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Ammonium-Mediated Reduction of Plasmid Copy Number and Recombinant Gene Expression in *Escherichia coli*

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The effect of ammonium as a medium supplement on plasmid-encoded recombinant β-galactosidase synthesis was explored in *Escherichia coli* cells during aerobic growth in complex medium. After induction, only doses of ammonium chloride below 1 g/L are able to transiently enhance the yield. However, the presence of nontoxic ammonium chloride concentrations of up to 10 g/L results in lower values of β-galactosidase in a concentration-dependent fashion. A significant reduction in plasmid DNA content explains the decrease in the yield by a gene-dosage-involving mechanism.

Introduction

The improvement of culture media is one of the requisites to achieve acceptable yields of a recombinant protein in production processes, especially when high-cell-density procedures are assayed (Riesenberg, 1991). Apart from the precise supplements required for a specific biological system or strain, the general influences of some medium components on recombinant gene expression have been described and studied. Among them, glucose-mediated inhibition of cell growth (Andersen and von Meyenburg, 1980) and product yield (Rinas et al., 1989; Vila and Villaverde, 1993) is of relevant interest because this sugar is often employed as a carbon source in defined media or even as a supplement in complex media. In general, all of the essential substrates for bacterial growth are toxic to the cells beyond critical thresholds, which are specific for each particular strain, medium, and growth conditions.

In recent years, we have produced engineered viral proteins of interest by using *Escherichia coli* thermoinducible systems based on the CI857 λ repressor (Vidal et al., 1991; Benito et al., 1993a; Corchero et al., manuscript in preparation) and we have studied the accuracy of temperature for the control of gene expression levels (Villaverde et al., 1993). In order to optimize batch and fed-batch protocols, we have explored, in this work, the influence of supplementary nitrogen sources on cell growth and *lacZ* gene expression in a complex medium, searching for stimulator effects that could improve the yield. The results obtained indicate that, even at nontoxic concentrations, ammonium significantly reduces recombinant β-galactosidase synthesis after thermal induction and that only at very low doses can it fairly increase the yield. This inhibition of β-galactosidase production is due to a reduction in plasmid content in cultures growing in the presence of supplementary ammonium, which takes place even under noninducing conditions.

Materials and Methods

Construction of Recombinant Plasmids. A recombinant plasmid based on pJLA602 (Schauder et al., 1987) was constructed to express the *E. coli lacZ* gene under the control of p_L and p_R tandem promoters. The *lacZ* DNA fragment was obtained by PCR amplification from pAZe3 (Zaballos et al., 1987) using the upstream BEG3 (5′-GGGGATCCCCGTCGTTTTACA-3′) and downstream BEG2 (5′-GGGAATTCTTTTTGACACCAGA-3′) primers, in which *Bam*HI and *Eco*RI restriction sites were, respectively, introduced. PCR reactions were performed using the high-fidelity Vent^R polymerase. Both plasmid and PCR product were digested to completion with these two enzymes and ligated further. The resulting vector pJCO46 (Figure 1) was introduced into the *E. coli* MC1061 Lac$^-$ strain (Casadaban and Cohen, 1980) by standard transformation procedures (Sambrook et al., 1989). The synthesis of β-galactosidase was induced thermally at 42 °C thanks to the thermosensitive CI857 repressor encoded by the plasmid.

Media and Culture Conditions. The experiments were usually performed in Luria–Bertani (LB) broth (Sambrook et al., 1989) with 100 μg/mL ampicillin. Only when indicated was M9 (Maniatis et al., 1982) with 0.2% glucose, 25 μg/mL leucine, and 100 μg/mL ampicillin employed. Cells were grown after a 1:50 inoculum in shake flasks at 28 °C and 250 rpm until OD_{550} reached 0.3–0.4 and then shifted to 42 °C and incubated further for several hours. Appropriate volumes of 10% ammonium chloride dissolved in LB (or M9) were added to the fresh medium just before the inoculum to achieve the desired final concentrations. The wild-type CGSC5073 *E. coli* strain was grown at 37 °C, and chromosomal *lacZ* gene expression was induced by using 0.1 M IPTG.

