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ORIGINAL PAPER

Protein production by *Escherichia coli* wild-type and *ΔptsG* mutant strains with IPTG induction at the onset

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Abstract During *Escherichia coli* growth on glucose, uptake exceeds the requirement of flux to precursors and the surplus is excreted as acetate. Beside the loss of carbon source, the excretion of a weak acid may result in increased energetic demands and hence a decreased yield. The deletion of *ptsG*, the gene coding for one of the components ($IICB^{Glc}$) of the glucose-phosphoenolpyruvate phosphotransferase system (Glc-PTS) reduced glucose consumption and acetate excretion. Induction of protein production at the onset of cultivation decreased growth rate and glucose consumption rate for both the WT and the mutant strains. The mutant strain produced β-galactosidase at higher rates than the wild-type strain while directing more carbon into biomass and CO_2 and less into acetate.

Keywords *E. coli* · *ptsG* deletion · Protein production · Batch-cultivation · Induction · Carbon-flux · Acetic acid formation

Introduction

Growth of *Escherichia coli* on a single carbon source implies that the carbon input has to fulfill a dual function: provision of the correct spectrum of precursors and supply of the energy required to transform these precursors into new biomass. During growth on several carbon sources, their uptake exceeds the requirement of flux to precursors and the surplus has to be excreted as acetate [8]. Although glucose has been widely used as carbon and energy source in *E. coli* recombinant fermentation technology, one of the major problems of glucose-containing media is the excretion of 10–30% of carbon supply as acetate into the medium [7]. Acetate accumulation not only results in dissipation of energy but also has an adverse effect on growth yield, induces the RpoS regulon, which is normally associated with entry into stationary phase [2] and has been correlated with a reduced production of recombinant protein [11]. It has been shown that the observed expression of the RpoS regulon is mainly triggered by a gradual decline in specific growth rate [9].

Several strategies have been tested to control acetate formation: the use of fed-batch techniques, genetic engineering approaches to direct carbon flux into a less harmful byproduct and the modulation of glucose uptake rate. Focusing on this last strategy, the first example of modulation of glucose uptake rate has been the use of the glucose analog methyl alpha-glucoside (αMG) by Chou et al. [4]. This compound is transported by the same phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) as glucose (Glc-PTS), thereby acting as a non-toxic competitive inhibitor and resulting in a reduction of acetate excretion. A strain bearing a mutation in one of the components of Glc-PTS, the *ptsG* gene, led to a reduction in acetate excretion together with an improvement in biomass and protein production [5]. Deletion of *ptsG* resulted in a strain that directed more carbon into biomass and carbon dioxide, and less into acetate. At the same time, this strain produced protein in amounts comparable to the wild-type strain [13].

This manuscript is dedicated to the memory of Pieter Postma.

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Here, we report on the production of *β*-galactosidase by a wild-type *E. coli* strain and its *ΔptsG* derivative when induction was performed at the onset of cultivation. Data recorded on specific consumption and production rates were subjected to statistical analyses. Our results show that IPTG induction at the onset of cultivation significantly decreased the growth rate ($P < 0.001$) and the glucose consumption rate ($P < 0.05$) of both strains. In spite of the low growth rate recorded for the mutant strain, the *β*-galactosidase production and yield on glucose were significantly ($P < 0.001$) higher than in the parent strain.

Materials and methods

Bacterial strains and cultivation media

The bacterial strains and plasmids used in this study are listed in Table 1. The medium used (PC) was a mineral salt medium composed of 2 g $(NH_4)_2SO_4$, 1.6 g KH_2PO_4, 6.6 g $Na_2HPO_4 \cdot 2H_2O$ and 0.5 g $(NH_4)_2$-H-citrate per litre. Glucose was autoclaved separately and added to the medium together with 1 mL L^{-1} sterile trace element solution and 1 M $MgSO_4$ solution, respectively. The trace element solution was composed of 0.5 g $CaCl_2 \cdot 2H_2O$, 16.7 g $FeCl_3 \cdot 6H_2O$, 0.18 g $ZnSO_4 \cdot 7H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, 0.15 g $MnSO_4 \cdot 4H_2O$, 0.18 g $CoCl_2 \cdot 6\ H_2O$ and 20.1 g NaEDTA per litre. If necessary, chloramphenicol and tetracycline were added to the medium at a final concentration of 34 µg mL^{-1} and 25 µg mL^{-1}, respectively.

