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Impact of Heterologous Expression of *Escherichia coli* UDP-Glucose Pyrophosphorylase on Trehalose and Glycogen Synthesis in *Corynebacterium glutamicum*

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Trehalose is a disaccharide with a wide range of applications in the food industry. We recently proposed a strategy for trehalose production based on improved strains of the gram-positive bacterium *Corynebacterium glutamicum*. This microorganism synthesizes trehalose through two major pathways, OtsBA and TreYZ, by using UDP-glucose and ADP-glucose, respectively, as the glucosyl donors. In this paper we describe improvement of the UDP-glucose supply through heterologous expression in *C. glutamicum* of the UDP-glucose pyrophosphorylase gene from *Escherichia coli*, either expressed alone or coexpressed with the *E. coli ots* genes (*galU otsBA* synthetic operon). The impact of such expression on trehalose accumulation and excretion, glycogen accumulation, and the growth pattern of new recombinant strains is described. Expression of the *galU otsBA* synthetic operon resulted in a sixfold increase in the accumulated and excreted trehalose relative to that in a wild-type strain. Surprisingly, single expression of *galU* also resulted in an increase in the accumulated trehalose. This increase in trehalose synthesis was abolished upon deletion of the TreYZ pathway. These results proved that UDP-glucose has an important role not only in the OtsBA pathway but also in the TreYZ pathway.

Trehalose (α-glucopyranosyl-α-glucopyranoside) is a stable, odor-free, nonreducing disaccharide that is widespread in nature (34). Its protein-stabilizing properties have suggested a wide range of commercial applications, ranging from cosmetics to the agricultural food sector (18). In the 1990s, the high cost of this disaccharide triggered the development of methods based on immobilized enzymes to obtain trehalose from maltodextrins (20, 21). This achievement drastically reduced the commercial price of trehalose (34). A microbiological alternative for large-scale production of trehalose relying on *Corynebacterium glutamicum* (19), a gram-positive bacterium able to synthesize and excrete this sugar (37, 41), was recently proposed (27).

Trehalose synthesis in *C. glutamicum* during growth on glucose proceeds through two major pathways (Fig. 1): (i) from UDP-glucose and glucose-6-phosphate (OtsAB pathway) and (ii) from malto-oligosaccharides or α-1,4 glucans (TreYZ pathway) (36, 42). Recently, a sixfold increase in OtsBA pathway activity in *C. glutamicum* (27) was achieved through heterologous expression of the *Escherichia coli otsBA* operon encoding the trehalose-synthesizing enzymes trehalose-6-phosphate synthase (OtsA) and trehalose-6-phosphate phosphatase (OtsB) (17). However, this approach resulted in only a twofold increase in the amount of excreted trehalose (27). These results suggested that further improvements in trehalose synthesis were hampered by the low activity of UDP-glucose pyrophosphorylase, which is responsible for UDP-glucose synthesis (17) according to the following reaction: glucose-1-P + UTP → UDP-glucose + pyrophosphate. This enzyme is critical in *Lactococcus lactis* and *Streptococcus thermophilus* during exopolysaccharide accumulation (3, 22), a process analogous to trehalose synthesis.

The objective of this work was to increase the specific activity of UDP-glucose pyrophosphorylase in *C. glutamicum* through heterologous expression of the corresponding *Escherichia coli* gene *galU* (14, 40), either expressed singly or coexpressed with the *E. coli ots* genes (*galU otsBA* synthetic operon). The heterologous expression obtained in both configurations, in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG), as well as in the presence of increasing amounts of the inducer, was examined. The effect of such expression on glycogen accumulation and trehalose excretion during cultivation of *C. glutamicum* is discussed below.

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MATERIALS AND METHODS

Growth of bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) medium was used as the standard medium for *C. glutamicum* Δ*mluI* (33), Δ*treS*, Δ*treY* (42), and derived strains. The composition of the defined medium used for *C. glutamicum* shake flask experiments (DMCG I) was as follows: sodium citrate, 1.1 g/liter; NaCl, 1 g/liter; $MgSO_4 \cdot 7H_2O$, 200 mg/liter; $FeSO_4 \cdot 7H_2O$, 25 mg/liter; $CaCl_2 \cdot 2H_2O$, 50 mg/liter; K_2HPO_4, 8 g/liter; KH_2PO_4, 1 g/liter; $(NH_4)_2SO_4$, 5 g/liter; $MnSO_4$, 2 mg/liter; $Na_2B_4O_7 \cdot 10H_2O$, 0.2 mg/liter; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1 mg/liter; $FeCl_3 \cdot 6H_2O$, 2 mg/liter; $ZnSO_4 \cdot 7H_2O$, 0.5 mg/liter; $CuCl_2 \cdot 2H_2O$, 0.2 mg/liter; biotin, 1 mg/liter; thiamine hydrochloride, 1 mg/liter; and deferoxamine mesylate, 3 mg/liter. Glucose (20 g/liter) was used as the carbon source. Chloramphenicol was added to final concentration of 20 μg/ml. IPTG was added to final concentrations of 0.1, 0.2, and 0.5 mM. For batch bioreactor cultivation the defined medium (DMCG II) of Delaunay et al. (8) was used, with a few modifications, as follows: nitrileacetic acid, 0.5 g/liter; NaCl, 2 g/liter; $MgSO_4 \cdot 7H_2O$, 400 mg/liter; $FeSO_4 \cdot 7H_2O$, 40 mg/liter; $CaCl_2 \cdot 2H_2O$, 84 mg/liter; Na_2HPO_4, 3 g/liter; KH_2PO_4, 6 g/liter; $(NH_4)_2SO_4$, 8 g/liter; $MnSO_4$, 3.9 mg/liter; $Na_2B_4O_7 \cdot 10H_2O$, 0.3 mg/liter; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.1 mg/liter; $FeCl_3 \cdot 6H_2O$, 3.9 mg/liter; $ZnSO_4 \cdot 7H_2O$, 0.9 mg/liter; $CuCl_2 \cdot 2H_2O$, 0.3 mg/liter; biotin, 4 mg/liter; thiamine hydrochloride, 20 mg/liter; deferoxamine mesylate, 3 mg/liter; and polypropylene glycol (molecular weight, 2,000), 1 ml/liter. Glucose (100 g/liter) was used as the carbon source. Antibiotics were added to final concentrations of 50 μg/ml (ampicillin)

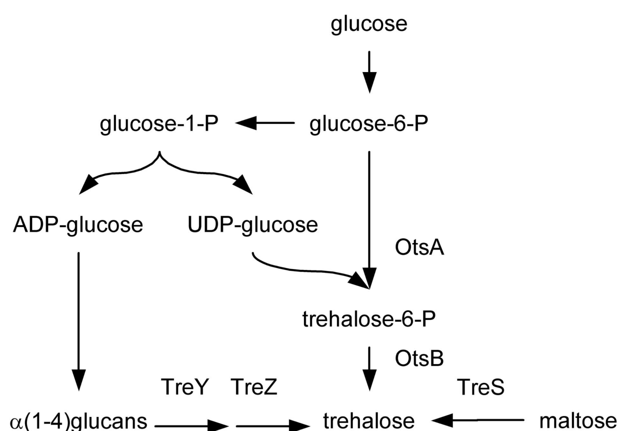

FIG. 1. Three pathways for trehalose synthesis in *C. glutamicum* and *Mycobacterium tuberculosis*. The *otsBA* pathway leads to trehalose from glucose 6-phosphate through UDP-glucose and trehalose 6-phosphate as intermediates. In the *treYZ* pathway, isomerization and hydrolysis of a α(1-4)glucan leads to trehalose. The synthesis of such glucans proceeds in a way fairly similar to the *otsBA* pathway, with ADP-glucose as the glucosyl donor. Finally, the *treS* pathway leads to trehalose after isomerization of maltose, when this sugar is available as a carbon source.

and 20 μg/ml (chloramphenicol). IPTG was added to a final concentration of 1.0 mM.

