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Overproduction of Recombinant Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase from *Synechococcus* sp. Strain PCC6301 in Glucose-Controlled High-Cell-Density Fermentations by *Escherichia coli* K-12

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A predictive and feedback glucose feed controller, previously developed for nutrient-sufficient growth of *Escherichia coli* to high cell densities, was used to produce large quantities of a heterologous, cyanobacterial recombinant hexadecameric (L_8S_8) protein, ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) in *E. coli*. Culture and plasmid stability conditions were optimized to yield the production of approximately 1 g of soluble, active recombinant RubisCO per liter. Recombinant RubisCO also was produced in lactose-induced high-cell-density fermentations of *E. coli* K-12.

We have previously developed a predictive glucose-controlled (GC), high-cell-density (HCD) fermentation procedure for the substrate-sufficient growth of *Escherichia coli* at high densities (13, 14, 17) and have used this system to study the physiology of *E. coli* under HCD fed-batch growth conditions (17).

Form I ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) is a hexadecameric (L_8S_8) enzyme with an M_r of approximately 550,000 (27). In previous studies, only low levels (ca. 50 mg/liter) of recombinant RubisCO were produced in large-scale fermentations (24). The heat shock proteins GroES and GroEL have been shown to be required for the proper folding and subsequent assembly of recombinant RubisCO in *E. coli* (9), and recent evidence indicates that other chaperonins such as DnaK may have a significant role in proper folding of RubisCO (5). It is well-known that the induction of heat shock proteins occurs under a wide variety of stresses encountered by *E. coli* (4). Because HCD fermentation conditions would be expected to induce stress responses in *E. coli* due to high osmolarity, cell densities, nutrient inconsistencies due to difficulties with mixing, and shear forces, we predicted that foreign RubisCO, the assembly of which requires GroES and GroEL, should be well expressed and correctly folded under these types of fermentation conditions without the requirement for overexpressed *groES* and *groEL* genes. Herein, we examine the ability of recombinant *E. coli* to overproduce *Synechococcus* sp. strain PCC6301 RubisCO in GC HCD fed-batch fermentations of wild-type *E. coli* K-12. This source of cyanobacterial RubisCO is especially important for structure-function studies, as its structure has been solved (25) and this protein is quite amenable to site-directed mutagenesis manipulation (26).

Bacterial strains and plasmids. *E. coli* K-12 (ATCC 10798) was obtained from the American Type Culture Collection. For routine genetic manipulations, *E. coli* was grown in Luria-Bertani (LB) medium (21). To maintain selective conditions for the various plasmids, the agar and liquid media contained 100 μg of ampicillin per ml.

Plasmid pBGL710 (Fig. 1A) (20), containing the RubisCO genes (*rbcL* and *rbcS*) from *Synechococcus* strain PCC6301, was isolated and purified from *E. coli* MV1190 as described by Kraft et al. (18). Plasmid pANT900 (Fig. 1B) was constructed by subcloning the 2.2-kb *Pst*I DNA fragment containing the *rbcL* and *rbcS* genes from pBGL710 into the *Pst*I site of pTRC99A (Pharmacia Biotech, Uppsala, Sweden) (1). Orientation of the insert was confirmed by restriction digestion. Plasmid pANT900 contains *lacI*^{q}, a *lacI* promoter up-mutation that yields about 10-fold oversynthesis of the repressor (22), as well as a strong transcription terminator downstream of *rbcS*, whereas pBGL710 does not contain *lacI* and lacks known vector terminators after the inserted genes. Both plasmids contain the pBR322 origin of replication, indicating that their copy numbers should be approximately 30 (8). *E. coli* K-12 strain ATCC 10798, transformed with either pBGL710 or pANT900 by standard procedures (21), was used for these studies.

