Use of Glucose Starvation to Limit Growth and Induce Protein Production in *Escherichia coli*

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The use of glucose starvation to uncouple the production of recombinant β-galactosidase from cell growth in *Escherichia coli* was investigated. A *lacZ* operon fusion to the carbon starvation-inducible *cst-1* locus was used to control β-galactosidase synthesis. β-Galactosidase induction was observed only under aerobic starvation conditions, and its expression continued for 6 h following the onset of glucose starvation. The cessation of β-galactosidase expression closely correlated with the exhaustion of acetate, an overflow metabolite of glucose, from the culture medium. Our results suggest the primary role of acetate in *cst-1*-controlled protein expression is that of an energy source. Using this information, we metered acetate to a glucose-starved culture and produced a metabolically sluggish state, where growth was limited to a low linear rate and production of recombinant β-galactosidase occurred continuously throughout the experiment. The *cst-1* controlled β-galactosidase synthesis was also induced at low dilution rates in a glucose-limited chemostat, suggesting possible applications to high-density cell systems such as glucose-limited recycle reactors. This work demonstrates that by using an appropriate promoter system and nutrient limitation, growth can be restrained while recombinant protein production is induced and maintained.
Key words: carbon starvation • *Escherichia coli* • growth control

INTRODUCTION

For several years our research group and others (see references in 14 and 15) have investigated the use of immobilization techniques to cultivate cells to high densities. Typically, the objectives of cell immobilization are to increase the volumetric reactor productivity, the outlet product concentration or purity, or the substrate conversion to product.[14] In many cases, however, immobilized cell reactor operation is hindered by continued cell growth. This is because growing cells within a confined volume can exert significant stress (e.g., up to 3 atm of pressure in the case of *Escherichia coli*[35]) on their surroundings, often resulting in structural failure of the physical barriers used to immobilize or contain them.[13,15,35] In addition, uncontrolled growth in both immobilized and suspension culture diverts valuable substrate(s) from synthesis of the desired product to biomass production. Finally, the root of genetic instability in continuous recombinant protein production systems lies in the use of growing cells, where nonproducing mutants or plasmid-free cells grow faster and overwhelm the culture. Given these concerns, it is prudent to investigate means whereby biomass proliferation might be partially or wholly uncoupled from product synthesis.

One method used to attenuate growth in both immobilized and suspension cell culture is to restrict the supply of a required nutrient. The most common strategies have employed nitrogen[3,9,17,18,33] or phosphate[10,16,17] limitation. Others have limited the supply of vitamins to restrict growth[8] or, in some cases, both nitrogen and vitamins.[30,31] In the case of secondary metabolites, such as antibiotics produced by *Streptomyces*[16] or citric acid from yeast,[3] production itself is initiated in response to a growth limitation caused by starvation for a required nutrient. In bacteria, it has long been recognized that nutrient deprivation induces the production of numerous natural proteins (see ref. 24 for a review). As an industrially significant example, *Bacillus* species produce and excrete large amounts of subtilisin, an important proteolitic detergent additive, upon entry into the stationary phase.[29]

We have investigated the genetic coupling of growth control and recombinant protein production in *E. coli* by using the regulatory region of a carbon starvation-inducible protein under conditions of glucose starvation or limitation. In this report, β-galactosidase synthesis under control of the *cst-1* regulatory region was used as our model system. The Cst-1 protein belongs to a subset of recently discovered starvation-inducible proteins, the Cst proteins, which have been defined by their dependence on adenosine 3′, 5′-cyclic monophosphate (cAMP) for postexponential phase induction, as determined by absence of expression in Δ*cya* strains.[32] For these studies, we have confined ourselves to suspension culture reactor operation and used a single chromosomal *cst-1*::*lacZ* fusion. Obviously, the production of any pro-

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tein using starved cells cannot be continued indefinitely, regardless of the particular nutrient starvation employed. We have therefore focused on using carbon limitation to produce a metabolically sluggish state, where cell growth is controlled to a low rate while carbon-limitation-induced β-galactosidase production is maintained.

MATERIALS AND METHODS

Bacterial Strains and Media

AMS3 (*cst-1*::Mu dX [Ap^r Cm^r *lacZ*]) was previously constructed in the *E. coli* K12 derivative MC4100 (F$^-$ *araD139* Δ(*lacIPOZYAargF*)*U169 rpsL150 relA thi ptsF25 flbB5301 deoC1*),[6] as described by Groat et al.[11] β-Galactosidase was produced from the Mu dX transcriptional fusion to *cst-1* and is presented as β-galactosidase specific activities (see assays). Throughout this report, we employ β-galactosidase production as a measure of *cst-1* regulatory region activity. The transcriptional fusion used herein, which by definition produces native *lacZ* message, permits direct measurement of *cst-1* activity independent of any special translational control to which *cst-1* mRNA may be subject.