Growth conditions

All the *E. coli* strains were inoculated from frozen stocks into 5 mL Luria-Bertani broth and grown for 6 h at 37 °C prior to inoculation in defined media. Cells were grown overnight at 37 °C in PC medium with 0.2% (wt/vol) glucose as carbon source. The next morning, exponentially growing cells were harvested, resuspended in 10 mL fresh PC medium with 0.2% glucose, and used as inoculum for batch-fermentors containing 400 mL mineral medium and 0.1 or 0.2% glucose as carbon source. Initial optical density at 600 nm in the bioreactor was around 0.1–0.15. Bioreactor cultivations were performed at 37 °C, with a stirring speed of 1,000 rpm and an aeration rate of one volume of air per vessel volume per minute. *β*-galactosidase production was induced by adding 100 µM isopropyl-*β*-D-thiogalactopyranoside (IPTG) in the reactor medium prior to cell inoculation. During cultivation, the pH of the culture was monitored and it did not deviate more than 0.1 unit from the preset value of 7.0.

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
AF1000	MC4100, *relA*$^+$, wild-type	Sandén et al. [15]
AF1016	AF1000/F'lacIqAmp^R/pAF1016	A. Farewell
PPA652	AF1000, *ΔptsG*::Km	Picon et al. [13]
pACYC184	p15A-replicon, Cm^R, Tc^R	Chang and Cohen [3]
pAF1016	pACYC184-derivative carrying P_{lacUV5}-*lacZ*, Cm^R	Sandén et al. [15]

Analytical determinations

Samples were withdrawn every hour throughout the cultivation. The cell growth was followed by optical density at 600 nm (Novaspec II, Amersham Pharmacia, Buckinghampshire, UK). Biomass concentration was determined as previously described [13]. Samples for the determination of extracellular metabolites were prepared by adding 100 µL 35% perchloric acid (v/v) to 1 mL of the culture supernatant. Samples were neutralized with 55 µL 7 M KOH. After centrifugation (3 min at 14,000*g*), the supernatant was filtered through 0,45 µm nylon syringe filters (Alltech, Capelle aan den IJssel, The Netherlands). Fermentation product concentrations (pyruvate, succinate, lactate, formate, acetate, acetoin and ethanol) and glucose content were determined by HPLC (LKB, Bromma, Sweden), using a Rezex organic acid column (Phenomenex, Torrance, CA) maintained at 65 °C. The mobile phase consisted of 7.2 mM H_2SO_4, with a flow rate of 0.5 mL/min. Peaks coming off the column were detected with a refractive index detector (LKB2142). The detection limits were 5 µM for pyruvate, 70 µM for succinate, 60 µM for lactate, 100 µM for formate, 80 µM for acetate, 140 µM for acetoin and 50 µM ethanol. Peak integration and data processing were done with Borwin (Le Fontanil, France) chromatography software.

Oxygen consumption and carbon dioxide production were determined by passing the gas from the batch-fermentors through an oxygen analyser (Taylor Servomex type OA 272, Zoetermeer, NL) and a carbon dioxide analyser (Servomex IR Gas analyser PA404).