Fermentation procedures. Batch cultures were grown in 250-ml flasks containing 50 ml of either LB medium (21) or fermentation medium (FM), which consists of the following components (per liter): casein hydrolysate, 40 g; KH_2PO_4, 7.5 g; Na_2HPO_4, 7.5 g; K_2SO_4, 0.85 g; glucose, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.17 g; and trace elements solution (14), 0.8 ml. The glucose, $MgSO_4 \cdot 7H_2O$, and trace elements solution were autoclaved separately and added after cooling. The cultures were inoculated with a 1% (vol/vol) exponential-phase inoculum, grown at 37°C for 4 h on a rotary shaker (250 rpm), induced with 100 mg of isopropyl-β-D-thiogalactoside (IPTG) per liter, and then grown with induction for an additional 3 h prior to harvesting by centrifugation.

Ten-liter GC HCD fermentations were performed at 35°C with the predictive glucose feedback computer-controlled system and the optimized algorithm parameters (proportional constant, 0.5; number of datum points used to calculate glucose consumption, 5) described previously (14). Inocula were 5% (vol/vol) exponential-phase cells growing in LB medium containing 100 μg of ampicillin per ml. Dry cell weight (DCW) and acetate concentration were measured for each time point shown as described previously (14). To maintain selective pressure for plasmid retention during the fermentations, ampicillin was added with the inoculum to a final concentration of 100 mg/liter. An additional 1.0 g of ampicillin was added to the fermentor in the casein hydrolysate feed. To determine the

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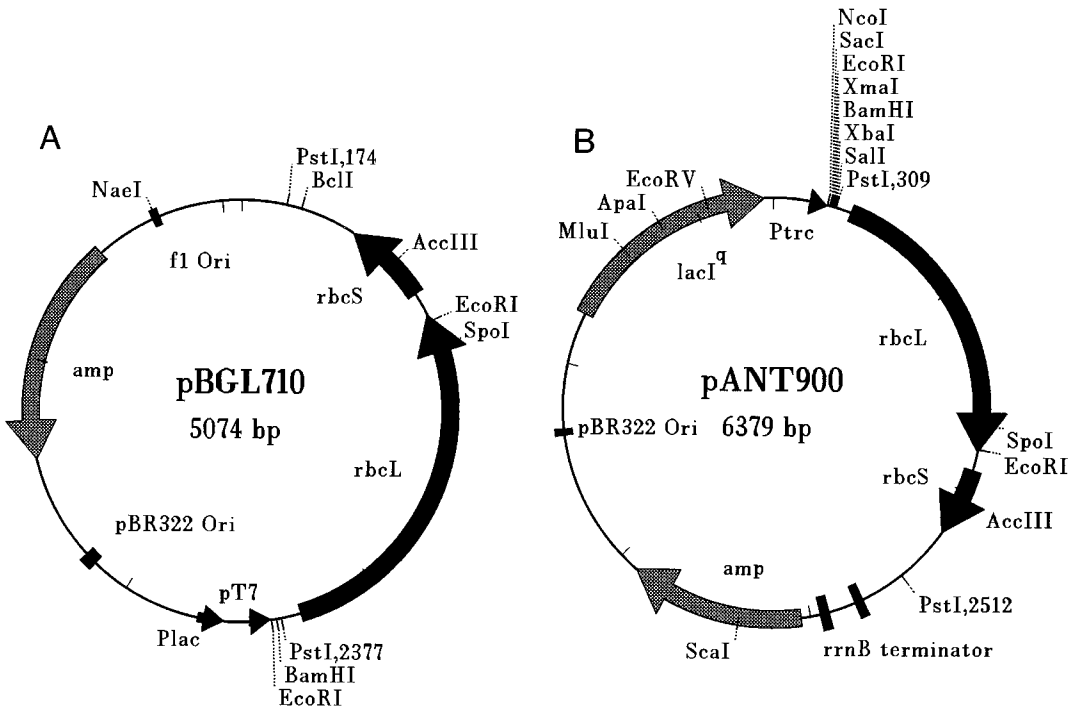

FIG. 1. Plasmid maps. (A) Plasmid pBGL710 (20), containing *rbcL* and *rbcS*, encoding large and small subunits of *Synechococcus* strain 6301 RubisCO, respectively, driven by the *lac* (Plac) promoter. (B) Plasmid pANT900, constructed in this work, contains *rbcL* and *rbcS* in pTRC99A (*lacI*^{q}) under expression of the strong *trc* (Ptrc) promoter.

fraction of cells retaining the plasmid, duplicate samples from the fermentor were diluted in sterile 0.1% (wt/vol) peptone and plated onto the LB agar plates with or without 100 μg of ampicillin per ml.