Western Blot, Plasmid DNA Content, Dry Weight, and β-Galactosidase Activity Analyses. Culture samples of 0.4 mL were centrifuged, and the pellets were processed for SDS–PAGE and additional Western blot. Anti-β-galactosidase monoclonal antibody from Boehringer Mannheim was employed to detect the recombinant protein. The amounts of β-galactosidase were calculated as described (Benito et al., 1993a) by densitometric analysis of the nitrocellulose sheets using dilutions of pure enzyme as a standard. Cell pellets from 1.5 mL samples were processed to quantify extractable plasmid DNA by densitometry of ethidium bromide-stained agarose gels as described (Benito et al., 1993b). Dry weight was calculated by the filtration of small volume culture samples, using carefully preweighed filters that were

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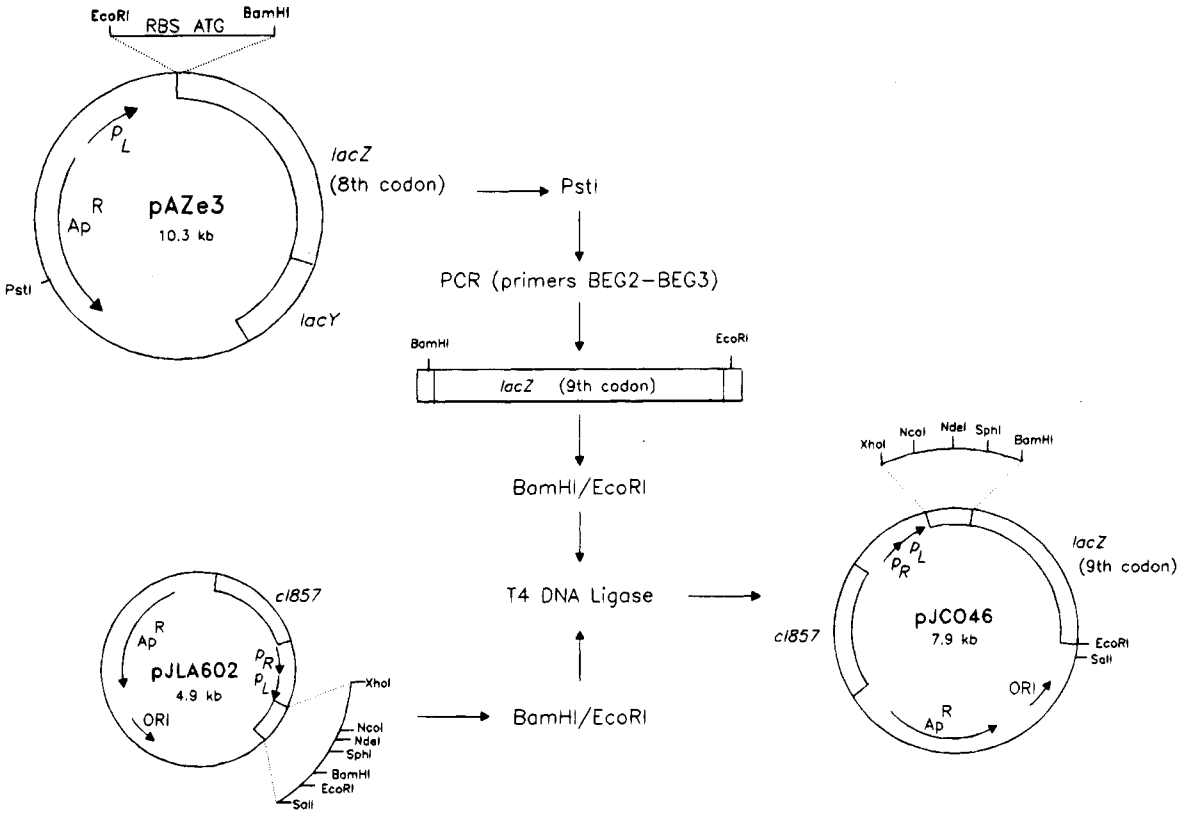

Figure 1. Construction of plasmid pJCO46. The *lacZ* DNA fragment was obtained by amplification from plasmid pAZe3, further digested with *Bam*HI and *Eco*RI restriction enzymes, and inserted into plasmid pJLA602.

