



Short communication

Enhanced UDP-glucose and UDP-galactose by homologous overexpression of UDP-glucose pyrophosphorylase in *Lactobacillus casei*

Jesús Rodríguez-Díaz, María.J. Yebra*

Laboratorio de Bacterias Lácticas y Probióticos, Instituto de Agroquímica y Tecnología de los Alimentos, CSIC. Av. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

ARTICLE INFO

Article history:

Received 28 February 2011

Received in revised form 18 May 2011

Accepted 25 May 2011

Available online 1 June 2011

Keywords:

UDP-glucose

UDP-galactose

UDP-glucose pyrophosphorylase

UDP-galactose 4-epimerase

Lactobacillus casei

ABSTRACT

UDP-sugars are widely used as substrates in the synthesis of oligosaccharides catalyzed by glycosyltransferases. In the present work a metabolic engineering strategy aimed to direct the carbon flux towards UDP-glucose and UDP-galactose biosynthesis was successfully applied in *Lactobacillus casei*. The *galU* gene coding for UDP-glucose pyrophosphorylase (GalU) enzyme in *L. casei* BL23 was cloned under control of the inducible *nisA* promoter and it was shown to be functional by homologous overexpression. Notably, about an 80-fold increase in GalU activity resulted in approximately a 9-fold increase of UDP-glucose and a 4-fold increase of UDP-galactose. This suggested that the endogenous UDP-galactose 4-epimerase (GalE) activity, which inter-converts both UDP-sugars, is not sufficient to maintain the UDP-glucose/UDP-galactose ratio. The *L. casei galE* gene coding for GalE was cloned downstream of *galU* and the resulting plasmid was transformed in *L. casei*. The new recombinant strain showed about a 4-fold increase of GalE activity, however this increment did not affect that ratio, suggesting that GalE has higher affinity for UDP-galactose than for UDP-glucose. The *L. casei* strains constructed here that accumulate high intracellular levels of UDP-sugars would be adequate hosts for the production of oligosaccharides.

© 2011 Elsevier B.V. All rights reserved.

Sugar nucleotides, such as UDP-glucose and UDP-galactose, are the most commonly used activated sugars in many enzymatic glycosylation processes, which give rise to the oligosaccharide moieties in the glycoconjugates or to soluble oligosaccharides. Both types of oligosaccharides play important roles in many biological processes, such as tumor metastasis, inflammation, cell–cell adhesion, and they also serve as recognition sites for bacteria, viruses, toxins, antibodies and hormones (Appelmek et al., 2003; Levander et al., 2009; Misonou et al., 2009; Newburg et al., 2005). Despite their relevance, research efforts and possible applications of oligosaccharides have been hampered by the inherent difficulties to obtain them using chemical or enzymatic synthesis. The latest utilizes glycosyltransferases that require expensive substrates such as UDP-sugars. Therefore, to avoid this limitation one alternative is using a whole-cell system, which would allow the regeneration in situ of sugar nucleotides.

Lactic acid bacteria are a group of microorganisms commonly used in food fermentations. In these bacteria the glycolytic intermediate glucose-6P is converted to glucose-1P by α -phosphoglucomutase activity, and this sugar phosphate is further metabolized to UDP-glucose and UDP-galactose by the consecutive action of enzymes UDP-glucose pyrophosphorylase (GalU)

and UDP-galactose 4-epimerase (GalE) (Boels et al., 2001). The sugar nucleotides are precursors for exopolysaccharides and cell wall components. Until now engineering strategies aimed to increase these activated sugars have been performed in *Lactococcus lactis* (Boels et al., 2001, 2003) and *Streptococcus thermophilus* (Levander et al., 2002) and they have been mainly focused on evaluating their impact in exopolysaccharide production. *Lactobacillus casei* is a facultative heterofermentative lactic acid bacterium widely used in the food industry as a dairy starter culture, and some strains are also utilized as probiotics. Recently, metabolic engineering strategies have been successfully applied to *L. casei* strain BL23 directed to enhance the production of the flavor-related compounds diacetyl and acetoin (Nadal et al., 2009), and to produce a new end product such as the low-calorie sweetener sorbitol (De Boeck et al., 2010). *L. casei* strain BL23 does not produce exopolysaccharides, hence it would be an adequate host for the production of oligosaccharides. This process will require a suitable glycosyltransferase, an acceptor and efficient supply of activated sugars. In this work, we report a metabolic engineering strategy in *L. casei* intended to increase the intracellular levels of UDP-glucose and UDP-galactose by homologous overexpression of UDP-glucose pyrophosphorylase (GalU). In addition, the functionality of the *L. casei galU* and *galE* genes has been demonstrated.