Minimal M9 medium[26] (pH 7) was supplemented with glucose (0.1–0.3%) and/or sodium acetate (10–60 m*M*) as defined in the text. The NH_4Cl concentration (0.2%) was double the normal M9 value[26] to prevent NH_4 starvation in experiments with cell densities greater than 1.5 g dry mass/L. $NaHCO_3$ (0.5 m*M*) and $FeSO_4$ (0.01 m*M*) were added to prevent a growth lag between serial dilutions and to provide adequate iron for respiratory chain enzyme activity,[27] respectively. Ampicillin (50 μg/mL) and chloramphenicol (20 μg/mL) were included in all culture media and plates to ensure retention of the fusion.

Growth and Starvation Protocol

Permanent cell stocks, in 20% glycerol, were stored at −70°C. Several days before the start of an experiment, cells were streaked on LB agar plates.[26] Cells from these plates were then grown in M9 medium, under conditions of excess nutrients, for three serial dilutions before initiating experiments. Except as noted below, all experiments were batch cultures grown in a Queue orbital shaker at 360 rpm. For batch experiments, the culture-volume-to-flask-volume ratio was maintained below 12% (v/v) to ensure adequate oxygen supply. All experiments were conducted at 37°C. The onset of starvation was determined by analyzing the supernatant for glucose, and experimental results are plotted in figures versus glucose starvation time where appropriate. In one experiment, carbon starvation was artificially induced by a centrifugation-based washing procedure. Centrifuged cells (3200*g*, 10 min room temperature) were rinsed twice in the resuspension medium and resuspended in their own supernatant (control) or fresh M9 medium containing either sodium acetate or no carbon sources. In cAMP addition experiments, an aliquot of an exponentially growing culture was added to an equal volume of cAMP-containing growth medium to yield a final concentration of 0 (control) or 30 m*M* cAMP. The continuous culture work with a glucose-limited chemostat (0.2% glucose M9 feed) was operated as previously described.[25]

Experiments using acetate as the sole carbon source, anaerobic and aerobic glucose experiments, and an acetate fed-batch experiment were conducted in a 2-L pH-controlled Setric fermentor equipped with dissolved oxygen and pH probes. Air (aerobic cultures) or 5% CO_2 in N_2 (anaerobic cultures)[34] was sparged at 25 L/h with agitation (600 rpm). In aerobic fermentor experiments, the dissolved oxygen concentration was maintained above 65% of air saturation. The pH was maintained at 7.0 by the controlled addition of KOH (4.0*M*) or HCl (4.0*M*).

Sampling Procedure

For batch and fermentor cultures, 1-mL culture samples were added to 0.5 mL of sodium azide (1.2 g/L) and pelleted by centrifugation (8800*g*, 5 min). The supernatant was removed and retained for glucose and organic acids assays. The cell pellet was stored at −70°C and assayed for β-galactosidase within 5 days. For chemostat and cAMP addition experiments, two drops of chloroform and one of sodium dodecyl sulfate (SDS) (0.1%) were added to 1-mL culture samples,[26] which were then stored on ice and subsequently assayed for β-galactosidase within 1 day.

Assays

Cell density was determined by 660 nm light scattering using a Gilford spectrophotometer 250 and converted to grams dry mass per liter. The dry cell mass concentration was calculated from a previously measured correlation between light scattering and cell mass. One absorbance unit at 660 nm corresponded to 0.38 g dry mass/L and did not significantly vary between growth and starvation periods of cell culture. Supernatant glucose concentrations were determined using a glucose analysis kit (Sigma Glucose HK-50). Organic acids were analyzed by high-performance liquid chromatography (HPLC) (Hewlett Packard 1082B liquid chromatograph) using the Hamilton PRP-X300 and Brownlee Labs Polypore H ion exclusion columns. The mobile phase was 5m*N* H_2SO_4 at 40°C and the acids detected by UV absorption at 210 nm. The frozen cell pellets or the samples stored on ice were assayed for β-galactosidase at 37°C as described previously.[11,32] Specific activity units are defined as the amount of enzyme which hydrolyzes 1 μmol *o*-nitrophenyl-β-D-galactoside (ONPG)/min/g

dry mass. Both the above methods of sample storage yielded equivalent specific activities. The final specific activity produced by the wild type *E. coli* K12 *lac* operon in response to isopropyl β-D-thiogalactopyranoside (IPTG) induction was 1300 specific activity units by our definition. This can be compared to the standard "Miller units" often reported where an IPTG-induced *E. coli* culture produces approximately 1000 Miller units[26] (AU_{420} change/min/mL culture/AU_{600}, where an increase in AU_{420} reflects ONPG hydrolysis). Differential rate values were calculated as the slope of a plot of ONPG hydrolyzed per minute per sample volume versus the culture cell density and used when comparing enzyme production during exponential growth.