DNA manipulation and bacterial transformation. All DNA cloning steps were performed according to standard procedures (32). *E. coli* DH5α-*mcr* was transformed by using a high-efficiency method (15). *C. glutamicum* was transformed by a protocol described elsewhere (38). The restriction mutant *C. glutamicum* Δ*mluI* was used to obtain higher electroporation yields throughout this study.

Plasmid constructs. The sequence of *E. coli galU* (accession no. M98830) was obtained from GenBank. The following oligodeoxynucleotides for PCR amplification were designed by using the Primer Premier software (Premier Biosoft International, Palo Alto, Calif.): GALU1(5′ G<u>CC CGG G</u>GA TAC AGA AAT ATG AAC ACG 3′) and GALU2 (5′ GTT <u>CCC GGG</u> ATA ACA CGA TAT CGG ATG 3′) (the underlining indicates SmaI cut sites created in both primers for subsequent cloning steps).

The cloning procedures are summarized in Fig. 2. The 994-bp fragment which contained the coding sequence of *galU*, flanked upstream to the start codon by a 58-bp sequence (harboring the ribosomal binding site without the putative promoter [see Fig. 2 for details]) and downstream to the stop codon by a 27-bp sequence, was amplified by PCR from chromosomal DNA of *E. coli* JM109 (Promega Corporation, Madison, Wis.). The PCR product was ligated into the pGEM-T vector (Promega Corporation). The resulting construct (pLPI*galU*00) was transformed into *E. coli* DH5α-*mcr*, a DNA methylation mutant (10). The *galU* fragment was excised from pGEM-T constructs with restriction enzyme SmaI. The excised fragment was ligated into the *E. coli*-*C. glutamicum* shuttle vector pXMJ19 (16), previously digested with restriction enzyme SmaI. The resulting ligation mixture was used to transform *E. coli* DH5α-*mcr*. The transformants were selected on chloramphenicol-containing LB medium plates. Two kinds of *E. coli* clones were obtained: direct clones, carrying the gene in the right orientation, and reversed clones, carrying the gene in the inverted sense, which were used as controls (see Fig. 2). Plasmid DNA was extracted from clones and used to electroporate *C. glutamicum* strains Δ*mluI* (33) Δ*treY*, and Δ*treS* (41). The transformants were selected on LB medium plates. In this way we obtained *C. glutamicum* strains harboring the *galU* gene. The synthetic operon *galU otsBA* was assembled as follows. The *galU* fragment excised from the pLPI*galU*00 construct was ligated into the vector pLPI*otsBA*01 (harboring the *otsBA* operon from *E. coli* [27]) previously digested with restriction enzyme SalI and filled in with DNA polymerase I (Klenow fragment). The ligation mixture was used to transform *E. coli* DH5α-*mcr*. Then the procedure was exactly the same as the procedure used for *galU* cloning in the pXMJ19 vector. The resulting *C. glutamicum* strains are summarized in Table 1.

Shake flask experiments. For each induction experiment, 1.5 ml of a fresh overnight LB medium culture was inoculated into 30 ml of DMCG I. After 2 h of growth, the appropriate amount of IPTG was added. Samples were withdrawn at regular intervals (2 h) for trehalose, glycogen, and enzymatic analyses.

Bioreactor experiments. For batch cultivation, a preinoculum of each strain was made in 100 ml of tryptone soy broth. After overnight growth in a rotatory shaker at 30°C and 250 rpm, the cells were centrifuged and resuspended in 100 ml of defined medium. The cell suspension obtained was added to a 1-liter Bio-Flo IIc bioreactor (New Brunswick Scientific, Edison, N.J.) containing 900 ml of the defined medium. Cultures were grown at 30°C with agitation at 700 rpm. The pH was kept at 7.0 with 6 M NaOH.

Sample preparation for sugar measurement. Ten-milliliter samples were withdrawn at regular intervals from a culture. The samples were immediately centrifuged at 4°C at 1,600 × *g* for 10 min. Each supernatant was collected and used directly for high-performance liquid chromatography analysis of extracellular metabolites.

Quantitative high-performance liquid chromatography analysis of carbohydrates was performed with a Merck-Hitachi L7100 pump system coupled to a Merck-Hitachi L7490 refraction index detector. An HPX-87H column (Bio-Rad Laboratories, Hercules, Calif.) was used with 5 mM sulfuric acid as the eluant (5). The column temperature was kept at 55°C by using a Merck-Hitachi L7350 column oven.

Preparation of cell extracts and enzyme assays. Cultures (100 ml) were harvested by centrifugation, washed twice in 20 ml of buffer containing 100 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM $MnSO_4$, and 1 mM dithiothreitol. The

TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Genotype and/or description	Source or reference
Plasmids		
pGEM-T	Amp^r	Promega
pXMJ-19	Cf^r	16
pLPI*galU*00	*galU* Amp^r	This study
pLPI*galU*01	$galU^+$ (direct insert) Cf^r	This study
pLPI*galU*02	*galU* (inverted insert) Cf^r	This study
pLPI*otsBA*01	$otsBA^+$ (direct insert) Cf^r	27
pLPI*galUotsBA*01	$galU^+$ $otsA^+$ $otsBA^+$	This study
Strains		
E. coli DH5α-*mcr*	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA mcrA* Δ(*mrr-hsdRMS-mcrBC*)	10
C. glutamicum Δ*mluI*	Δ*mluI* derivative of *C. glutamicum* ATCC13032	33
C. glutamicum Δ*treY*	Δ*treY* derivative of *C. glutamicum* ATCC13032	42
C. glutamicum Δ*treS*	Δ*treS* derivative of *C. glutamicum* ATCC13032	42
C. glutamicum Δ*mluI*/pLPI*galU*01	Δ*mluI* $galU^+$, Cf^r	This study
C. glutamicum Δ*mluI*/pLPI*galU*02	Δ*mluI galU,* Cf^r	This study
C. glutamicum Δ*mluI*/pLPI*galUotsBA*01	Δ*mluI* $galU^+$ $otsA^+$ $otsB^+$, Cf^r	This study
C. glutamicum Δ*treY*/pLPI*galU*01	Δ*treY* $galU^+$, Cf^r	This study
C. glutamicum Δ*treY*/pLPI*galUotsBA*01	Δ*treY* $galU^+$ $otsA^+$ $otsB^+$, Cf^r	This study
C. glutamicum Δ*treS*/pLPI*galU*01	Δ*treS* $galU^+$ $otsA^+$ $otsB^+$, Cf^r	This study

