Engineering sorbitol-6-phosphate Dehydrogenase encoding gene in the lactose operon of Lactobacillus casei.

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Abstract

Sorbitol is a sugar polyol claimed to have health-promoting properties.
D-sorbitol-6-phosphate dehydrogeanse (StolPDh) is required for sorbose and sorbitol metabolism in *Lactobacillus casei.* StolPDh catalyzes the oxidation of Sorbitol-6-phosphate and also the reverse reaction, or reather, the reduction of fructose-6-phosphate with NAD^+ regeneration. In order to test this function *in vivo*, a new food grade recombinant strain of *L. casei* was constructed by the integration of a StolPDh-encoding gene (*gutF*) in the chromosomal lactose operon. Lactose induction of *gutF* expression was achieved and sorbitol production could be detected by ^{13}C-nuclear magnetic resonance analys, upon addiction of glucose to resting cells. Additional inactivationn of L-lactate dehydrogenase led to higher sorbitol production. These outcomes indicates that the accumulation of the glycolytic precursor, fructose-6-phosphate and an high NAD^+/NADH ratio were the limiting factors for sorbitol accumulation.

Introduction

Materials

Bacterial strains, plasmids, primers and growth conditions
L. casei strains were grown in MRS medium or MRS fermentation medium supplemented different carbohydrates at 37ºC under static conditions. *Escherichia coli* DH5 was grown LB medium at 37ºC under agitation. When required erythromycin and ampicillin were added to the media.

Construction of strains
Total DNA was isolated from *L. casei* as described before (Posno et al. 1991), and was used as template in a PCR reaction with primers gutNdeI and gutEcoRI (Table 1). ThePCR product was isolated and cloned into plasmid pllac (Table 1) giving plasmid pIlgutF. This plasmid, pVBldh or pRVgut3 were electroporated (Posno et al. 1991) into
L. casei cells with a Bio-Rad Genepulser apparatus. General recombinant DNA techniques, plasmid DNA isolation and PCR experiments were performed by standard procedures (Sambrook et al. 1989). DNA sequencing was carried out by using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase and an automatic ABI 310 DNA sequencer (Applied Biosystems).

Methods

Enzymatic assays
Crude extracts were prepared as described before (Yebra and Pérez-Martínez 2002) and StolPDh activity was determined with 2 mM sorbitol-6-phosphate and 2mM NAD+ in 10 mM Tris-HCl, pH 8.5, as previously reported (Yebra and Pérez-Martínez 2002). The reduction of fructose-6-phosphate was assayed in 10 mM Tris-HCl, pH 7.5, with 0.1 mM NADH and 2 mM fructose-6-phosphate. The rate of NAD+ reduction or NADH oxidation was determined by measuring the rate of absorbance variation at 340 nm.

13C-nuclear magnetic resonance (NMR) analysis
L. casei strains were grown in MRS fermentation medium supplemented with 0.5 % lactose, cells were collected and suspended in 150 mM potassium phosphate buffer pH 5.5 to 10 mg (dry mass) of cells per ml. [1-13C]glucose (Sigma Aldrich) was added to a final concentration of 10 mM and after a 30 min incubation at 37ºC the supernatants were analyzed by NMR. 13C-NMR spectra were recorded at 75.47 MHz by using a
Bruker Avance DPX-300 spectrometer. Acquisition parameters were: spectral width, 18kHz; data size, 32K; repetition delay, 1s; 30º pulse angle (5.5 µs); number of scans, 14.000. NMR tubes with a 5 mm diameter were used and 10 % (vol/vol) 2H2O was added to provide a lock signal. The 13C chemical shifts were relative to dioxane designated at 67.19 ppm.

Quantification of sorbitol and glucose
Sorbitol was quantified by the enzymatic method previously described (Bergmeyer et al. 1983) with some modifications. Sorbitol oxidation by sorbitol dehydrogenase (RocheDiagnostics GmbH) was determined in a reaction mixture containing 100 mM Tris-HCl, pH 9, 2 mM NAD+ and 10 times diluted supernatant of resting cells. The rate of NAD+ reduction was determined by measuring the rate of absorbance change at 340 nm. Glucose consumption was determined as described previously (Kunks et al. 1984). Sorbitol production and glucose consumption were measured in resting cells, which were prepared as mentioned above and incubated at 37ºC with 50 mM glucose. Samples were taken, centrifuged and supernatants were stored at -20ºC until they were analyzed.