RESULTS AND DISCUSSION

Induction under Aerobic or Anaerobic Conditions

We initially investigated the expression of the *cst-1*::*lacZ* fusion and biomass production in pH-controlled aerobic and anaerobic batch culture (Figs. 1A and B). In all experiments, the glucose concentration decreased exponentially from its initial value to zero as the cell mass increased exponentially to its final value at the point of glucose starvation. Since this report focuses on the behavior of cultures following the onset of glucose starvation, glucose concentrations are not shown, and our results are plotted versus glucose starvation time. In aerobic culture, the *cst-1* regulatory region had an induction ratio (ratio of basal expression level of β-galactosidase during exponential growth to the final level achieved during starvation) of 12.6 after 6 h of glucose starvation with no further increase in cell mass, in agreement with a previous report from one of our laboratories.[32] In contrast, anaerobically cultured cells failed to exhibit a *cst-1*-directed β-galactosidase response upon glucose starvation. When air was introduced into the previously anaerobic culture approximately 4 h following glucose exhaustion, however, the *cst-1* regulatory region was activated (Fig. 1B). In addition to the β-galactosidase response, a 35% increase in cell mass occurred following oxygen addition. In a separate experiment where anaerobic conditions were maintained over a 10-h glucose starvation period, no β-galactosidase induction above the basal growth level was observed (data not shown).

While our aerobic results do demonstrate the uncoupling of cell growth and recombinant product synthesis, it is not surprising that production was transient in nature. To achieve extended production, it was necessary to determine what factor(s) ultimately limited *cst-1*-directed protein production in glucose-starved cells and supply the cells with this factor(s). The requirement of oxygen for induction indicated the limitation was related to cellular energy metabolism, and suggested that the by-products of glucose catabolism were used as energy

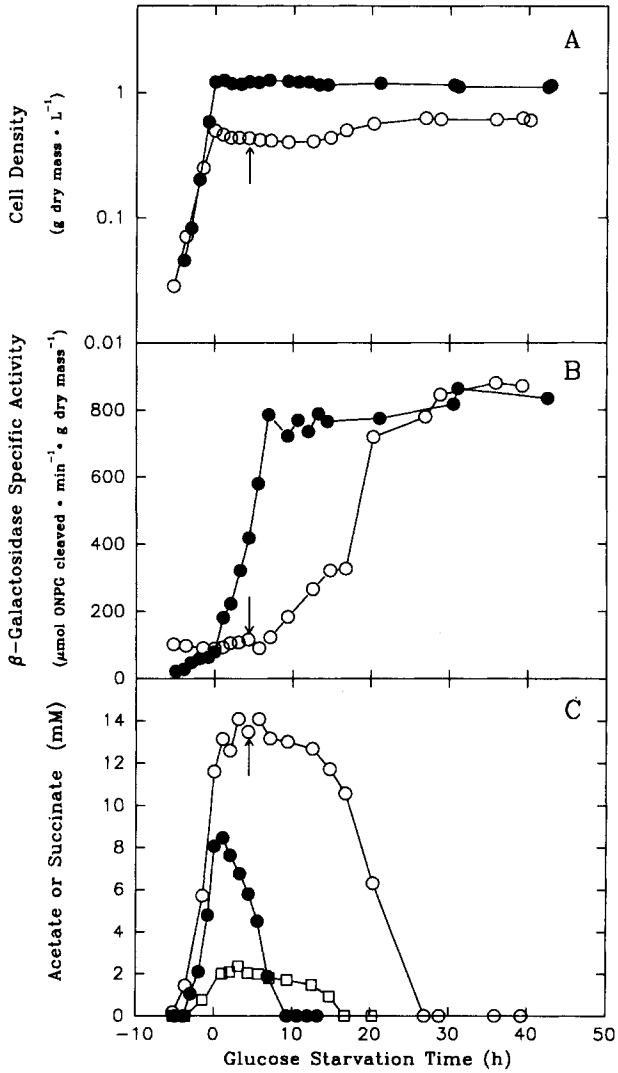

Figure 1. (A) cell density, (B) β-galactosidase production, and (C) acetate (○) and succinate (□) concentrations during aerobic (filled) and anaerobic (hollow) fermentor culture. Initial glucose 0.3%. At 4 h following glucose starvation, air was introduced into the anaerobic culture (see arrows). Zero glucose starvation time is the time at which the reactor glucose concentration reached zero as determined by a glucose assay.

sources for the production of glucose-starvation-induced β-galactosidase. We therefore analyzed the time course of organic acid production and consumption in our culture samples by HPLC.