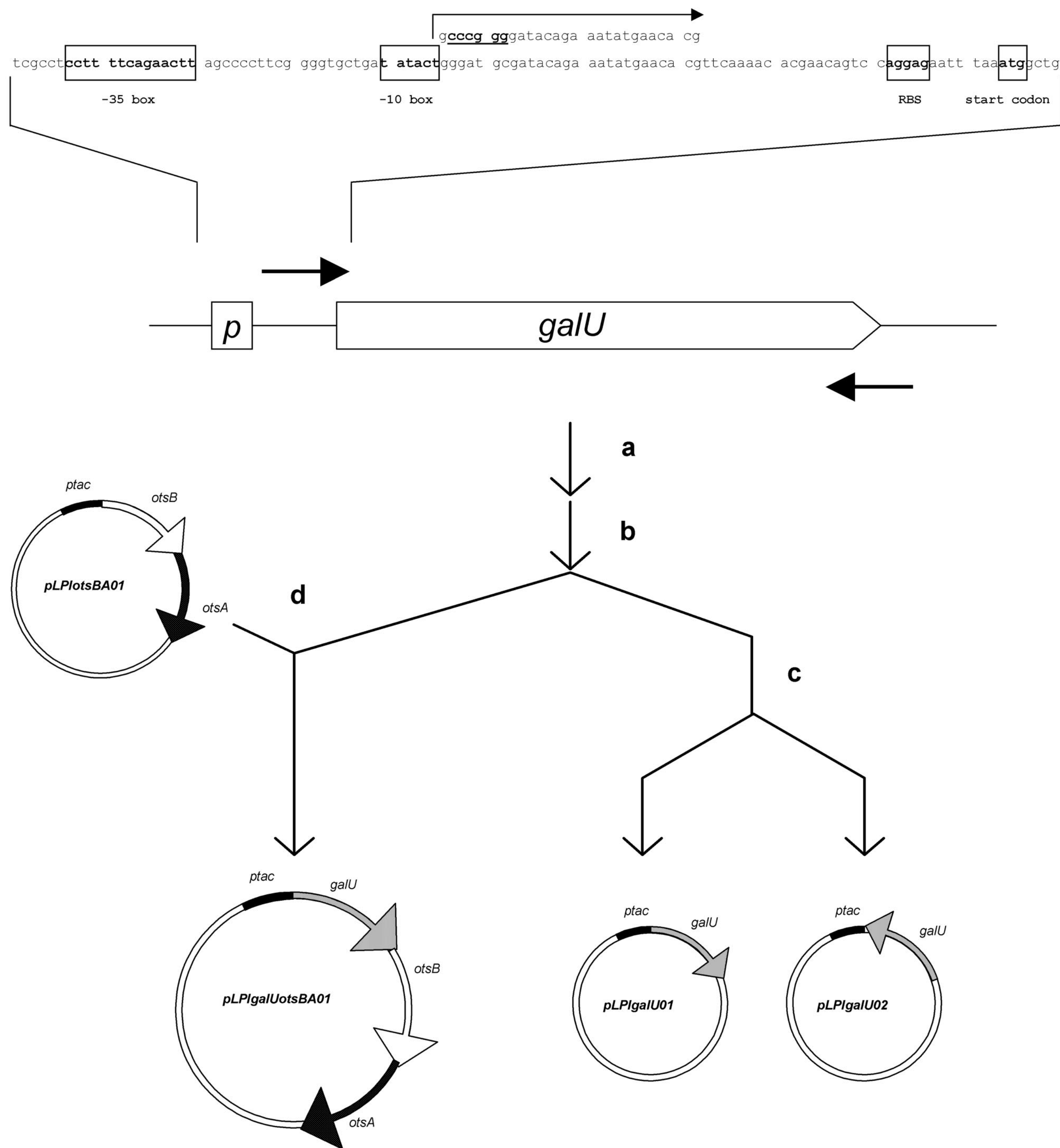

FIG. 2. Construction of expression vectors for the *galU* gene. A DNA fragment containing the *galU* coding sequence and its ribosomal binding site (RBS) was amplified by PCR from genomic DNA of *E. coli* JM109 (step a). The PCR primers (arrows with solid heads) were designed to avoid amplification of the putative *E. coli* promoter sequence indicated by *p* (−10 and −35 boxes indicated in the enlarged sequence). Both primers also contained SmaI cutting sites to facilitate subsequent cloning steps (in the enlarged sequence the cutting site in the sense primer is underlined). The PCR product was cloned into the pGEM-T vector (step b); then the *galU* fragment was excised from pGEM constructs with SmaI and ligated into the pXMJ19 vector previously digested with the SmaI enzyme (step c). Since the blunt fragments generated by SmaI digestion could ligate in both orientations, two constructs were obtained: pLPI*galU*01 (*galU*-expressing vector) and pLPI*galU*02 (control). To achieve simultaneous expression of the *galU* and *ots* genes from *E. coli*, the *galU* fragment obtained in step c was ligated into the vector pLPI*otsBA*01 (harboring the *otsBA* operon [see text for further details]) previously digested with SalI and filled in with the Klenow enzyme (step d). In this way the vector pLPI*galUotsBA*01 was obtained.

TABLE 2. Specific activities of some enzymes related to UDP-glucose metabolism in crude extracts from *C. glutamicum*[a]

Strain	Induction with 1 mM IPTG	Enzyme	Sp act (pkat · mg of protein^{-1})
Δ*mluI*/pLPI*galU*01	+	UDP-glucose pyrophosphorylase	18,910
Δ*mluI*/pLPI*galU*01	−	UDP-glucose pyrophosphorylase	212
Δ*mluI*/pLPI*galU*02	−	UDP-glucose pyrophosphorylase	178
Δ*mluI*	−	UDP-glucose pyrophosphorylase	25
Δ*mluI*	−	Trehalose-6-P phosphatase	206
Δ*mluI*/pLPI*galUotsBA*01	+	UDP-glucose pyrophosphorylase	9,808
Δ*mluI*/pLPI*galUotsBA*01	+	Trehalose-6-P phosphatase	243
Δ*mluI*/pLPI*galUotsBA*01	−	UDP-glucose pyrophosphorylase	201
Δ*mluI*/pLPI*galUotsBA*01	−	Trehalose-6-P phosphatase	367
Δ*mluI*	−	Phosphoglucomutase	1,330
Δ*mluI*/pLPI*galU*01	+	Inorganic pyrophosphatase	14,718
Δ*mluI*/pLPI*galU*01	+	UDP-glucose-4-epimerase	57
Δ*mluI*/pLPI*galU*01	+	UDP-galactose:glucose-1-P uridylyltransferase	36

[a] The specific activities are the means of at least two independent measurements, and the standard deviation was always less than 10%. Extracts were prepared from cells grown in shake flasks with DMCG I and were induced for 6 h with 1 mM IPTG. The inducer was added when the culture reached an optical density at 600 nm of 0.3 to 0.4.

cells were resuspended in 1 ml of the same buffer, mixed with 300 μl of 0.1-mm-diameter glass beads, and broken by eight 20-s cycles with a Mini Bead-Beater apparatus (Biospec Products Inc., Bartlesville, Okla.). The total protein contents of the extracts were determined by the dye binding assay method (4) with bovine serum albumin as the standard.