For lactose induction, two additional feeds were used. The first feed contained 70 g of lactose per liter, and the second feed contained 300 g of lactose per liter. Lactose concentration was maintained at 2.0 g/liter by using a model 2700 biochemical analyzer (YSI, Inc., Yellow Springs, Ohio) and the same algorithm as used for the glucose control.

All batch and GC HCD fermentations were duplicated; the results shown in this paper are for representative fermentations.

Assays. For quantitation of RubisCO and β-galactosidase (β-Gal), crude extracts were prepared from samples taken from the fermentor. The cells were pelleted by centrifugation for 8 min at 8,000 × *g* (4°C). The supernatant was removed, and the cells were resuspended in TEM buffer (25 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 5 mM β-mercaptoethanol) and pelleted again by centrifugation. The pelleted cells were frozen immediately at −70°C. For analyses, the samples were thawed and resuspended in TEM buffer. The cells were then lysed using an automated French pressure cell at 12,600 lb/in^2. Unbroken cells and large debris were pelleted by centrifugation for 20 min at 20,000 × *g* (4°C), and the supernatants (crude cell extracts) were used for analyses. The protein concentration was determined by the Bradford method (3) using the Bio-Rad (Hercules, Calif.) reagent.

The levels of RubisCO and β-Gal protein in crude extracts were quantitated by rocket immunoelectrophoresis as described previously (11). Purified RubisCO and polyclonal antibody against native RubisCO were as described previously (20). Pure *E. coli* β-Gal and anti-β-Gal polyclonal antibody were purchased from Sigma Chemical Co. (St. Louis, Mo.). RubisCO enzyme assays were performed as described previously (28). Western blotting (immunoblotting) was performed as described by Gallager et al. (7) after fractionation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Antibody-antigen complexes were visualized by using biotin-labeled goat anti-rabbit antibody (Kirkegaard & Perry Laboratories [KPL], Gaithersburg, Md.) and peroxidase-labeled streptavidin (KPL). The reaction was performed using 4-chloro-naphthol (KPL) as a substrate for the peroxidase enzyme.

Batch culture results. Batch shake flask cultures of *E. coli* K-12(pBGL710) and *E. coli* K-12(pANT900) grown in LB medium resulted in the synthesis of recombinant RubisCO at 4.3 and 3.6% of the total soluble protein, respectively (Table 1). This compares to a value of ca. 13% obtained for *E. coli* MV1190 (pBGL710) (20); this figure, however, was obtained with overnight induction rather than the 3-h induction period used in this study. In batch shake flask cultures using FM, which is the initial base medium used for the predictive GC HCD fed-batch fermentation procedure (14), recombinant RubisCO was synthesized at levels of 14.3 and 12.9% of total soluble protein, respectively, by *E. coli* K-12(pBGL710) and *E. coli* K-12(pANT900) (Table 1). Thus, batch cultures of *E. coli* K-12(pBGL710) and *E. coli* K-12(pANT900) produced comparable levels of RubisCO in either medium.

IPTG-induced fed-batch fermentations of *E. coli* K-12(pBGL710). On the basis of batch culture data, it appeared that *E. coli* K-12(pBGL710) was a good candidate strain for use in the GC HCD fermentations. The GC HCD fermentation of *E. coli* K-12(pBGL710) had an initial specific growth rate of 0.87 h^{-1} (48-min generation time) and resulted in a final biomass concentration of nearly 62 g/liter, with only 7.6 g of acetate accumulated per liter (Table 1). Approximately 80% of the plasmid was retained in the fermentations of *E. coli* K-12(pBGL710) (Table 1). RubisCO was produced at a level of 109 mg/liter,