Samples for *β*-galactosidase analyses were spun down at 5,000*g* for 10 min. Pelleted cells were washed once in cooled mineral medium, resuspended in 25 mM potasium phosphate buffer pH 7.0 supplemented with 0.5 mM EDTA and 0.5 mM DTT, frozen in liquid nitrogen and stored at −80 °C until analyses were performed. Cell extracts were prepared by disrupting cells at 800 psi in a French press cell (SLM Instruments, Inc., New York, USA). Cell debris was removed by centrifugation at 7,000*g* for 10 min. *β*-Galactosidase activity was assayed by measuring the rate of hydrolysis of o-nitrophenol-*β*-D-galactopyranoside (ONPG) as described [12]. *β*-Galactosidase activity was expressed as

micromole ONP mg protein^{-1} min^{-1}. β-Galactosidase was quantified by rocket inmunoelectrophoresis [10], using β-galactosidase (Sigma, St. Louis, MO, USA) as standard and 0.1% anti-β-galactosidase antibodies (Sigma). β-Galactosidase amount was expressed as percentage of total protein. Total protein content was determined on a COBAS-BIO automatic analyzer (Roche, Basel, Switzerland), using the bicinchoninic acid assay [16] with bovine serum albumin as standard.

Calculations

All measurements obtained from bioreactor cultivations were expressed as specific consumption or production rates (q values). These specific rates were calculated by dividing the difference of the measured concentrations between two consecutive time points by the mean dry weight and by the interval of time. Rates are expressed in millimoles g DW^{-1} h^{-1}.

Data were subjected to analysis of variance by means of the SPSS Windows version 8.0 (SPSS, Chicago, IL, USA). Comparison of means was carried out using Tukey's test.

Results

Growth rates in bioreactor cultivations

Averages of optical density and glucose present in the medium are plotted in Fig. 1. The maintenance of plasmid pAF1016 in the wild-type (WT) derivative strain did not alter growth rate (Table 2). However, the presence of IPTG

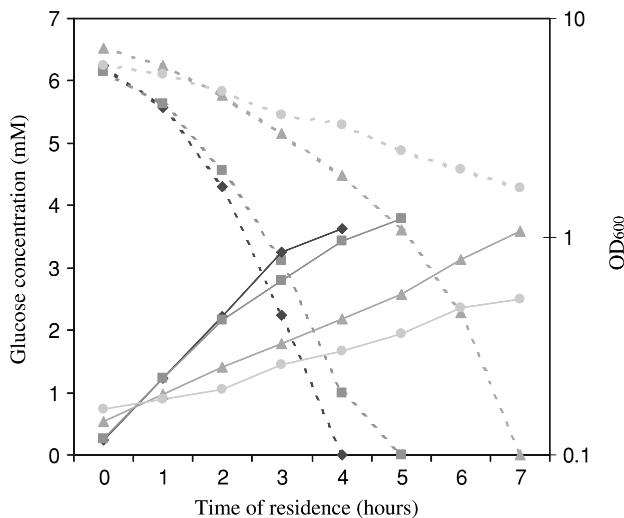

Fig. 1 Glucose (*dotted lines*) concentration and bacterial optical density (*full lines*) during batch-cultivations in PC minimal medium with 10 mM glucose as C-source. Markers: uninduced AF1016 cells (*filled diamond*), induced AF1016 cells (*filled square*), uninduced PPA652/pAF1016 cells (*filled triangle*) and induced PPA652/pAF1016 cells (*filled circle*)

Table 2 Growth rates and yields in bioreactor cultivations

Plasmid present	IPTG	Growth rate (h^{-1})		Yield (g DW/g glucose)	
		WT-der	PPA652-der	WT-der	PPA652-der
none	–	0.69*	0.46*	0.30*	0.39*
pAF1016	–	0.66*	0.27†	0.32*	0.37*
pAF1016	+	0.52†	0.20‡	0.33*	0.52†

Means of duplicate determinations on two trials. Mean values within each column with the same superscript (*, †, ‡) do not differ ($P < 0.001$)

in the bioreactor medium significantly ($P < 0.001$) decreased growth rate. The maintenance of the same plasmid in the PPA652-derivative without IPTG resulted in a strong and significant ($P < 0.001$) reduction of the growth rate [13]. When IPTG was present in the bioreactor medium, the growth rate of the PPA652-derivative was further reduced ($P < 0.001$). These results show that the presence of IPTG at the onset of cultivation caused a significant reduction in growth rate for both WT- and PPA652-derivatives.