The trehalose-6-phosphate phosphatase (OtsB) activity was assayed by monitoring phosphate release from trehalose-6-P (9). The reaction was carried out in a mixture (final volume, 1 ml) containing 50 μmol of Tris-HCl (pH 7.2), 5 μmol of $MgCl_2$, and 1 μmol of trehalose-6-P. Samples were withdrawn at regular time intervals and assayed for phosphate by the zinc acetate method (1) as follows: 300 μl of a sample was mixed with 900 μl of a solution containing 100 mM zinc acetate and 15 mM ammonium molybdate (pH 5.0) and incubated at room temperature for exactly 15 min, and then the absorbance at 350 nm was quickly measured.

The inorganic pyrophosphatase activity was also assayed by monitoring the phosphate release from pyrophosphate. The reaction was carried out in a mixture (final volume, 1 ml) containing 50 μmol of Tris-HCl (pH 7.5), 8 μmol of $MgCl_2$, 1.58 mg of cysteine hydrochloride, 0 to 5 μl of crude extract, and 0.5 μmol of sodium pyrophosphate. Samples were withdrawn at regular time intervals (5 min) and assayed for phosphate by the zinc acetate method.

The UDP-glucose pyrophosphorylase (GalU) reaction assay used was a spectrophotometric coupled assay (7) in which the reaction mixture (final volume, 1 ml) contained 50 μmol of Tris-HCl (pH 7.5), 8 μmol of $MgCl_2$, 1.58 mg of cysteine hydrochloride, 0.5 μmol of NAD, 1.25 μmol of UTP, 50 mU of UDP-dehydrogenase (Calbiochem), and 0 to 5 μl of crude extract. The reaction was started by adding 1 μmol of glucose-1-P, and the increase in absorbance at 340 nm was monitored.

UDP-galactose-4-epimerase was assayed by the method of Degeest and De Vuyst (7) by using a mixture (final volume, 1 ml) containing 40 μmol of glycylglycine (pH 8.5), 5 μmol of $MgCl_2$, 0.6 μmol of NAD, 50 mU of UDP-glucose dehydrogenase, and 0 to 1 μl of cell extract. The reaction was started by addition of 0.2 μmol of UDP-galactose, and the increase in absorbance at 340 nm was monitored.

UDP-galactose:glucose-1-P uridylyl dehydrogenase was assayed like UDP-galactose-4-epimerase (23) by using a mixture (final volume, 1 ml) containing 40 μmol of glycylglycine (pH 8.5), 5 μmol of $MgCl_2$, 0.2 μmol of UDP-galactose, 0.6 μmol of NAD, 50 mU of UDP-glucose dehydrogenase, and 0 to 1 μl of cell extract. The reaction was started by addition of 0.2 μmol of glucose-1-P, and the increase in absorbance at 340 nm was monitored.

Cell carbohydrate analysis. Analysis of the internal trehalose and glycogen contents was performed as described by Parrou and François (28). Cells (1 ml of a culture having an optical density at 600 nm of ~20) were collected by centrifugation (15 min in a bench centrifuge at 4°C), carefully drained to remove the culture medium, resuspended in 0.25 ml of 0.25 M Na_2CO_3 in screw-cap Eppendorf tubes, and incubated at 95°C for 4 h. The pH of the mixture was adjusted to 5.2 by addition of 0.15 ml of 1 M acetic acid and 0.6 ml of 0.2 M sodium acetate (pH 5.2). One hundred microliters of the suspension was incubated overnight with trehalase (0.05 U/ml) at 37°C, and 100 ml was incubated with an *Aspergillus niger* amyloglucosidase preparation (1.2 U/ml) at 57°C with constant agitation in an Eppendorf thermomixer. The suspensions were centrifuged for 4 min at 4°C in a bench centrifuge, and the glucose in 30 μl of supernatant was determined by addition of 1 ml of a glucose oxidase mixture (Human, Wiesbaden, Germany) by following the supplier's instructions.

RESULTS

Heterologous expression of the *galU* gene and *galU otsBA* synthetic operon. Our first goal was functional expression of the *galU* gene and the *galU otsBA* synthetic operon in *C. glutamicum*. To check the accomplishment of this goal, the activities of the GalU and OtsB enzymes were measured in crude extracts prepared from the recombinant strains obtained in this work.

We first tested the UDP-glucose pyrophosphorylase (GalU) activity in extracts prepared from uninduced and induced (0.5 mM IPTG) *C. glutamicum* Δ*mluI*/pLPI*galU*01 cells. The specific activity of GalU increased from 212 pkat/mg of protein in uninduced cells to almost 12,000 pkat/mg of protein after addition of the inducer (Table 2). To determine if the activity found in the absence of inducer was a consequence of native GalU activity, we tested the enzyme activity in crude extracts prepared from parental strain Δ*mluI*. The specific activity in this strain was 25 pkat/mg of protein, which was 10-fold lower than the activity found in the uninduced Δ*mluI*/pLPI*galU*01 cells. This demonstrated that the plasmid-encoded *galU* gene was expressed in the absence of inducer. To test if this basal expression resulted from residual activity of the *tac* promoter (*Ptac*), we also tested extracts of the control strain *C. glutamicum* Δ*mluI*/pLPI*galU*02 harboring the plasmid with an inverted *galU* insert. The specific activity in Δ*mluI*/pLPI*galU*02 cells was 178 pkat/mg of protein, which was very close to the activity in the Δ*mluI*/pLPI*galU*01 extract. This result suggested the presence of a cryptic promoter in the insert, which was responsible for *galU* expression in the absence of *Ptac*.

Then the GalU and OtsB activities in Δ*mluI*/pLPI*galUotsBA*01 cells were assayed under conditions identical to those described above for Δ*mluI*/pLPI*galU*01 cells. The specific activity of GalU increased from 201 pkat/mg of protein in uninduced cells to 9,808 pkat/mg of protein after addition of 0.5 mM IPTG (Table 2). Surprisingly, the behavior of OtsB was opposite that of GalU; the activities were 367 pkat/mg of protein in uninduced cells and 243 pkat/mg of protein upon addition of IPTG. The activities in the parental strain *C. glutamicum*