Acetate as a Post-Exponential Phase Energy Source

While many chemical species have been reported in the conditioned media of *E. coli* cultures,[1] the principle by-product of aerobic glucose catabolism in our experiments was found to be acetate (Fig. 1C), with low, but detectable, levels of pyruvate and lactate (not shown). Neijssel and Tempest refer to glucose by-products such as these as overflow metabolites since glucose catabolism results in a carbon surplus with respect to the energetic require-

ments of the cell.[36] A recent model also indicates that if the capacity of either the Krebs cycle or the respiratory chain is limited during aerobic growth, the rate of ATP production can be increased by metabolizing a portion of the available glucose to acetate.[22] Under aerobic conditions, the consumption of acetate (Fig. 1C) and induction of the *cst-1* regulatory region, as measured by β-galactosidase translation (Fig. 1B), commenced concomitantly following glucose starvation. Furthermore, the *cst-1* regulatory region became inactive after acetate was exhausted from the medium.

Under anaerobic culture conditions, the principal glucose by-product which, with the addition of oxygen, could be used as a carbon and energy source was again acetate (Fig. 1C). In addition to the acetate, formate (24 m*M*) and succinate (2.5 m*M*) were also excreted along with trace amounts of pyruvate. Following glucose starvation, none of these were consumed during the 4-h anaerobic starvation period. Upon oxygen addition, the trace pyruvate immediately disappeared, and both the acetate and succinate were concomitantly consumed as β-galactosidase synthesis proceeded (Figs. 1B and 1C). Formate, which cannot be used as a carbon or energy source, remained constant following glucose starvation (not shown). As in the entirely aerobic culture, the exhaustion of acetate and cessation of *cst-1*-driven β-galactosidase production were linked.

The correlation of acetate exhaustion with the cessation of β-galactosidase production and the oxygen requirement of *cst-1* expression demonstrated the particular importance of acetate as an energy source following glucose starvation. Before attempting to produce a metabolic state where cell growth is attenuated and recombinant protein production extended by feeding glucose-starved cells acetate, it was necessary to verify that acetate could act as the sole extracellular metabolite required for β-galactosidase production following glucose starvation. To accomplish this, exponentially growing *E. coli* containing the *cst-1*::*lacZ* fusion were subjected to glucose starvation by a centrifugation technique (see Materials and Methods). Cells resuspended in carbon-free M9 medium showed virtually no *cst-1* induction, as measured by β-galactosidase production, above the basal expression level, while those suspended in medium containing sodium acetate exhibited induction of the *cst-1* regulatory region (Fig. 2). Substantial induction of *cst-1* by cells resuspended in medium containing only acetate as a carbon source demonstrates that other excreted by-products of glucose catabolism are not required. In both the glucose exhaustion control, where cells were resuspended in their own supernatant, and in cells resuspended in acetate, the production of *cst-1*-driven β-galactosidase ceased once acetate was exhausted from the medium (not shown). These results indicate that the provision of acetate to β-galactosidase producing, glucose-starved cells should allow extension of the *cst-1*-driven β-galactosidase production period. This will be demonstrated below. It can be expected that this requirement of a secondary external energy source for production of *cst-1* starvation protein, which in our system was satisfied by acetate, will also apply to at least some of the other genes induced following glucose starvation.