β-Galactosidase production rate

To evaluate the efficiency of WT- and PPA652-derivatives to direct glucose into recombinant protein, the specific activity and amount of β-galactosidase produced in bioreactor cultures were determined. No significant differences were recorded for specific β-galactosidase activity measurements (expressed as µmol ONP mg protein^{-1} min^{-1}) between both strains (Fig. 2a). When specific β-galactosidase production rate was calculated from the specific activity (Fig. 2c), the PPA652-derivative strain nonetheless produced β-galactosidase at a significantly ($P < 0.001$) higher rate than the WT-derivative strain. The highest specific β-galactosidase production rate for the WT-derivative was recorded after 2 h of residence. From there on, a linear ($r^2 = 0.9916$) decrease in time was found. The specific β-galactosidase production rate in the PPA652-derivative increased slightly from 2 to 3 h. From 3 h onwards, a decrease in time was observed, although this did not fit so well ($r^2 = 0.9189$) into a linear decay.

Apart from measuring β-galactosidase production by specific activity, its amount was also determined by rocket immunoprecipitation assay [10]. No significant differences were recorded for β-galactosidase amounts (expressed as percentage of total protein) between WT- and PPA652-derivatives after 1 and 2 h of residence. From there on, all values recorded for the PPA652-derivative strain were significantly ($P < 0.001$) higher than the ones for the WT-derivative strain (Fig. 2b). When β-galactosidase production rate was calculated from these data (Fig. 2d), the values obtained for the PPA652-derivative strain were