Results

The StolPDh-encoding gene of *L. casei*, *gutF*, was amplified by PCR using total DNA as the template and primers gutNdeI and gutEcoRI (Table 1). Amplified DNA fragments of the expected size were cloned in the integrative vector pllac (Table 1) and the plasmid obtained pIlgutF was used to transform *L. casei* BL155. The selection strategy for recombinant colonies was the same as the previously described for pllac (Gosalbes et al. 2000). All the cloning and recombination events were checked by PCR analysis and DNA sequencing. Among several possible double recombinant clones, erythromycin sensitive and lactose positive, one was selected that contained *gutF* integrated between *lacG* and *lacF* and it was designated BL232. This strain did not contain any heterologous DNA fragments, for which it could be considered food-grade.
In order to determine if the expression of *gutF* was following the regulatory pattern of the *lac* operon, StolPDh activity was measured in BL232 grown on MRS fermentation medium supplemented with ribose, lactose or glucose plus lactose.

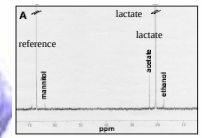
Table 2. Sorbitol 6-phosphate dehydrogenase activity in *L. casei* strains

L. casei strain	Sugar	Activity (nmol/min/mg protein)[a]		
		Not GF	+Rha GF	+Xyl GF
BL23	Ribose	2.07 ± 1.06	17.56 ± 8.18	
	Lactose	1.80 ± 0.77	ND	
	Glucose + Lactose	8.57 ± 4.36	3.69 ± 3.67	
BL232	Ribose	29.82 ± 2.98	67.4 ± 17.27	
	Lactose	181.59 ± 0.46	206.76 ± 70.24	
	Glucose + Lactose	5.58 ± 3.26	5.89 ± 0.45	
BL233	Ribose	2.62 ± 1.16	6.59 ± 2.03	
	Lactose	181.77 ± 11.43	221.47 ± 18.26	
	Glucose + Lactose	5.97 ± 0.31	ND	
BL234	Ribose	36.03 ± 4.13	67.58 ± 23.26	
	Lactose	116.97 ± 11.42	200.89 ± 40.93	
	Glucose + Lactose	7.65 ± 3.64	5.41 ± 3.85	

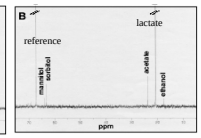
ND not detected. [a]Results are from at least three independent experiments.

Data in Table 2 show that lactose induced StolPDh activity in BL232, but not in the wild-type BL23, and also that the presence of glucose repressed the activity in BL232. These results showed that *gutF* has not just become physically part of the lactose operon, but it has also become a functional part of it following the same regulation..
Lactose grown cells of *L. casei* BL23 (wild-type) and BL232 were used to analyse by NMR the pattern of fermentation products secreted in a resting cells system, by addition of [1-13C]glucose. Consumption of glucose by lactose-grown cells is possible because, in *L. casei*, glucose is constitutively transported by the mannose-specific PTS (PTSMan) (M.J. Yebra, V. Monedero and G. Pérez-Martínez, unpublished data). The 13C-NMR spectra of the metabolites formed by BL23 (Fig. 2A) and BL232 (Fig. 2B) clearlyshowed resonances due to lactate (at 20.7 ppm), ethanol (17.5 ppm), acetate (23.7 ppm) and mannitol (63.9 ppm). A resonance peak due to sorbitol (63.1 ppm) was only observed in the supernatant of BL232 cells, due to the overexpression of *gutF* on lactose (Table 2). This would mean that StolPDh would catalyze the conversion of fructose-6-phosphate into sorbitol-6-phosphate, which then would need to be dephosphorylated and exported (Fig. 1). To our knowledge sorbitol-6-phosphatase activity has not yet been studied in bacteria.

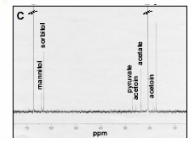
^{13}C-NMR spectra of liquid supernatants containing the fermentation products formed from [1-^{13}C]-Glucose.



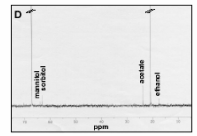
WT: *Lactobacillus casei* BL23 (CECT 5275)



BL232: *L.casei* BL 155 with *gutF* in *lac* operon



BL233: *L.casei* BL232 with *ldhL*::Prv300



BL234: *L.casei* BL232 with *gutF*::pRV300 in *gut* operon

Conclusion

The engineering strategy presented here led to the construction of *L. casei* strains with the StolPDh-encoding gene integrated in the lactose operon and subjected to its regulation. Lactose induction of *gutF* expression conducted to sorbitol synthesis, which might be of considerable interest in the food industry due to the claimed physiological values of this polyol. Based on this work we will design new metabolic strategies toenhance sorbitol production.

Licterature cited

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For further information