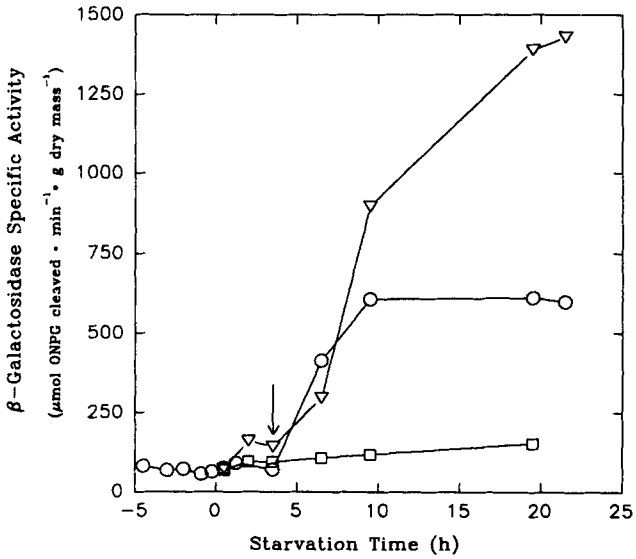

Figure 2. The *cst-1*::*lacZ* fusion induction following centrifuge washing. A mid-log culture in 0.3% glucose M9 medium was centrifuged and washed, and aliquots were resuspended in (○) the culture supernatant or fresh M9 containing (□) zero or (▽) 6 m*M* sodium acetate as the sole carbon source. Zero starvation time is the time at which the cell aliquots were resuspended in the above media. The point of glucose exhaustion is shown for the control aliquot resuspended in its own supernatant (arrow).

Due to the important role of acetate for production of our recombinant β-galactosidase, we investigated the expression of the *cst-1*::*lacZ* fusion following acetate starvation of a culture growing on acetate. Since acetate metabolism does not produce fermentable by-products,[7] little or no induction of *cst-1*::*lacZ*-directed β-galactosidase production should be observed following its exhaustion. Accordingly, upon complete exhaustion of acetate from a culture growing exponentially on acetate (initial concentration 50 m*M*), no induction of the *cst-1*::*lacZ* fusion above the basal β-galactosidase expression level was observed (data not shown). Interestingly, the basal expression level during exponential growth on acetate was substantially higher than observed during growth on glucose, 420 versus 60 specific activity units. *Escherichia coli* has been reported to have increased cAMP levels during exponential growth on acetate compared to glucose,[4] which may explain this comparatively high basal expression level of β-galactosidase observed during growth on acetate. To test this explanation, we considered the effects of cAMP addition to cells growing exponentially in our glucose M9 medium.

Effects of Exogenously Added cAMP

While the Cst class of proteins are defined by their requirement of a functional cAMP–CRP complex for induction following glucose starvation, in the case of at least two, Cst-4 and Cst-8, other additional unknown

factors appear to be necessary.[2] To assess the degree of dependence of the *cst-1* regulatory region upon cAMP, we added cAMP (at a final concentration of 0 or 30 m*M*) to exponentially growing cells. A fivefold increase in the β-galactosidase specific activity (22 to 107 specific activity units) following the addition of 30 m*M* cAMP was measured. No change in the activity of the control was observed. This result cannot rule out the possibility that other intracellular postexponential phase factors modulate induction of the *cst-1* regulatory region. It does, however, support our interpretation of the comparatively high basal β-galactosidase levels during growth on acetate versus glucose as due to the increased intracellular cAMP levels in cells growing on acetate. A similar relationship between reported cAMP levels and basal carbon starvation regulatory region activities during exponential growth has been previously described for the *cstA* and *cstB* promoters in cells growing on glucose, succinate, or glycerol.[32]

Establishment of a Metabolically Sluggish Production State

Our final objective was to produce a growth-limited or metabolically sluggish state during which a recombinant protein could be produced for extended periods. Having determined that the presence of acetate following glucose starvation was sufficient to allow production of *cst-1*-driven β-galactosidase, we investigated two strategies to prolong the period of β-galactosidase production and yet limit cell growth by providing acetate to glucose-starved cells. The report that acetate metabolism promotes high intracellular cAMP levels,[4] which we have shown stimulates the *cst-1* regulatory region, further confirms acetate as an appropriate choice for our system.

First we examined simple diauxic culture conditions where the growth medium was modified to initially contain both glucose and acetate. The acetate was provided at significantly higher concentrations (10–60 m*M*) than normally produced (see Fig. 1B) by the aerobic catabolism of glucose alone. A representative experiment where *cst-1*::*lacZ*-containing cells were initially supplied with 0.3% glucose and 40 m*M* acetate is shown in Figure 3. The cells grew first on glucose until its exhaustion, experienced a lag in cell growth, and then commenced growth on acetate, with a specific growth rate of 0.1 h^{-1}. Surprisingly, β-galactosidase production from the *cst-1*::*lacZ* fusion was not induced immediately following glucose starvation. Instead, a lag period similar to that observed for cell growth was evident. Following the lag period, β-galactosidase was produced at approximately four times the rate observed following simple glucose exhaustion (Fig. 1A), and the final induction ratio was approximately threefold higher. The *cst-1* regulatory region activity was again coupled to the presence of acetate. Once acetate was exhausted from the culture medium, β-galactosidase production ceased (Fig. 3). As the initial acetate concentration in the growth medium was increased, the lag following glucose exhaustion of both *cst-1* regulatory region induction and growth on acetate increased similarly, ranging from 2 to 7 h, but the final induction ratio was also significantly enhanced with modest increases in cell mass (Table I). Since production of the glycolytic bypass enzymes is necessary before growth on acetate can occur,[28] the growth lag after the diauxic shift from glucose to acetate was not surprising. The lag in β-galactosidase production, however, was not anticipated based on our previous results. The observation that the production and growth lags increased with increasing initial acetate concentra-