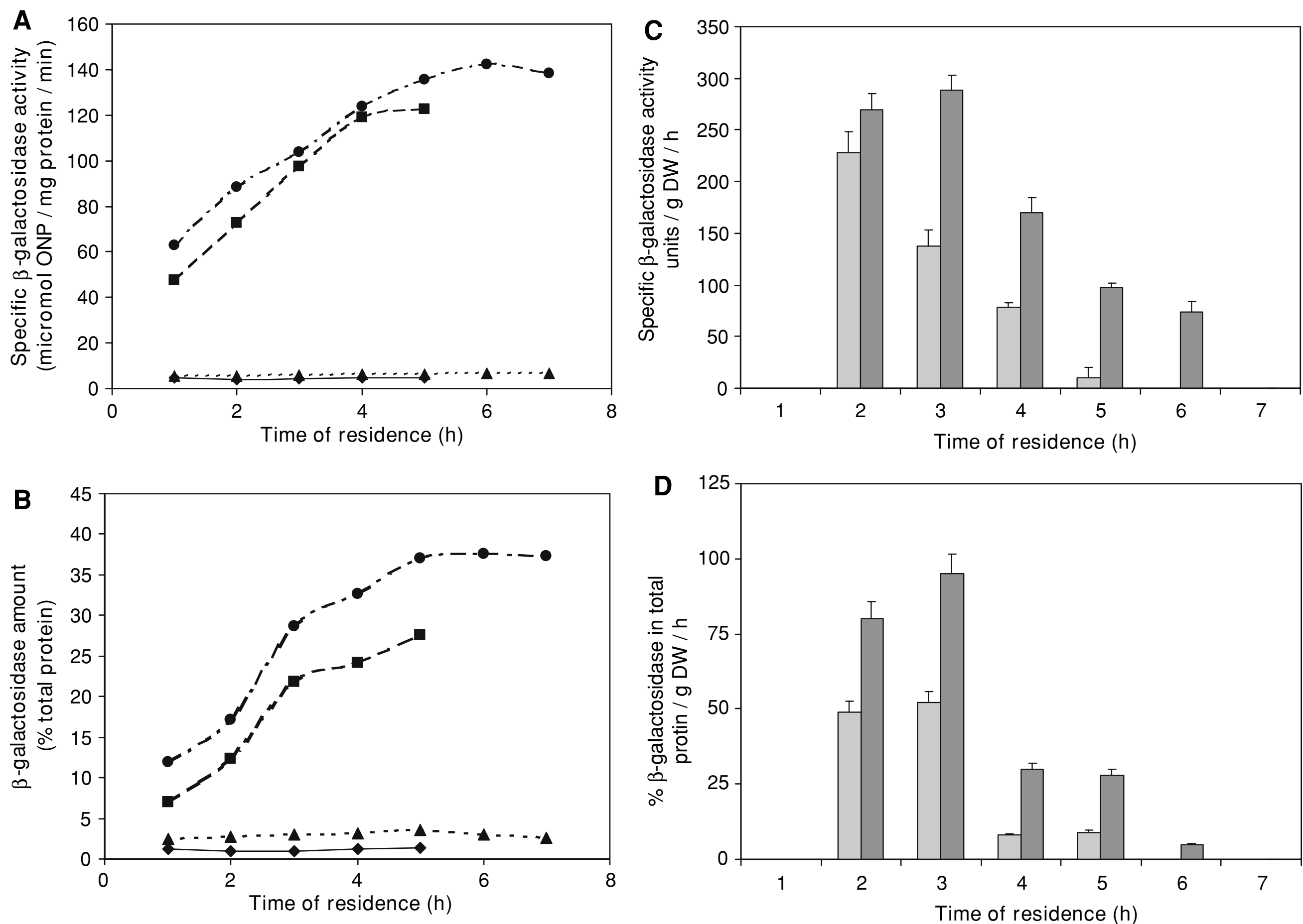

Fig. 2 *β*-Galactosidase production values during cultivations: **a** activity measurements (expressed as μmol ONP mg protein^{-1} min^{-1}) and **b** amount (expressed as percentage of total protein). Markers: uninduced AF1016 cells (*filled diamond*), induced AF1016 cells (*filled square*), uninduced PPA652/pAF1016 cells (*filled triangle*) and induced PPA652/pAF1016 cells (*filled circle*). Specific *β*-galactosidase production rate during cultivations: **c** activity rates (expressed as activity units g dry weight^{-1} h^{-1}) and **d** amount rates (expressed as percentage of *β*-galactosidase on total protein. g dry weight^{-1} h^{-1}). *Bars in light gray* correspond to induced AF1016 cells and in *dark grey* to induced PPA652/pAF1016 cells. All values are means of duplicate analyses from duplicate experiments. *Error bars* represent the standard error of the mean

significantly ($P < 0.001$) higher than the ones obtained for the WT-derivative strain. These results show that the PPA652-derivative strain was able to produce more *β*-galactosidase than the WT-derivative strain when protein production was induced at the onset of cultivation. While total *β*-galactosidase amount increased during cultivation, its production rate decreased in both strains.

Glucose consumption, external metabolites production and respiration rates

To determine the efficiency of WT- and PPA652-derivatives in directing glucose into cellular protein, the specific glucose consumption, external metabolite production and respiration rates were evaluated in bioreactor cultures. The WT-derivative strain consumed a significantly ($P < 0.05$) higher amount of glucose than the PPA652-derivative strain (Fig. 3). The presence of IPTG in the bioreactor medium significantly ($P < 0.05$) reduced glucose consumption rate for both strains. When comparing yields on glucose (Table 2), the WT-derivative strain produced similar amounts of dry weight per gram of glucose consumed in all experimental conditions. Upon induction, the values recorded for the PPA652-derivative strain were significantly ($P < 0.001$) higher than both the values for the PPA652-derivative without IPTG and the values for the WT-derivative in the presence of IPTG.