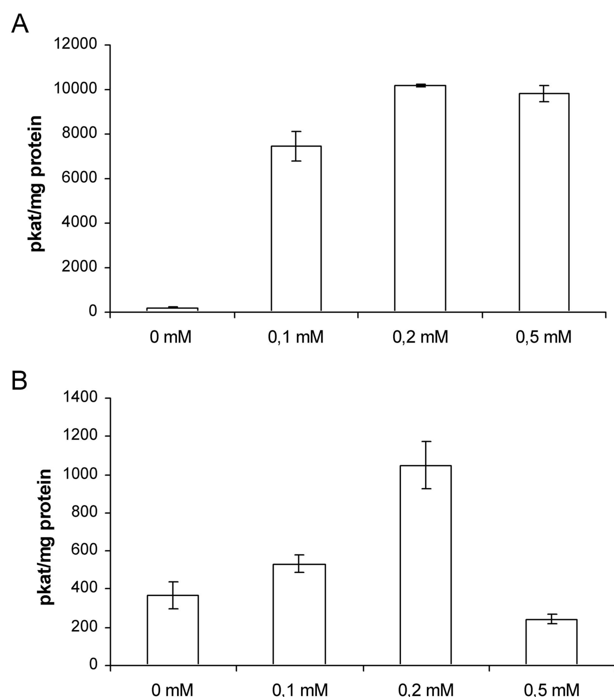

FIG. 3. Effect of increasing levels of expression of the *galU otsBA* operon on the specific activities of UDP-glucose pyrophosphorylase (A) and trehalose-6-phosphate phosphatase (B) in *C. glutamicum* strain Δ*mluI*/pLPI*galUotsBA*01 growing on DMCG I. The culture medium was supplemented with 15 mg of chloramphenicol per liter to avoid plasmid loss, and several concentrations of the inducer IPTG (0, 0.1, 0.2, and 0.5 mM) were added after 2 h of cultivation. Cells were harvested after 6 h of induction.

Δ*mluI* were as follows: 25 pkat/mg of protein for GalU and 206 pkat/mg of protein for OtsB. To clarify the observed differences, the specific activities of both enzymes were examined with different degrees of induction (0.1, 0.2, and 0.5 mM IPTG). The increase in GalU activity correlated with the level of induction (Fig. 3A). At low induction levels, a slight increase in OtsB activity was observed. However, when the IPTG concentration was increased to 0.5 mM, the tendency was reversed, and OtsB activity was diminished (Fig. 3B). The consequences of this enzymatic imbalance were studied in subsequent experiments.

Effect of *galU* and *galU otsBA* operon expression on the trehalose yield in batch cultures. The trehalose-synthesizing abilities of the recombinant strains were examined under controlled culture conditions (high cell density and high glucose concentration [100 g/liter] with proper aeration and pH control) during batch growth in a 1-liter bioreactor. The final titer of trehalose excreted by the Δ*mluI*/pLPI*galUotsBA*01 strain was 10 g/liter (5 g of residual glucose per liter) when the IPTG concentration was 0.1 mM. An increased level of inducer (1 mM) resulted in reduced disaccharide excretion (6.8 g/liter). Figure 4 shows the significant increase in the maximum trehalose specific productivity (q_{tre}) of the Δ*mluI*/pLPI*galUotsBA*01 strain (61 mg of trehalose · g [dry weight] of cells^{-1} · h^{-1} with 0.1 mM IPTG and 22 mg of trehalose · g [dry weight] of cells^{-1} · h^{-1}with 1 mM IPTG) compared to that observed in a strain expressing *otsBA* alone (12 mg of trehalose · g [dry weight] of cells^{-1} · h^{-1}) and in a control strain (4 mg of trehalose · g [dry weight] of cells^{-1} · h^{-1}). Also, strain Δ*mluI*/pLPI*galU*01 excreted significant amounts of trehalose (7 g/liter). In this case, the residual amount of glucose in the medium was 25 g/liter, an amount greater than that observed for the Δ*mluI*/pLPI*galUotsBA*01 strain. The profile of trehalose specific productivity was also different in the Δ*mluI*/pLPI*galU*01 strain; the maximum value occurred during the stationary growth phase (8 mg of trehalose · g [dry weight] of cells^{-1} · h^{-1}).

The observed similarity in the final titers of trehalose suggests that the native activity of the Ots enzymes is sufficient to increase trehalose synthesis through the OtsAB pathway during *galU* overexpression. However, the basal activity of OtsB in crude extracts of the Δ*mluI* strain was 206 pkat/mg of protein, a rather small value compared with the 18,000 pkat/mg of protein observed for GalU upon induction with 1 mM IPTG. This indicates that high *galU* expression does not necessarily lead to a high in vivo flux. Three major reasons might explain the deficiency: (i) a limiting supply of glucose-1-phosphate for UDP-glucose synthesis; (ii) a limiting activity of inorganic pyrophosphatase, which is required to push forward the otherwise thermodynamically unfavorable GalU reaction (ΔG°′, ~43 kJ mol^{-1}); and (iii) draining of UDP-glucose through alternate pathways (i.e., synthesis of cell wall precursors). To discriminate among these possibilities, we performed several enzyme and growth tests.

(i) Limiting supply of GalU substrates. We measured the activity of the glucose-1-phosphate-generating enzyme phosphoglucomutase in crude extracts of *C. glutamicum*. The activity of this enzyme was rather high (1,350 pkat/mg of protein) (Table 2), which in principle should have provided an appropriate amount of glucose-1-phosphate for UDP-glucose synthesis. Hence, limitation of glucose-1-phosphate is not a reason for a low flux through GalU.

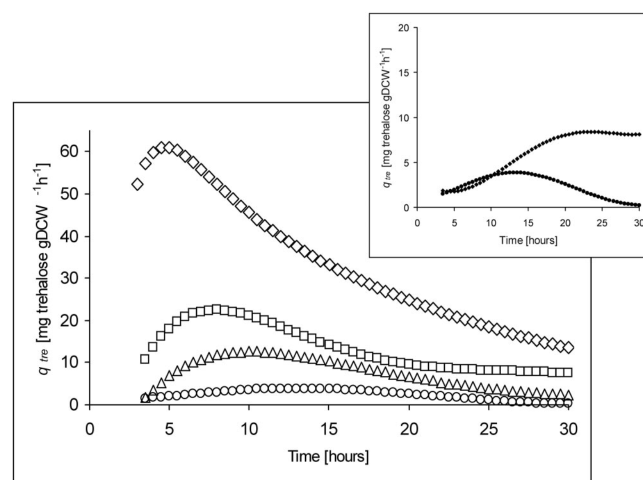

FIG. 4. q_{tre} during batch culture of *C. glutamicum* strains in DMCG II supplemented with 1 mM IPTG (added after 4 h of cultivation). The strains used were Δ*mluI*/pLPI*galUotsBA*01 induced with 0.1 mM IPTG (◇) or 1 mM IPTG (□), Δ*treS*/pLPI*otsBA*01 (△), and Δ*treS*/pLPI*otsBA*02 (control) (○). The insert shows the q_{tre} of Δ*mluI*/pLPI*galU*01 (◆) and Δ*treS*/pLPI*otsBA*02 control cells (●). gDCW, grams (dry weight) of cells.

(ii) Insufficient inorganic pyrophosphatase activity. The activity of inorganic pyrophosphatase in crude extracts of Δ*mluI*/pLPI*galU*01 cells was 14,718 pkat/mg of protein, which is the same order of magnitude as the GalU activity observed with strong induction (Table 2). This fact excludes the possibility that a thermodynamic barrier is an explanation for the observed results.