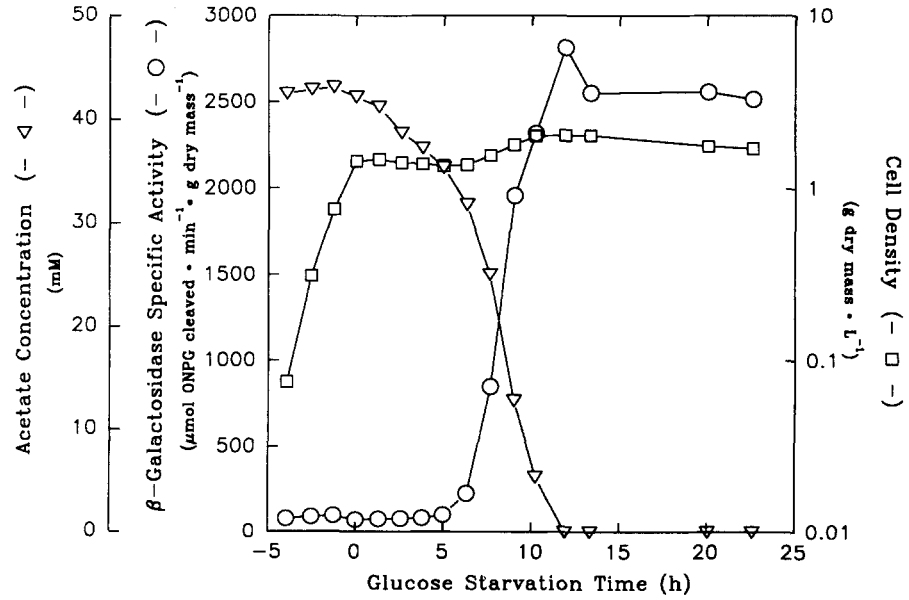

Figure 3. β-Galactosidase production, acetate concentration, and cell mass in an aerobic batch diauxic growth experiment. The culture medium originally contained 0.3% glucose and 40 m*M* sodium acetate. Glucose starvation time is defined in Fig. 1.

Table I. Cell growth and *cst-1*::*lacZ* fusion induction under diauxic growth conditions.

Initial acetate concentration (m*M*)[a]	Exponential growth specific activity[b]	Final specific activity[b]	Induction ratio[c]	β-Galactosidase production lag[d] (h)	Percentage increase in cell mass[e] due to growth on acetate
0	62.5	790	12.6	0	0
10	66.8	796	11.9	2.0	0
20	81.4	1870	23.0	4.6	21
40	81.9	2830	34.5	6.6	39
60	90.8	3590	39.5	7.0	48

[a]Acetate concentration at the onset of starvation for the 0–60 m*M* intial acetate experiments were 8.5, 13.3, 22.3, 42.2, and 63.0 m*M*, respectively.

[b]μ-mole ONPG cleaved/min/g dry mass.

[c]Ratio of exponential to final specific activities.

[d]Both production and growth lags were experimentally indistinguishable for the 20, 40, and 60 m*M* initial acetate experiments.

[e]Relative to the cell mass observed at the onset of glucose starvation.

tions suggests organic acid (acetate) inhibition.[12,34] This inhibition may be due to a decrease in the efficiency of oxidative phosphorylation, such as reported by Landwell and Holme for cells cultured in the presence of high concentrations of glucose catabolism by-products.[19]

Comparison of the diauxic growth culture (Fig. 3) and the batch glucose-starved culture (Fig. 1B) shows that the increased β-galactosidase levels were not a result of a significant extension of the production period, but instead were due to a roughly fourfold enhancement of the enzyme synthesis rate. Thus, while initially supplementing the growth medium with additional acetate is not appropriate for prolonging the period of *cst-1* activity, it substantially increases the amounts of recombinant protein which can be produced from this regulatory region using a simple batch reactor.