Acetate was the only external metabolite produced in measurable amounts. The WT-derivative strain produced significantly ($P < 0.05$) more acetate than the PPA652-derivative (Fig. 4). No significant effect on acetate production rate was recorded when IPTG was present in the bioreactor medium. The WT-derivative strain produced carbon dioxide at a higher rate ($P < 0.05$) than the PPA652-derivative (15–25 vs. 8–10 mmol CO_2 g DW^{-1} h^{-1}). These values were significantly ($P < 0.05$) different from each other

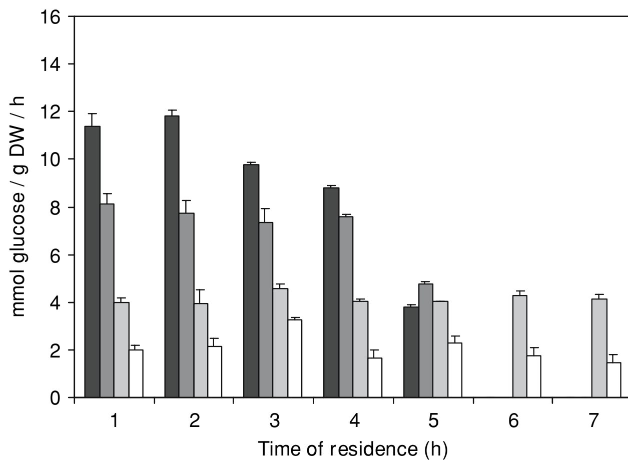

Fig. 3 Specific glucose consumption rate during cultivations. Values (expressed as millimole glucose g dry weight^{-1} h^{-1}) are means of duplicate analyses from duplicate experiments. *Bars* (from left to right, with decreasing gray intensity): uninduced AF1016 cells, induced AF1016 cells, uninduced PPA652/pAF1016 cells and induced PPA652/pAF1016 cells. *Error bars* represent the standard error of the mean

during the whole cultivation. Represented as fluxes, the PPA652-derivative strain directed less carbon into acetate than the WT-derivative (0.09 vs. 0.55 mol of acetate per mol of glucose consumed) and had a more aerobic metabolism (3.2 vs. 2.2 moles of CO_2 produced per mol of glucose consumed). PPA652-derivatives were more efficient in directing carbon-flux to biomass (0.80 vs. 0.64 mol of biomass produced per mol of glucose consumed). These results show that PPA652-derivatives could use glucose in a more efficient manner than WT-derivatives.

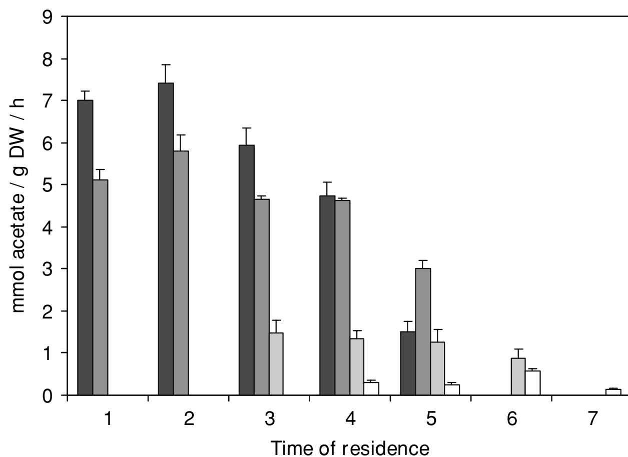

Fig. 4 Specific acetate production rate during cultivations. Values (expressed as millimole acetate g dry weight^{-1} h^{-1}) are means of duplicate analyses from duplicate experiments. *Bars* (from left to right, with decreasing gray intensity): uninduced AF1016 cells, induced AF1016 cells, uninduced PPA652/pAF1016 cells and induced PPA652/pAF1016 cells. *Error bars* represent the standard error of the mean

