	Target gene		
	ptcC	*celB*	*bglS*
MG1363	3.0	216.3	53.5
pBL1	1.2	43.5	10.2
Δ*celB*	7.6	-[a]	16.7

[a]-, *celB* is not present in this strain.

Table 5. Production of Lcn972 in CDM-glucose and CDM-cellobiose by *L. lactis* strains. Samples were taken at the transition to stationary phase. Lcn972 was quantified by ELISA using rabbit-polyclonal Lcn972 antibodies and corrected by OD_{600}.

L. lactis strain	Lcn972[a] (µg/OD)	
	CDM-glucose	CDM-cellobiose
MG1363/pBL1	5.4±0.0	6.6±0.0
MG1363/pBL1E	4.7±0.1	6.9±0.1
MG1363 Δ*celB*/pBL1E	4.4±0.3	11.1±1.1

[a] Mean ± standard deviation of two independent cultures.

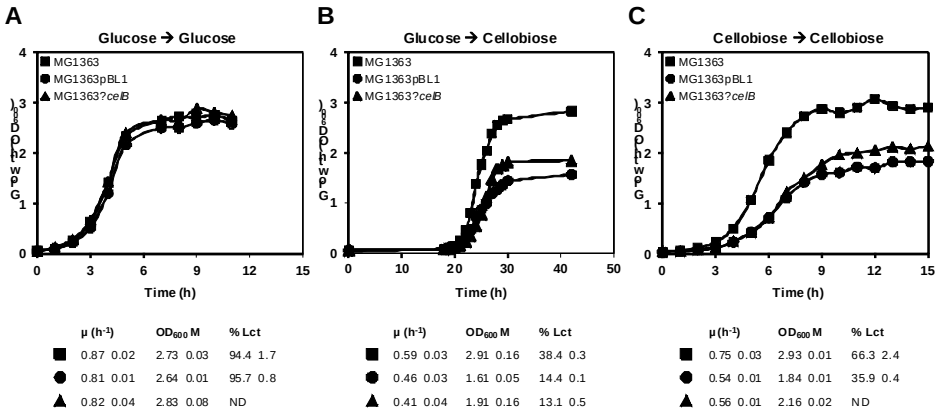

A
Glucose → Glucose
B
Glucose → Cellobiose
C
Cellobiose → Cellobiose
MG1363
MG1363pBL1
MG1363?celB
Time (h)
μ (h-1) OD600 M % Lct
0.87 0.02 2.73 0.03 94.4 1.7
0.81 0.01 2.64 0.01 95.7 0.8
0.82 0.04 2.83 0.08 ND
0.59 0.03 2.91 0.16 38.4 0.3
0.46 0.03 1.61 0.05 14.4 0.1
0.41 0.04 1.91 0.16 13.1 0.5
0.75 0.03 2.93 0.01 66.3 2.4
0.54 0.01 1.84 0.01 35.9 0.4
0.56 0.01 2.16 0.02 ND

Fig 1.

Fig. 2.

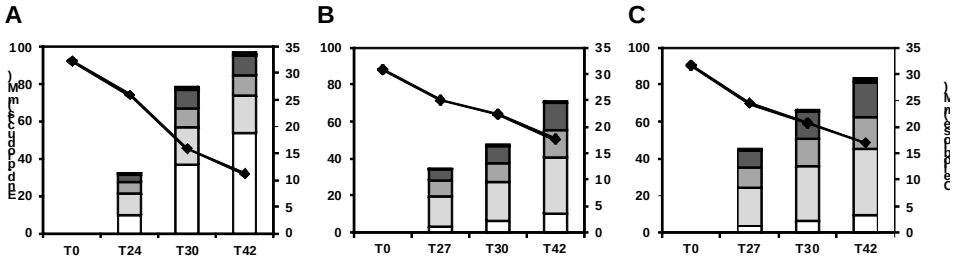


Fig. 3

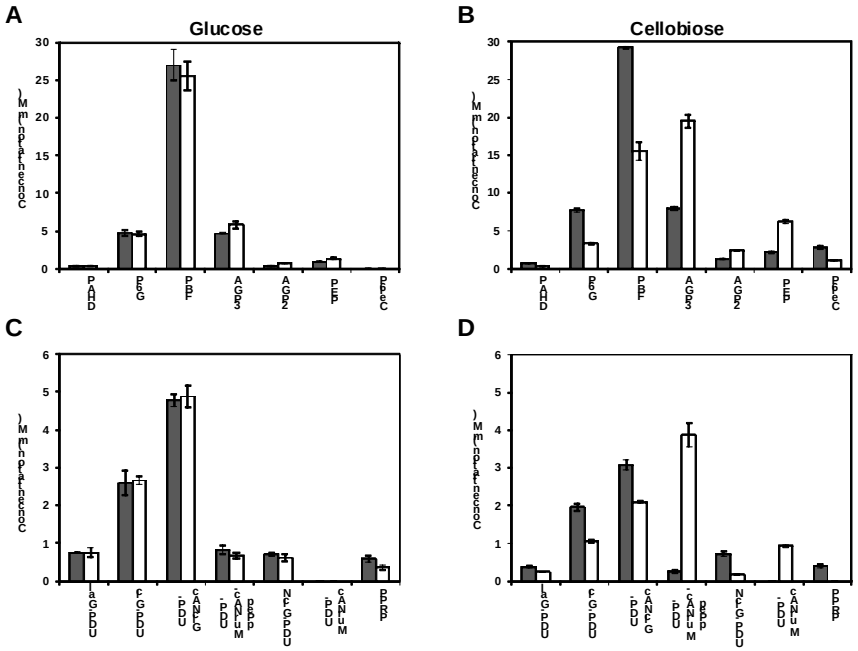
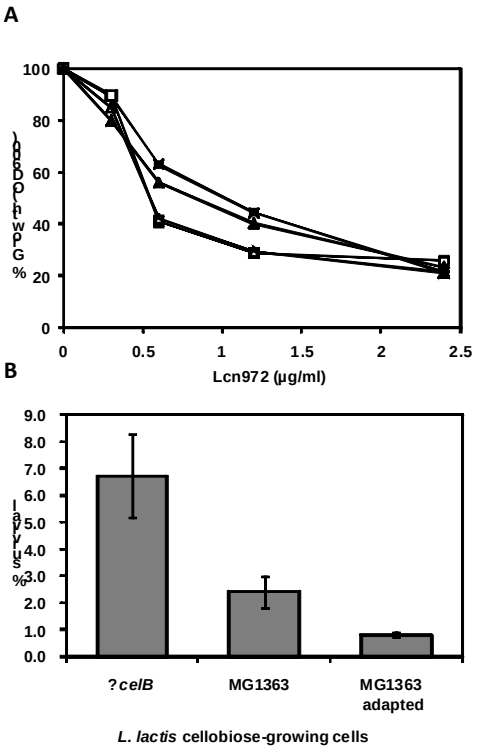


Fig. 4.


A
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
Lcn972 (µg/ml)
B
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
?celB
MG1363
MG1363 adapted
L. lactis cellobiose-growing cells