Second, we examined the expression of our *cst-1*::*lacZ* fusion in glucose-starved cells, which were continuously fed acetate at a low rate shortly following the onset of glucose starvation. The reactor was operated as an aerobic batch culture until 30 min after the exhaustion of glucose. The culture was then supplied with sodium acetate at a rate calculated to match the acetate consumption rate initially observed in stationary phase glucose-starved batch cultures (1.3 mmol acetate/h/g dry mass). Following exhaustion of the residual acetate in the culture (both that resulting from glucose metabolism and the acetate feeding), the cells exhibited a controlled linear growth rate of 3.4 mg dry mass/h on the supplied acetate, which resulted in only a 25% increase in the cell mass with continued β-galactosidase production over a 44-h period (Fig. 4). Using this fed-batch culture strategy, the β-galactosidase activity increased from an exponential growth value of 85 specific activity units to a final level of 2170 specific activity units after 58 h of acetate-fed glucose starvation. At the end of the experi-

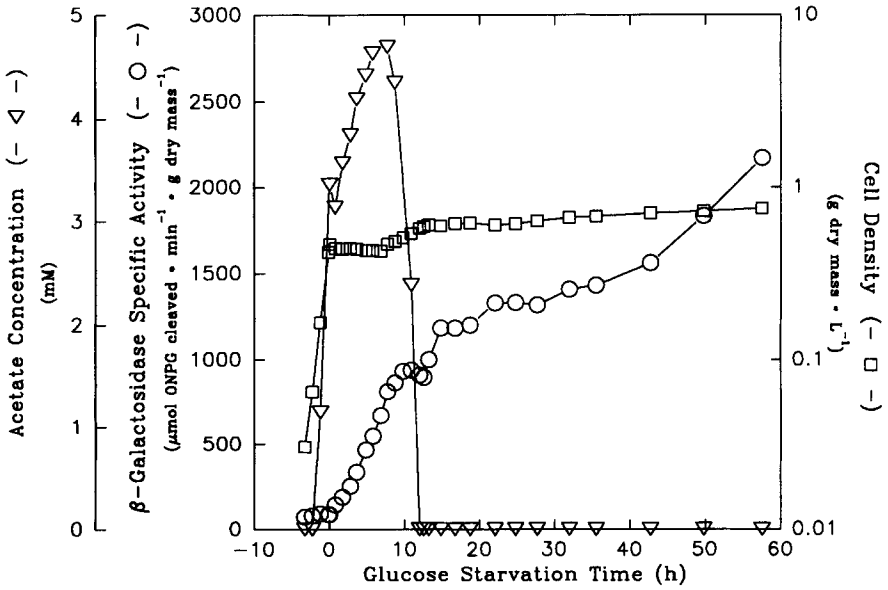

Figure 4. β-Galactosidase production, acetate concentration, and cell density in a pH-controlled acetate fed-batch culture. Acetate feeding was initiated 0.5 h after glucose (initial concentration 0.1%) exhaustion. Glucose starvation time is defined in Fig. 1.

ment, the β-galactosidase specific production rate (41 specific activity units/h), which while increasing, was considerably lower than that observed following simple glucose exhaustion in the presence of acetate (130 specific activity units/h; see Fig. 1A) or during the production period of the diauxic growth culture (440 specific activity units/h; see Fig. 3). The final high β-galactosidase activity measured, the continued β-galactosidase production which had not leveled off as in previous experiments (Figs. 1–3), and the controlled or sluggish growth of the cell mass, however, were all promising (Fig. 4) and future work is in progress to test the limits of this system.

β-Galactosidase Production in Chemostat Culture

As a final application of carbon-starvation-inducible promoters, we considered the performance of our *cst-1*::*lacZ* fusion in a chemostat where the steady-state growth rate is equal to the dilution rate. In this system, glucose in the feed, instead of acetate, provides energy and carbon for recombinant protein production, while the negligible concentration of glucose in the reactor at low dilution rates supplies the necessary stimulus for substantial expression of the *cst-1*::*lacZ* fusion. As the dilution rate was lowered, the β-galactosidase specific activity increased substantially, whereas the specific production rate reached a maximum at a dilution rate of 0.2 h^{-1} (Fig. 5). This peak in the production rate can be better understood if one considers the extracellular cAMP concentrations observed by Matin and Matin[25] for the parent strain of our MC4100 cell line, *E. coli* K12 in a chemostat (Fig. 5). Both the cAMP concentration and the production rate peaked at the same dilution rate, as expected based on our demonstration that cAMP alone is sufficient to stimulate the *cst-1* promoter. The maximum specific production rate of 260 specific activity units/h compares favorably with the 130 specific activity units/h observed following simple aerobic glucose exhaustion (Fig. 1). Thus, by using a glucose-limited reactor strategy known to promote high cAMP levels, a twofold improvement in productivity was made on a per cell basis compared with simple batch culture glucose exhaustion.