(iii) Draining of UDP-glucose mediated by alternative UDP-glucose-utilizing enzymes. The specific activities of the UDP-glucose-utilizing enzymes UDP-glucose-4-epimerase and UDP-galactose:glucose-1-phosphate uridylyltransferase were measured in crude extracts of Δ*mluI*/pLPI*galU*01 cells. The results (Table 2) showed that the values were low: 57 pkat/mg of protein for the epimerase and 36 pkat/mg of protein for the uridylyltransferase. Assuming that these values are representative of the in vivo situation, these reactions do not seem to be significant sinks of UDP-glucose, and in principle, this hypothesis can be ruled out. An alternative explanation might be an increased flux through the TreYZ pathway during *galU* overexpression. This hypothesis argues for the importance of a proper supply of glycogen, the substrate of this pathway of trehalose synthesis. Therefore, we designed a series of shake flask experiments to monitor the internal contents of glycogen and trehalose in *C. glutamicum* upon expression of the *galU* gene and the *galU otsBA* operon, respectively.

(a) Expression of the *galU* gene. First, we tested the effect of increasing *galU* expression on Δ*mluI*/pLPI*galU*01 cells. Figure 5A shows that increasing the level of *galU* expression by increasing the IPTG concentration from 0 to 0.5 mM led to a growth delay upon addition of the inducer. This delay depended on the presence of inducer but not on its concentration. The glycogen and trehalose contents were measured in the exponential phase. Almost twofold increases in the glycogen content (21 to 55 μmol of glucose residues/g [dry weight] of cells) and the trehalose content (28 to 59 μmol/g [dry weight] of cells) were observed upon induction, but again the change was independent of the IPTG concentration (Fig. 6A and B). We did not observe differences in the final titer of trehalose. The concomitant increases in the trehalose and glycogen contents agree with the hypothetical involvement of the TreYZ pathway. To obtain further proof of this hypothesis, a mutant strain, *C. glutamicum* Δ*treY* having an impaired TreYZ pathway, was transformed with plasmid pLPI*galU*01. The resulting strain, Δ*treY*/pLPI*galU*01, grew faster than Δ*mluI*/pLPI *galU*01 (Fig. 5B) in the absence of inducer. The fast growth was abolished by addition of IPTG (Fig. 5B), which also resulted in strong aggregation of the cells at the end of the growth phase. The glycogen content in induced cells was eightfold greater than that in the Δ*mluI*/pLPI*galU*01 cells under similar conditions (Fig. 6A). The trehalose content was reduced from 20 to 13 μmol/g [dry weight] of cells in induced cells (Fig. 6B), a value which was closely correlated to the ~50% reduction in the final titer of the disaccharide in the broth (Fig. 6C). These results fully agreed with the hypothetical increase in the flux through the TreYZ pathway and also explain the q_{tre} profile observed during bioreactor growth of the Δ*mluI*/pLPI*galU*01 strain, since glycogen synthesis increased during the stationary phase (29, 36).

(b) Expression of *galU otsBA* synthetic operon. To test whether there was similar TreYZ involvement during overexpression of the *galU otsBA* synthetic operon, the Δ*mluI*/pLPI *galUotsBA*01 strain was tested under the conditions described above for *galU*-expressing cells. Figure 5C shows the effect of increasing the concentration of IPTG (from 0 to 0.5 mM) on cell growth. As observed for the Δ*mluI*/pLPI*galU*01 strain, there was a significant delay in growth upon addition of the inducer. Significant increases in the external and intracellular

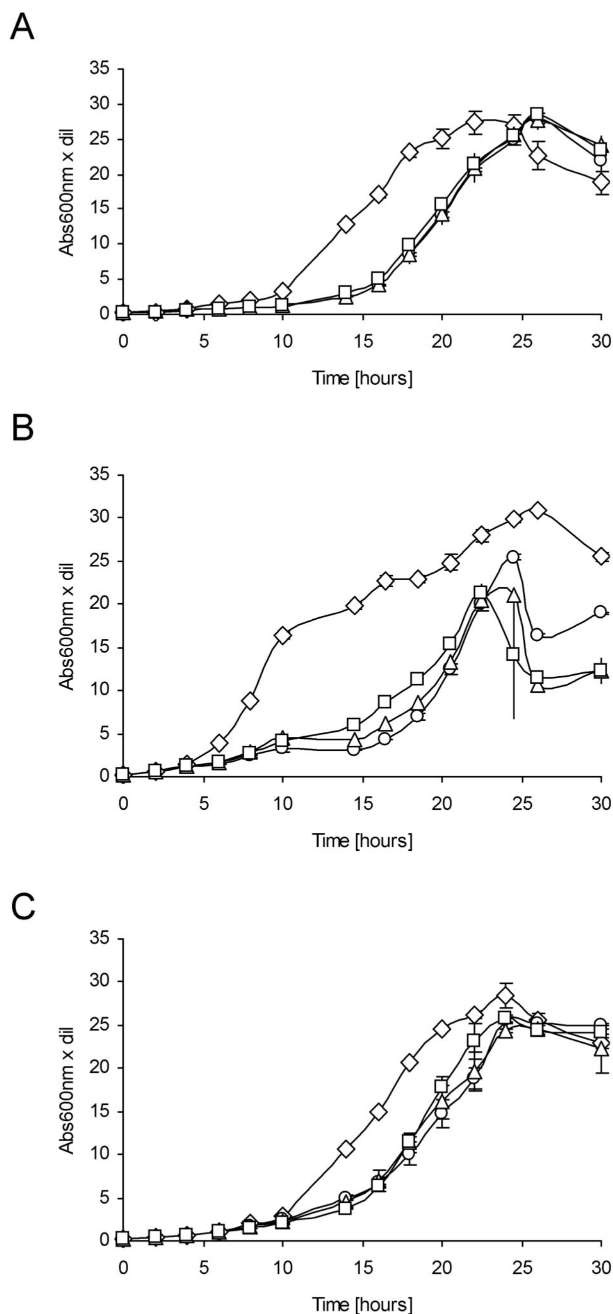

FIG. 5. Effects of inducer addition (no inducer [◇], 0.1 mM IPTG [○], 0.2 mM IPTG (△), and 0.5 mM IPTG [□]) on the growth curves of *C. glutamicum* strains Δ*mluI*/pLPI*galU*01 (A), Δ*treY*/pLPI*galU*01 (B), and Δ*mluI*/pLPI*galUotsBA*01 (C) in DMCG I. The culture medium was supplemented with 15 mg of chloramphenicol per liter to avoid plasmid loss, and IPTG (inducer) was added after 2 h of cultivation.