TABLE 1. Comparison of *E. coli* K-12 shake flask and GC HCD fed-batch fermentations producing recombinant RubisCO[a]

Plasmid	Fermentation condition	Medium used	Inducer used	Plasmid stability (%)[b]	DCW (g/liter)	Acetate produced (g/liter)	RubisCO concn (mg/liter)	% RubisCO[c]	mg of RubisCO/g (DCW)	β-Gal concn (mg/liter)	% β-Gal[c]
pBGL710	Flask	LB	IPTG	NA[d]	2.5	NA	41.6	4.3	16.6	NA	NA
pANT900	Flask	LB	IPTG	NA	2.7	NA	37.8	3.6	14.0	NA	NA
pBGL710	Flask	FM	IPTG	NA	3.4	NA	274	14.3	80.6	NA	NA
pANT900	Flask	FM	IPTG	NA	6.4	NA	366	12.9	57.2	NA	NA
pBGL710	GC HCDF[e]	FM-FB[f]	IPTG	80	61.6	7.6	109	0.4	1.8	400	1.6
pANT900	GC HCDF	FM-FB	IPTG	86	45.7	7.0	960	3.6	21.0	ND	ND
pBGL710	GC HCDF	FM-FB	Lactose	88	34.3	5.4	40	0.2	1.2	180	0.9
pANT900	GC HCDF	FM-FB	Lactose	97	23.0	4.0	ND[g]	ND	ND	ND	ND

[a] All fed-batch fermentations were controlled at pH 7.0 except for that with IPTG-induced *E. coli* K-12(pBGL710), which was controlled at pH 7.5. In replicate experiments, similar data were obtained with *E. coli* K-12(pBGL710) at pH 7.0 (13).
[b] Percentage of cells retaining plasmid as analyzed by antibiotic resistance.
[c] Percentage of total soluble protein.
[d] NA, not assayed.
[e] HCDF, HCD fed-batch fermentation.
[f] FM-FB, FM with glucose-controlled feed regimen as previously described (14, 17).
[g] ND, none detected.

0.4% of the total soluble protein. Since *E. coli* K-12 is Lac^+, β-Gal was produced to a final concentration of 400 mg/liter, or 1.5% (wt/wt) of total soluble protein (Table 1). This level is below the level that can be attained in typical nonrecombinant strains of *E. coli* (5% [wt/wt] of total soluble protein) (29). In preliminary experiments, identical GC HCD fermentations of *E. coli* MV1190(pBGL710) and *E. coli* JM103(pBGL710) resulted in unacceptably high levels of plasmid loss with concomitant reproducibly negligible levels of recombinant RubisCO production (13).

In the GC HCD fermentations of *E. coli* K-12(pBGL710), both RubisCO and β-Gal were produced at low levels prior to induction with IPTG, indicating that repression of the *lac* operon was not complete. Approximately 10 *lac* repressor molecules are typically present in wild-type *E. coli* cells carrying only a chromosomal copy of *lacI* (2). Thus, the additional repressor binding site carried on the medium-copy-number plasmid (ca. 30 copies per genome), pBGL710, would likely titrate out available repressor and allow weak transcription from the *lac* promoters. This does not explain, however, why only low levels of RubisCO were produced after induction by IPTG. In terms of plasmid stability, growth rate, biomass achieved, acetate produced, reproducibility, and RubisCO produced, HCD cultures of *E. coli* K-12(pBGL710) outperformed those of *E. coli* MV1190(pBGL710) and *E. coli* JM103 (pBGL710) (13). None of these recombinant cultures, however, resulted in reproducibly high levels of RubisCO production in GC HCD fermentations (13).

IPTG-induced fed-batch fermentations of *E. coli* K-12 (pANT900). Since *E. coli* K-12(pBGL710) yielded such poor levels of RubisCO in IPTG-induced, GC HCD fermentations, a new plasmid, pANT900 (Fig. 1B), was constructed for RubisCO expression in the GC HCD fermentations of recombinant *E. coli* K-12. Plasmid pANT900 contains a plasmid-borne copy of $lacI^q$, which should decrease preinduction expression, and the strong promoter *trc*, which is a hybrid *trp-lac* promoter similar in strength to the *tac* promoter but containing a 17-nucleotide spacer region between the −10 and −35 regions (1).