Discussion

One of the problems often encountered in recombinant protein production is the accumulation of acetate, which results in dissipation of energy and reduced growth yield and protein production. In this study, we have used a mutant engineered to redirect carbon flux away from acetate generation and towards β-galactosidase production. Protein induction at the onset of cultivation resulted in a decreased growth rate and glucose consumption rate for both the WT and the mutant strains. Although the growth rate of the PPA652-derivative strain was lower than the WT-derivative, its protein production rate and yield on glucose were higher than those of the parent strain. On the contrary, glucose consumption and acetate production rates for this strain were lower than the ones recorded for the WT-derivative strain.

Many studies have been devoted to the reduction of acetate accumulation by designing optimal feeding strategies to maintain low residual glucose concentration. However, those nutrient feeding optimization strategies (fed-batch operations) require very elaborate techniques to maintain glucose below levels not giving rise to acidogenesis [1]. Moreover, implementation of complicated nutrient feeding profiles requires constant supervision, which makes process operation less convenient and more susceptible to errors [4].

In order to measure the in vivo flux distribution, we had to use a mineral medium, although it is not commonly used in practical applications. When cells are growing on a minimal medium, they have to synthesize all their amino acids de novo. Under those harsh conditions, we have shown that the mutant strain results in an improved performance per unit of cell mass. The molecular basis for the regulatory processes that take place in the mutant strain, and lead to the effects observed in this study, will be discussed further.

During growth on glucose, several regulation processes involving the specific components of the Glc-PTS (enzymes IIA^{Glc} and $IICB^{Glc}$), the pleiotropic transcriptional regulator Mlc and the catabolite repressor-activator protein Cra results in an activation of the glycolytic pathway and the common PTS components together with an inhibition of the TCA cycle [13] giving place to an imbalance situation as described by El-Mansi and Holms [6]. In our PPA652-derivative strain the deletion of the *ptsG* gene (coding for $IICB^{Glc}$) modifies all these complex regulations resulting in a less active glycolytic pathway together with a more active TCA cycle and glyoxylate shunt. The lower specific glucose consumption rates and acetate production rates recorded for this strain together with the lower levels of some glycolytic enzymes identified by mass spectrometry on 2D gel protein spots [13] are in good agreement with this hypothesis.

The higher production rate in the mutant strain is not due to its low specific growth rate, as have been shown by

Sandén et al. [15] in fed-batch cultivations with strain AF1016. In those experiments β-galactosidase production was studied with respect to the specific growth rate at induction. Although a high growth rate ($0.5\ h^{-1}$) was accompanied by a high-level acetate accumulation vs no acetate accumulation at $0.1\ h^{-1}$, protein production rate was approximately 100% higher at the higher growth rate. Similar results were obtained during carbon-limited continuous cultivation with strain AF1016 in our laboratory.

When WT-derivative and PPA652-derivative strains were induced after reaching an optical density at 600 nm of 0.4 we concluded that β-galactosidase was produced in amounts not significantly different from each other [13]. In this study we have shown that the PPA652-derivative strain produced β-galactosidase at higher rates than the WT-derivative strain. We have also observed that the presence of IPTG in the bioreactor medium significantly ($P < 0.05$) reduced glucose consumption rate for both strains. Levels of internal glucose in both type of cells are probably lower than when induction took place at an OD of 0.4, and that may have an effect on the level of cAMP-CRP, which exerts a positive control on the *lac* operon, resulting in the higher protein production rate observed in the PPA652-derivative strain. Other authors also found that aminolevulinate synthase production by strain AFP111 (*ΔptsG*−derivative of strain MG1655) was higher than its production by WT when glucose was used as carbon source [17].

The work presented in this article (the characterization of this mutant strain) has to be seen as a first step necessary for the further use of the strain in protein production. Further experiments to produce other types of proteins and to elucidate the metabolic routes in the mutant strain are in progress.

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