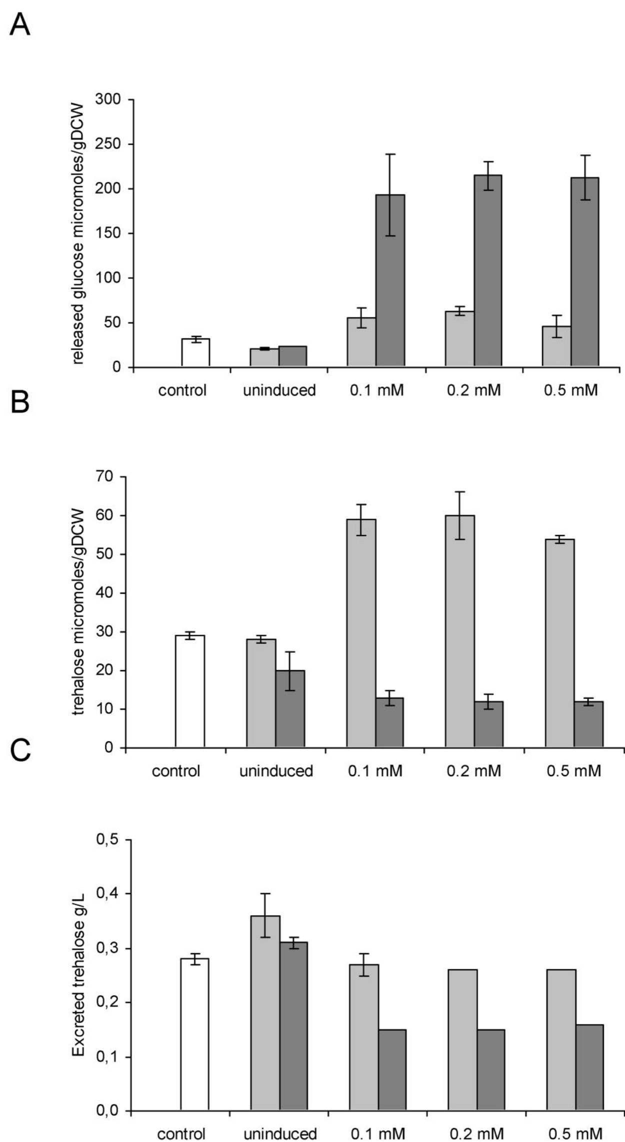

FIG. 6. Effects of several levels of *galU* gene expression on the cellular contents of glycogen (A) and trehalose (B) at the exponential growth phase and the final titers of trehalose (C) in *C. glutamicum* strains Δ*mluI*/pLPI*galU*01 (light grey bars) and Δ*treY*/pLPI*galU*01 (dark grey bars) growing on DMCG I. The culture medium was supplemented with 15 mg of chloramphenicol per liter to avoid plasmid loss, and IPTG (inducer) was added after 2 h of cultivation. For comparison, the open bars indicate the corresponding values for the Δ*mluI* strain. gDCW, grams (dry weight) of cells.

trehalose contents were observed in the absence of the inducer compared to the contents of a similar sample of the control strain, Δ*mluI* (Fig. 7B and C), as a result of the basal expression of the operon. The increases were inversely proportional to the amounts of IPTG added to the cultures. Also, the initial increase was significantly greater than that observed for the induced *galU* expression in the *C. glutamicum* Δ*mluI* strain. We did not detect an increase in the glycogen content under these conditions (Fig. 7A). These results suggest that there is marginal involvement of the TreYZ pathway during synthesis of trehalose in Δ*mluI*/pLPI*galUotsBA*01 cells. To validate this hypothesis, *C. glutamicum* Δ*treY* was transformed with plasmid pLPI*galUotsBA*01. The resulting strain, Δ*treY*/pLPI*galUotsBA*01, also showed increases in the internal and external concentrations of trehalose during shake flask experiments (Fig. 7), but the level of trehalose synthesis was lower than that observed in the Δ*mluI*/pLPI*galUotsBA*01 strain (there were 66 and 50% decreases in the internal and external trehalose concentrations, respectively). However, in the absence of inducer, there was no difference between the two strains. This result demonstrated

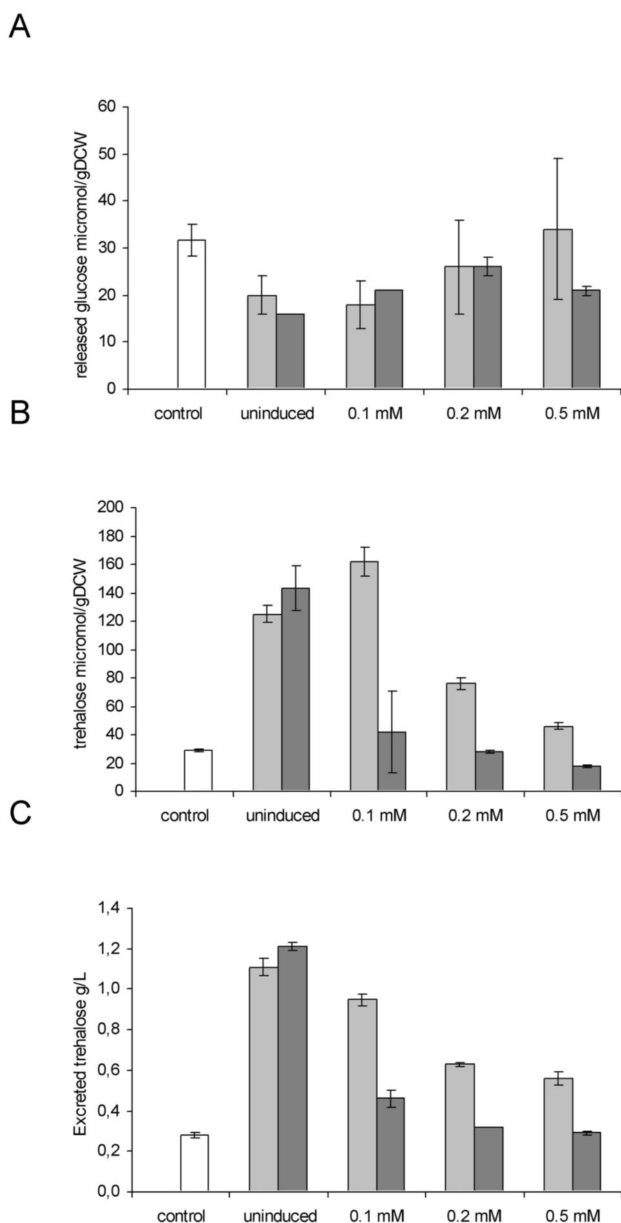

FIG. 7. Effects of several levels of *galU otsBA* gene expression on the cellular contents of glycogen (A) and trehalose (B) at the exponential growth phase and the final titers of trehalose (C) in *C. glutamicum* strains Δ*mluI*/pLPI*galUotsBA*01 (light grey bars) and Δ*treY*/pLPI *galUotsBA*01 (dark grey bars) growing on DMCG I. The culture medium was supplemented with 15 mg of chloramphenicol per liter to avoid plasmid loss, and IPTG (inducer) was added after 2 h of cultivation. For comparison, the open bars indicate the corresponding values for the Δ*mluI* strain. gDCW, grams (dry weight) of cells.

that there was significant involvement of the TreYZ pathway in trehalose synthesis only upon addition of IPTG.

DISCUSSION

In this study we investigated the impact of heterologous expression of the *E. coli galU* gene on trehalose synthesis in *C. glutamicum*. Interestingly, the metabolic consequences (i.e., whether the OtsAB pathway or the TreYZ pathway was predominantly involved) depended on the metabolic context studied (namely, expression of *galU* or *galU otsBA*).