The demonstration of substantial β-galactosidase expression by glucose-limited operation of a continuous culture suggests carbon starvation promoter production systems would be useful in glucose-limited recycle reactors where extremely high cell densities can be achieved. Under anaerobic conditions, Lee et al.[20] obtained cell densities up to 50 g dry weight/L with substantial production of acetate. By operating under aerobic conditions, Lee and Chang[21] were able to supply adequate oxygen to allow catabolism of much of the acetate produced from glucose metabolism and reached cell densities up to 145 g dry weight/L. With our *cst-1*-driven protein production system, the glucose limitation would induce protein production and aerobic acetate metabolism would supply the energy for protein synthesis.

CONCLUSIONS

Our results have demonstrated the feasibility of designing growth control strategies which induce recombinant DNA protein production. Using the *cst-1* regulatory region to control β-galactosidase synthesis, we have completely uncoupled cell growth and protein production in short-term glucose-starved batch cultures. The supple-

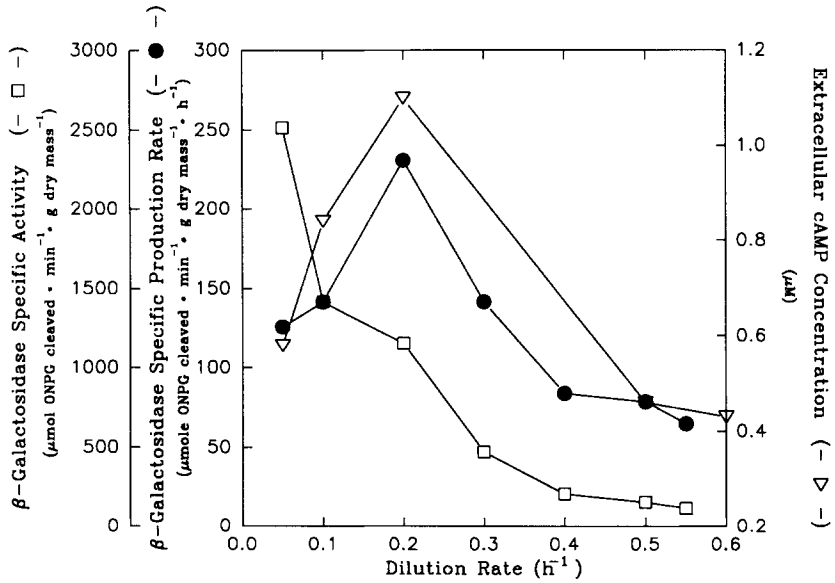

Figure 5. The *cst-1*::*lacZ* fusion expression and extracellular cAMP concentrations in a glucose-limited (0.2% feed) chemostat. The β-galactosidase specific production rate is the specific activity multiplied by the dilution rate. (The cAMP concentrations are reprinted from ref. 11 with permission.)

mentation of such cultures with acetate, allowing diauxic cell growth, resulted in over a fourfold increase in recombinant β-galactosidase production beyond the normal level observed following simple glucose starvation. By continuously feeding acetate to a glucose-starved culture, we produced the desired metabolically sluggish state where growth was limited to a low linear rate and continuous β-galactosidase production occurred throughout the experiment. In addition, we have shown the *cst-1* promoter can be used to produce recombinant β-galactosidase in a chemostat, suggesting that it and other carbon-starvation-inducible promoters may be applicable to high-density cell systems such as glucose-limited recycle reactors.

In addition to their utility in coupling growth control and recombinant protein production, starvation promoters may be of use as simple auto-inducing systems in industrial batch fermentations. By using a carbon starvation system, the complete aerobic use of the supplied carbon source could be insured and protein production induced without the addition of chemical inducers such a IPTG (*lac* and *tac* promoters) or changes in the fermentation temperature (λP_L and P_R promoters controlled by the temperature-sensitive cI_{857} repressor[5]). Finally, starvation promoters could be used in chemical waste biodegradation to induce the production of biodegradative enzymes in the environment, where the nutrient limitations required for starvation promoter induction often naturally exist.[23]

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