The *L. casei* BL23 genome was recently sequenced (GenBank accession no. **FM177140**; Maze et al., 2010). The product of the

* Corresponding author. Tel.: +34 963900022; fax: +34 963636301.

E-mail address: yebra@iata.csic.es (María.J. Yebra).

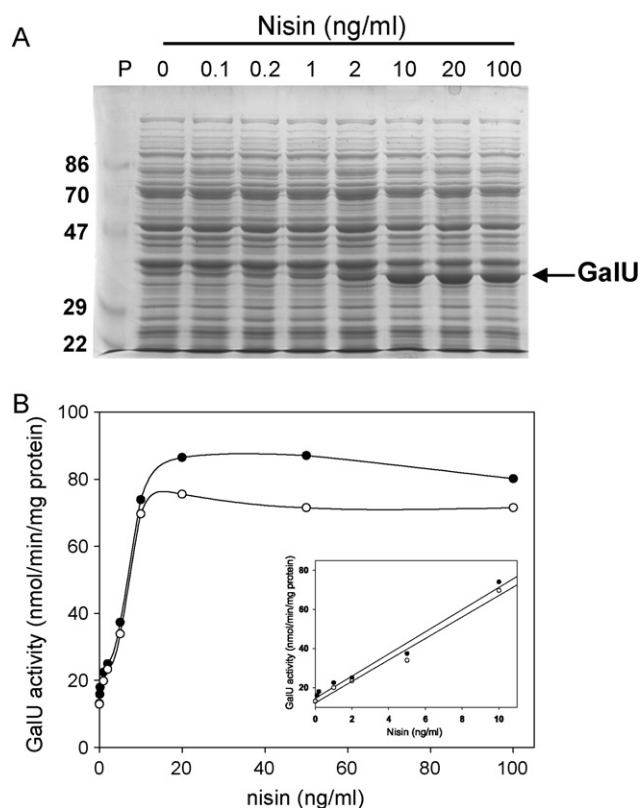


Fig. 1. Overproduction of UDP-glucose pyrophosphorylase (GalU) in *Lactobacillus casei* cell extracts from cultures induced with increasing concentrations of nisin. (A) Coomassie brilliant blue stained 10% SDS–PAGE showing *L. casei* strain PL28 (pNGgalU) cell extracts. The GalU band is indicated by an arrow. P indicates protein standards and the numbers on the left are the molecular weights. (B) Nisin-dependent GalU activity on *L. casei* strains PL28 (pNGgalU) (close circles) and PL29 (pNGgalUE) (open circles). The inner plot showed the linear relationship between GalU activity and nisin concentration (ranging from 0 to 10 ng/ml).

locus *galU* (LCABL.12560) is annotated as a putative GalU enzyme with 304 amino acids. To determine whether *galU* encodes a protein with GalU activity, the gene was amplified by PCR using chromosomal DNA from *L. casei* BL23 as a template and primers galURcaI (5'-GTGTA**CTCAT**GAAAGTTCGTAAG) and galUBamHI (5'-TGA**ACTTGGAT**CCTATGAATTTACTTC) (restriction sites introduced for cloning purposes are shown in *cursive*). The amplified DNA fragment was cloned into vector pNG8048E (Steen et al., 2007) under the control of the nisin-inducible promoter P_{nisA} and transformed in *L. lactis* NZ9000, which was used as an intermediary cloning host. The derivative pNGgalU was expressed in *L. casei* strain BL23 (*int:nisRK*) containing the lactococcal *nisRK* regulatory genes integrated into the chromosome (Hazebrouck et al., 2007). The newly created strain, named PL28, was cultured at 37 °C in MRS medium (Difco). An overnight culture of PL28 was diluted to an OD₅₅₀ of 0.4 in fresh media and after 90 min to restart bacterial growth, nisin was added at different concentrations and PL28 was grown for 3 h. Crude cell extracts were prepared as previously described (Yebra and Perez-Martinez, 2002) and they were analyzed by SDS–PAGE (Fig. 1A). A protein band of approximately 33 kDa appeared, which coincides with the predicted molecular weight of GalU (33,735 Da) and the protein amount seems to be modulated with increasing nisin concentrations. The GalU direct reaction mixture contained 50 mM Tris–HCl buffer, pH 7.5, 8 mM MgCl₂, 1.58 mg cysteine hydrochloride (pH 7.5), 1.25 mM UTP, 0.1 mg (0.015 U) of UDP-glucose dehydrogenase, 0.5 mM NAD⁺ and cell extract. The reaction was started by the addition of 1 mM glucose-1P (Grobben et al., 1996). Enzyme assays were performed