Table 1. Plasmid DNA Content in MC1061/pJCO46 Cells before and after Thermal Shift

induction time[a] (h)	plasmid DNA (μg/g of dry weight)		β-galactosidase activity (units/μg of plasmid DNA)	
	−[b]	+	−	+
0	0.70 ± 0.20	0.22 ± 0.05	184 ± 146	484 ± 363
1.5	0.99 ± 0.04	0.56 ± 0.06	18408 ± 834	11902 ± 1460
3	1.51 ± 0.01	0.82 ± 0.04	10593 ± 85	10685 ± 573

[a] Time 0 indicates the moment just before the temperature shift. [b] Cells were grown in LB (−) or LB plus 10 g/L NH_4Cl (+).

further dryed at 105 °C until they reached a constant weight. All of the samples were processed in triplicate. β-Galactosidase analysis was performed in duplicate by mixing 0.1 mL of the culture, 0.9 mL of Z buffer (Miller, 1972), and 0.1 mL of chloroform. After vortex mixing, the reaction was carried out (usually over 2 min), stopped, and quantified according the Miller's protocol. β-Galactosidase activity is expressed as corrected by the OD of the sample.

Results and Discussion

Effects of Ammonium on Thermally Induced β-Galactosidase Activity and Concentration. MC1061 cells carrying pJCO46 from the same overnight culture were inoculated in fresh LB medium containing different concentrations of ammonium chloride, from 0.05 to 10 g/L. Ammonium had no influence on cell density, neither before nor after a temperature shift, at any of the doses tested in any of the experiments (Figure 2A). Figure 2B shows the evolution of β-galactosidase activity in these induced cultures. A light stimulatory effect on gene expression was detected for up to 1 g/L ammonium chloride. However, a very evident inhibitory effect on β-galactosidase activity was also observed at concentrations above 1 g/L, in a dose-dependent fashion.

To explore the putative interferences of ammonium chloride in the Miller test, we analyzed the β-galactosidase activity of both pure enzyme and cell extracts in the presence of equivalent ammonium chloride concentrations. In all cases, the monitored activity was not influenced by the salt (not shown), suggesting that the reduced enzymatic activity in supplemented cultures shown in Figure 2 was due to a lower yield of recombinant protein. Western blot analysis of MC1061/pJCO46-induced cells with and without 10 g/L ammonium chloride (Figure 3) provided further evidence of a lower recombinant protein concentration. No degradation bands were detected in any of the samples analyzed.

Plasmid Content in MC1061/pJCO46 Cultures in the Presence of Ammonium. Although the accumulation of recombinant protein with and without ammonium tends to achieve similar values in long-term-induced cultures (Figure 2B), the observed influence on recombinant protein synthesis after induction suggests that ammonium inhibits gene expression. Since putative explanations for this fact could involve a gene dosage mechanism, we explored the plasmid DNA content in cultures growing in LB, with and without 10 g/L ammonium chloride. Table 1 shows that the presence of ammonium in the cultures significantly reduces the amount of extractable pJCO46 DNA to about 50%, and these differences are maintained quite constant after thermal induction. This fact is still evident even though some variations in plasmid content are promoted by an increase in temperature. In LB supplemented with ammonium, both the maximum β-galactosidase levels (achieved between 2 and 3 h after induction) and the amounts of recombinant β-galactosidase estimated from Western blot analysis represent about 50% of those from the culture in LB alone (Figures 2B and 3). Because no plasmid segregation was detected in any of the experiments (not shown), the reduced plasmid DNA content must result in a lower plasmid copy number.