GC HCD fermentations of *E. coli* K-12(pANT900), induced at 5 h with IPTG, resulted in a DCW of nearly 46 g/liter (Table 1; Fig. 2). The initial growth rate (0.93 h^{-1}), plasmid stability, and acetate production (Fig. 2) were very similar to those obtained in the same fermentation procedure using *E. coli* K-12(pBGL710) (Table 1). Prior to induction with IPTG, neither RubisCO activity (Fig. 2), RubisCO protein (Fig. 3), or β-Gal protein (Table 1) was measurable in fermentor samples, indicating that overproduction of LacI fully repressed the *lac* and *trc* promoters. After induction, the RubisCO concentration increased to a maximum of 960 mg/liter, or approximately 3.6% (wt/wt) of the total soluble protein (Fig. 2). The increase in specific activity of RubisCO (as a function of total soluble protein) closely followed the synthesis of the protein (Fig. 3). Figure 3 also shows that the heterologous protein produced by *E. coli* had the same molecular weight as the purified protein and that no observable inadvertent processing of the protein had occurred.

During the later stages of the fermentation, when the RubisCO concentration was greater than 3% of the total protein, the specific activity was approximately 2.8 μmol of CO_2 fixed per min per mg of total RubisCO, a level typical for the pure protein (20). This level of specific activity indicates that the RubisCO present was properly folded, fully assembled, and functional. No inactive, insoluble aggregates were observed.

To examine the affect of starvation conditions on recombinant protein production and stability, the addition of glucose to the culture was terminated shortly before 9 h (Fig. 2). At this point, the remaining glucose was consumed and the glucose concentration remained at 0 g/liter through the remainder of the fermentation (about 3 h). After 3 h under starvation conditions, the amount of RubisCO as a percentage of soluble protein decreased very little (3.6% [wt/wt] before starvation to 3.2% [wt/wt] after 3 h of starvation). The total amount of soluble protein in the fermentor, however, also decreased to 21 g/liter from 27 g/liter (13). Thus, the recombinant RubisCO protein was degraded at a rate approximately half that of the total cellular proteins in the culture.

To our knowledge, the results of large-scale recombinant fermentations of *E. coli* producing RubisCO have been published only once previously, when Newman and Gutteridge reported yields calculated to be ca. 50 mg of RubisCO per liter in 50 to 200-liter fermentations (24). The GC HCD method produced approximately 18-fold more RubisCO on a volumetric basis than the previously obtained productivities (24).

The intricacy and size of the RubisCO complex (a protein with an M_r of 550,000 comprising 16 subunits from two gene products) would make synthesis and assembly of an active enzyme much more difficult than most recombinant proteins.