in a volume of 0.2 ml at 37 °C in 96-well plates and the NADH formation was monitored by following the absorbance at 340 nm in a POLARstar Omega microplate reader (BMG Labtech). Protein concentrations were measured by the method of Bradford (1976). A linear relationship between GalU activity and nisin concentration was observed (Fig. 1B), ranging from 13.05 nmol/min/mg protein (0 ng/ml nisin) to 73.91 nmol/min/mg protein (10 ng/ml of nisin), thus showing an approximately 6 fold induction. These results demonstrated that *galU* encodes *L. casei* GalU and that the expressed protein was functional. In PL27 control strain, that contains the plasmid pNG8048E, no activity was detected, suggesting that the levels of GalU activity due to the *galU* gene copy present in the chromosome are under the detection limit of the GalU assay, which was calculated about 0.03 nmol/min (data not shown). The maximum activity determined was 87.1 nmol/min/mg protein at 50 ng/ml of nisin added to the culture medium. Therefore it can be inferred that the activity induction factor is over 80. The activity determined in PL28 without nisin is probably due to the already described less tight regulation of the nisin inducible promoter in *L. casei* (Hazebrouck et al., 2007; Renye and Somkuti, 2010).

To investigate whether GalU activity is essential for *L. casei* BL23 growth, the disruption of *galU* was attempted. An internal DNA fragment of *galU* (550 bp) was obtained by PCR using primers galU1 (5'-CAAAACGTTCTATTGAGGAC) and galU2 (5'-GGGTTTGGTTCAAGGTGT C), and *L. casei* BL23 chromosomal DNA as a template. The amplified DNA fragment was cloned into the integrative vector pRV300 and the resulted plasmid pRVgalU was used to transform *L. casei* BL23. Several efforts to inactivate *galU* gene by insertional inactivation with pRVgalU, failed, suggesting that *galU* has an indispensable role in *L. casei*.

In order to determine UDP-glucose and UDP-galactose production, GalU was induced in PL28 with increasing concentrations of nisin as described above. Cell crude extracts were prepared and immediately the enzymes were separated from the sugar-nucleotides and other small components by centrifugal filtration using filter units with a nominal molecular weight limit of 5,000 (Ultrafree-MC, Millipore). The concentration of UDP-sugars in the filtrates was measured by HPLC (JASCO Corporation, Tokyo, Japan) with an UV detector at 254 nm. Samples were loaded in a Supelcosil-LC-18-DB (Supelco) and 40 mM triethylamine acetate buffer, pH 6.0, was used as the mobile phase in isocratic conditions according to the method previously described (Ramm et al., 2004). The production of both UDP-sugars increased with the concentration of nisin until 50 ng/ml (Fig. 2). UDP-N-acetylglucosamine was used as a control since it is synthesized in bacteria using a different pathway (Mengin-Lecreux and van Heijenoort, 1994). As seen in Fig. 2, its concentration did not change with increasing amounts of nisin. In PL27 strain the concentration of UDP-glucose and UDP-galactose were lower than in PL28 without induction (Fig. 2). The results showed that overexpression of *galU* resulted in 4.4 fold increase in the levels of UDP-galactose and 9.5 fold increase in UDP-glucose. The UDP-glucose/UDP-galactose ratio is doubled when GalU is induced (Table S1), suggesting that the levels of *L. casei* UDP-galactose 4-epimerase (GalE), which catalyzes the inter-conversion of both UDP-sugars, would be insufficient to keep the ratio. The product of the locus *galE* in the genome of *L. casei* BL23 (LCABL.07310) is annotated as a putative GalE enzyme with 331 amino acids. The *galE* gene from *L. casei* BL23 was cloned downstream of *galU* in the vector pNGgalU. *galE* was amplified by PCR using chromosomal DNA from *L. casei* BL23 as a template and primers galEBamHI (5'-ACGGATCCAAAAGGGAGATATTTTATTATGAC) and galENotI (5'-AACGTGGCGCGCTCGCGGTCAATTCG) (restriction sites introduced for cloning purposes are shown in *cursive*, the

Download English Version:

<https://daneshyari.com/en/article/24017>

Download Persian Version:

<https://daneshyari.com/article/24017>

[Daneshyari.com](https://daneshyari.com)