Trehalose synthesis upon expression of the *galU* gene alone. The first scenario examined was heterologous expression of the *galU* gene alone. Induction of *galU* resulted in increases in the glycogen and trehalose contents of the cells, even at low concentrations of IPTG. The increase in the trehalose concentration was the result of TreYZ pathway activity, as demonstrated in the experiments in which Δ*treY* mutants were used. This relationship was also observed in a recent study (36) in which deletion of a putative glycogen synthase gene resulted in a significant decrease in the synthesis of trehalose by *C. glutamicum*. Although the link between glycogen and intracellular trehalose was clear, the relationship between *galU* expression and an increase in the glycogen content is puzzling. In principle, UDP-glucose utilization in glycogen synthesis is a straightforward explanation of the results observed. Unfortunately, there is no experimental support for this hypothesis, since the genes and enzymes involved in glycogen metabolism have not been characterized yet in *C. glutamicum*. Tzvetkov et al. (36) found two open reading frames encoding the putative enzymes ADP-glucose pyrophosphorylase and glycogen synthase, located adjacent to each other, indicating the presence of ADP-glucose-dependent glycogen synthesis in *C. glutamicum*, similar to that in most other bacteria (29). The only prokaryotic glycogen synthase that uses UDP-glucose exclusively known so far was found in the ruminal bacterium *Prevotella bryantii* (24).

An alternative explanation for the increase in glycogen content during induced *galU* expression is the interference by UDP-glucose with putative ADP-glucose futile cycling. In *E. coli*, the existence of an ADP-glucose pyrophosphohydrolase, encoded by the *aspP* gene, which hydrolyzes ADP-glucose to glucose-1-P and AMP, has been demonstrated (26). The simultaneous action of AspP and ADP-glucose pyrophosphorylase has been proposed to be required for regulation of the glycogen content. This was corroborated by an eightfold increase in the glycogen content of *E. coli* after deletion of the *aspP* gene (26). AspP belongs to the widespread family of Nudix (nucleoside diphosphate linked to an X moiety) hydrolases, which are enzymes involved in housecleaning of potentially toxic nucleoside compounds (2). These enzymes are characterized by a highly conserved motif, $GX_5EX_7REUXEEXGU$ (where X is any amino acid and U is usually Ile, Val, or Leu) in the catalytic domain (2). In the *C. glutamicum* chromosome database (http://gib.genes.nig.ac.jp/single/index.php?spid=Cglu_ATCC13032) we found five open reading frames harboring the Nudix motif and showing significant similarity to the *E. coli* AspP enzyme: Cgl1418 (25% similarity), Cgl0778 (41%), Cgl1318 (32%), Cgl2748 (30%), and Cgl1153 (36%). We also detected the corresponding activity accepting ADP-glucose as a substrate in crude extracts (data not shown). Inhibition of the (putative) pyrophosphohydrolase by UDP-glucose (based on observations of competitive inhibition by natural substrate analogues in some of these enzymes [30]) would result in the accumulation of ADP-glucose and therefore in an increase in the glycogen content. However, the studies performed with crude extracts were not fully conclusive in this respect. Further purification of the putative ADP-glucose pyrophosphohydrolase and optimization of the enzyme assay conditions are required to demonstrate inhibition of this enzyme by UDP-glucose. This hypothesis, in fact, offers a reasonable explanation for the puzzling observations that under strong *galU* overexpression conditions both glucose consumption and carbon dioxide evolution are reduced (a putative consequence of the reduced futile cycling of ADP-glucose) and glycogen accumulation is increased. The implications of this are significant not only for trehalose production but also for the production of other nonsugar metabolites, such as amino acids, where the elimination of such a putative futile cycle may be a feasible way to improve the yields of industrial processes in which *C. glutamicum* is employed.

Trehalose synthesis upon expression of the *galU otsBA* operon. The second scenario examined was coexpression of *galU* and the *otsBA* operon from *E. coli* in *C. glutamicum*. Upon expression of the synthetic operon *galU otsBA*, the increase in trehalose synthesis was dependent on the Ots pathway activity. The increase was more pronounced at low induction levels or even in the absence of IPTG in the medium, a fact explained by the occurrence of a cryptic promoter in the *galU* insert.

The inclusion of a promoter-like sequence in the PCR product corresponding to the *galU* gene occurred in spite of careful design of the PCR primers, which was intended to avoid amplification of the postulated RNA polymerase binding site of the *galU* gene (40). The occurrence of cryptic promoter-like sequences has been reported previously (25, 31). Although such sequences frequently interfere with induction experiments with reporter genes, in our case this fact turned out to be beneficial, since it saved addition of an expensive inducer during the process of trehalose synthesis. Moreover, a high level of induction negatively affected trehalose synthesis in cells expressing the synthetic operon. This effect may be ascribed to the formation of inclusion bodies biased towards Ots proteins or, more likely, to an imbalance in the translation of the enzymes encoded in the operon. This imbalance could be a consequence either of a polarity effect favoring the translation of the leading gene *galU* (39) or of a higher ribosomal binding efficiency in the *galU* gene (12). The observed predominance of GalU over OtsB activity, upon strong induction of *galU otsBA* operon, should result in a phenotype similar to that observed in the *galU*-expressing strain. However, such a result was not obtained. In fact, we observed neither accumulation of glycogen nor aggregation in the stationary growth phase of the Δ*treY*/pLPI*galUotsBA*01 strain, in sharp contrast to what was observed with the Δ*treY*/pLPI*galU*01 strain. A putative explanation for these results is the increase in the glycogen turnover rate during expression of the *galU otsBA* operon. This might also explain the decrease in trehalose synthesis observed upon strong induction of the operon, since trehalose synthesis through TreYZ pathway is affected by reduced glycogen accumulation. In terms of metabolic intermediates, a relevant difference between *galU* and *galU otsBA* overexpression might be

disturbance of the trehalose-6-phosphate pool. Therefore, we suggest that this compound is involved in the regulation of glycogen accumulation in *C. glutamicum*. In a previous study some evidence of the putative regulatory role of this compound was obtained; there was a significant reduction in the amount of glucose-6-phosphate/phosphogluconate dehydrogenases in *C. glutamicum* extracts upon expression of the *otsBA* operon (27), which was also ascribed to perturbation of the trehalose-6-phosphate pool. An effect on regulation of gene expression by this sugar phosphate has been demonstrated in *E. coli* (13), and a similar mechanism might also occur in *C. glutamicum*.

Additional targets in *C. glutamicum* trehalose metabolism. An interesting result obtained in this work is the observation of increased trehalose excretion upon an increase in the intracellular level of the disaccharide. This observation suggests that trehalose export is a limiting step, in contrast to previous observations (27). Unfortunately, both the mechanism and regulation of trehalose excretion in *C. glutamicum* are still unknown. Although the occurrence of trehalose uptake systems in some microorganisms has been described (11), the excretion of this disaccharide in microorganisms remains rather obscure (35). For *Saccharomyces cerevisiae*, evidence from genetic experiments points to the existence of a single gene expressing such a transporter (6). However, such studies have not been carried out so far with prokaryotic cells. Further research in this direction is required if we want to manipulate trehalose excretion in *C. glutamicum*.

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