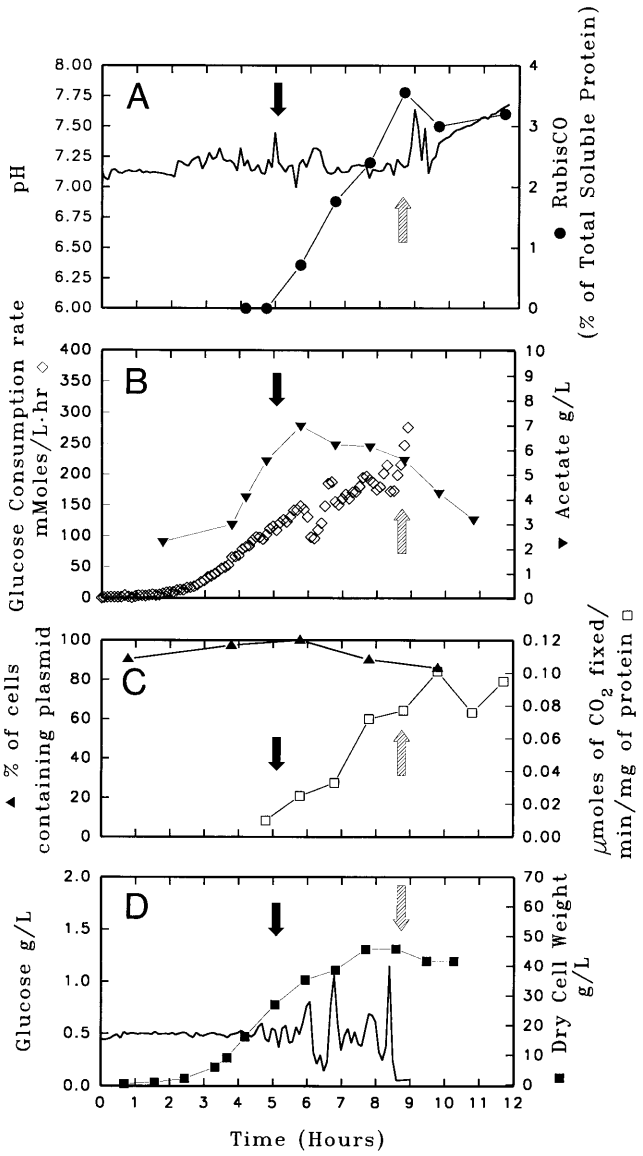

FIG. 2. Fed-batch fermentation of *E. coli* K-12(pANT900) grown to HCD with the pH controlled at 7.0 and the soluble glucose concentration controlled 0.5 g/liter. (A) pH and the amount of RubisCO present with respect to fermentation time; (B) glucose consumption rate and soluble acetate concentration with respect to time; (C) plasmid stability and activity of RubisCO with respect to time; (D) glucose concentration and DCW with respect to sampling time. The filled arrows indicate the time of induction, and the hatched arrows indicate the time at which the feed pumps were turned off.

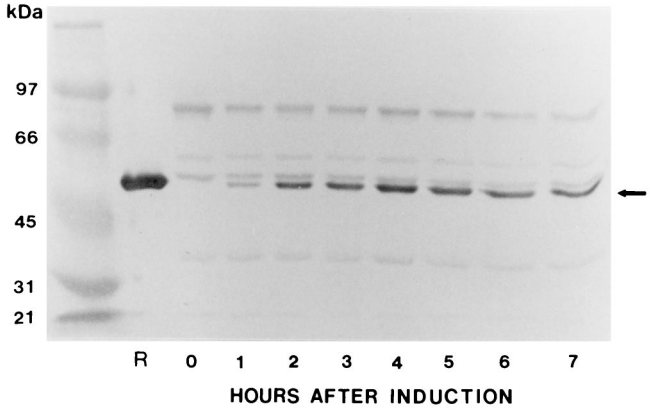

FIG. 3. Western blot of the large subunit of RubisCO (arrow). The first (unlabeled) lane contains prestained molecular size markers. Lane R contains the purified large subunit of RubisCO. The remaining lanes were loaded with extracts from different samples taken during the fermentation. The same amount (3 μg) of protein was loaded per lane in lanes 0 to 7. Lane 0 contains extract from a sample immediately prior to induction. Lanes 1 to 7 contain extract from samples taken every hour, 1 to 7 h after induction. The extraneous bands on the immunoblot are due to other biotinylated proteins in the extract.

Indeed, most proteins produced in *E. coli* HCD fermentations reported in the literature over the past several years are homomeric, and usually monomeric, in structure (15, 16). Thus, the production of such a large and complex protein as RubisCO in an active state in HCD fermentations is novel.

The proper folding and subsequent assembly of RubisCO in *E. coli* are known to be facilitated by the heat shock (chaperonin) proteins GroES and GroEL (9). When these proteins are overproduced, a 5- to 10-fold increase in recovery of active RubisCO proteins is observed (9). Thus, it may be possible to increase the amount of active RubisCO in our system when other sources of recombinant RubisCO are synthesized (19) by increasing the level of GroES and GroEL in the cells. The concentrations of these chaperonins, as well as other possibly required chaperonins such as DnaK (5), in the GC HCD fermentation of *E. coli*, however, appeared to be sufficient for the correct assembly of the *Synechococcus* strain 6301 RubisCO produced, as the specific activity of RubisCO with respect to RubisCO concentration in these fermentations was typical for that obtained with pure protein. This is particularly interesting, because the fermentations were run at 35°C, a temperature under which GroES and GroEL would be expected to be formed only at basal (i.e., uninduced) levels (4). It is possible that conditions within the GC HCD fermentations, including highly osmotic conditions, cell densities approximately 20-fold greater than those obtained in shake flasks of LB medium, and periods of fluctuation in nutrients (i.e., due to mixing limitations), induce stress responses, including the heat shock response.

Lactose as an inducer for recombinant GC HCD *E. coli* fermentations. The *lac* and *trc* promoters used in pBGL710 and pANT900, respectively, are inducible by either allolactose or IPTG. While the gratuitous inducer IPTG has been used traditionally, its cost can be prohibitive in large-scale fermentations. Lactose, on the other hand, is inexpensive and has been used successfully to induce cultures in small-scale batch fermentations (6) as well as large-scale fed-batch cultures (10, 12, 23). If lactose were used as the sole carbon and energy source by the culture as well as the inducer, this could prevent the repression of heterologous protein production by glucose catabolism, which is observed even when IPTG is used (12). Since the on-line YSI model 2700 biochemical analyzer is able to determine the concentrations of glucose and lactose simultaneously, we used this system in an attempt to induce GC HCD fermentations of *E. coli* K-12(pBGL710) and *E. coli* K-12(pANT900) with a continuous feed of lactose after initial growth to HCDs using glucose. The novelty of this approach is the induction of a Lac^+ *E. coli* strain using a continuous, controlled lactose feed.

In IPTG-induced GC HCD fermentations of *E. coli* K-12 (pBGL710) (Table 1), β-Gal was produced to a final concentration of 1.5% (wt/wt) of the total protein when the culture was induced with IPTG. Thus, this culture would be expected to be able to grow, and be induced, with a continuous feed of

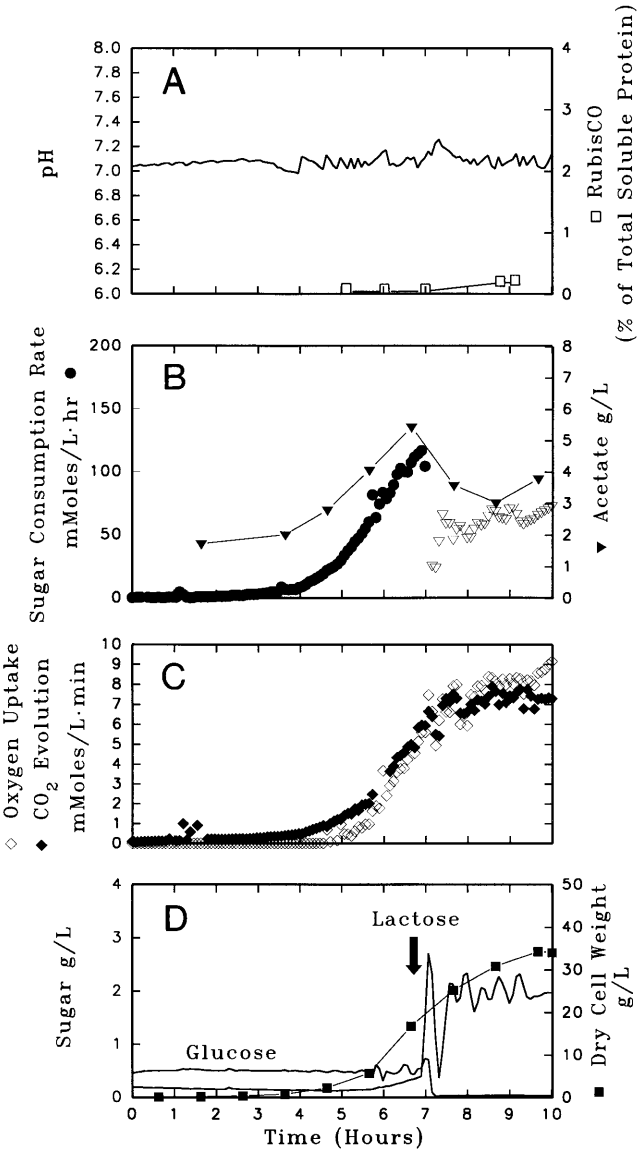

FIG. 4. Fed-batch fermentation *E. coli* K-12(pBGL710) grown to HCD with the soluble glucose concentration controlled at 0.5 g/liter and during the induction period the soluble lactose concentration controlled at 2.0 g/liter. (A) pH and the amount of RubisCO present versus time; (B) glucose consumption rate (●), lactose consumption rate (▽), and acetate concentration versus time; (C) oxygen uptake rate and carbon dioxide evolution rate versus time; (D) sugar concentration (glucose or lactose) and DCW versus time. The arrow indicates the switch at which the glucose feed was turned off and the lactose feed was turned on.

lactose. The results for *E. coli* K-12(pBGL710) grown in the computer-controlled fermentation system with induction using lactose are shown (Fig. 4). The glucose concentration was controlled at 0.5 g/liter to obtain HCDs, and during the induction period the lactose concentration was controlled at 2.0 g/liter. At approximately 7 h into the fermentation, the glucose feed was stopped and the lactose feeds and control program were started (Fig. 4). In this fermentation, the culture was readily able to utilize lactose as predicted, with the lactose consumption rate being approximately half the rate of glucose consumption on a millimolar basis (Fig. 4). The culture grew more slowly when utilizing lactose; however, the biomass continued to increase during this period to a final concentration of 35 g of DCW per liter. Because of the solubility limit of lactose in aqueous solutions (300 g/liter), fed-batch fermentations growing on lactose are not able to achieve the HCDs achievable on glucose, where the solubility is ca. 780 g/liter.

During the induction period when the culture was growing on lactose, 36 mg of RubisCO per liter, or 0.2% (wt/wt) of the total soluble protein, was produced. This was about half the heterologous protein produced when the same culture was induced with IPTG. The proportion of cells retaining the plasmid was above 88% throughout the fermentation (Table 1), so this decrease in productivity was not due to a loss of the plasmid. A decrease was also observed in the amount of β-Gal present in the cells growing on lactose (0.9% [wt/wt] of the total soluble protein) as compared to the culture induced with IPTG (1.5% [wt/wt] of the total soluble protein). Using lactose for induction for this strain and plasmid did not improve the productivity of RubisCO production compared with using IPTG; in fact, the amount of product may slightly decrease. In other systems, lactose induction has resulted in levels of recombinant protein expression equalling or exceeding that obtained with IPTG induction (6, 10, 12), indicating that lactose induction is a viable alternative to IPTG induction, if the host strain, plasmid, and medium conditions are optimized.

E. coli K-12(pANT900), which produced nearly 1 g of active, recombinant RubisCO per liter in IPTG-induced GC HCD fermentations, did not produce RubisCO or β-Gal in the lactose-induced fermentations (Table 1). During initial growth on glucose, growth rate, glucose consumption rate, oxygen uptake, and carbon dioxide evolution rate were identical to those observed in previous fermentations with this culture. At the 5-h point of the fermentation, at which the biomass was approximately 16 g (DCW)/liter, the glucose feed was halted, lactose was added to the vessel to a final concentration of 3 g/liter, and feed control of lactose at 2.0 g/liter was initiated. The culture did not utilize the lactose and ceased to grow, on the basis of oxygen consumption, CO_2 evolution, and lactose consumption data (13). These results were not surprising since even in IPTG-induced fermentations, no β-Gal was observed by rocket immunoelectrophoresis (Table 1). The inability of *E. coli* K-12 (pANT900) to grow on lactose, accompanied by the absence of RubisCO and β-Gal production, was likely due to the presence of *lacI*^{q} on pANT900, which would theoretically result in more than 3,000 copies of LacI repressor per cell.

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