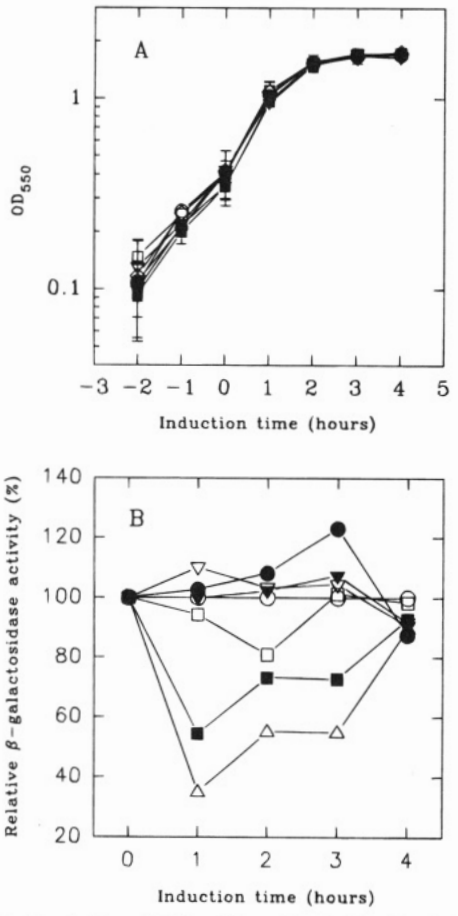

Figure 2. Evolution of OD_{550} (A) and relative β-galactosidase units (to those obtained in standard LB medium) (B) in MC1061/pJCO46 cells grown and induced in LB (○) and in LB plus ammonium chloride at 0.05 (●), 0.1 (▽), 0.5 (▼), 1 (□), 5 (■), and 10 g/L (△). The results presented are the mean of four independent experiments. Absolute values of β-galactosidase activity during typical induction experiments in LB can be found elsewhere (Vila and Villaverde, 1993).

To discard putative specific effects of ammonium over the expression of the particular *lacZ* gene, Lac operon transcription was stimulated by 0.1 M IPTG in an *E. coli* wild-type strain (CGSC5073 without any plasmid) growing in LB. In this case, β-galactosidase synthesis directed from a chromosomal gene was not influenced by the presence of ammonium in the medium. From some minutes after the addition of the inducer and for at least

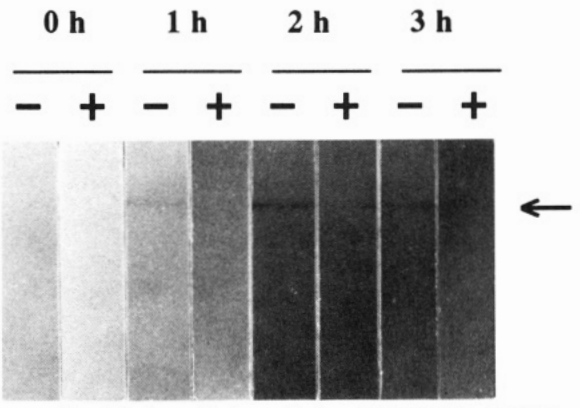

Figure 3. Western blot analysis of MC1061/pJCO46 cell extracts just before thermal induction (0 h) and at several times after induction, in LB (−) and in LB plus 10 g/L ammonium chloride (+). The arrow indicates the β-galactosidase monomer band at about 116 kDa.

4 h, the levels of enzymatic activity remained nearly constant at 7204 ± 218 and 7018 ± 363 units/mL·OD_{550} in LB and in LB plus 10 g/L ammonium chloride, respectively. The similarity of these results confirms the plasmid dependence of β-galactosidase yield reduction described above.

Ammonium Doses Influencing Gene Expression. Nitrogen sources in a typical LB medium probably are close to optimal concentration for cell growth and gene expression. Only a moderate stimulation in gene expression has been found when ammonium chloride is added at very low doses (Figure 2B). At higher amounts, ammonium becomes toxic for the cells, perhaps by more than one independent mechanism that would include alterations in the metabolism of DNA. As a result, plasmid DNA content and, subsequently, plasmid-encoded recombinant gene expression are modified even at doses at which the growth rate and biomass of the cultures remain unaffected. Results shown in Table 1 and those obtained with the CGSC5073 strain suggest that plasmid replication is much more sensitive to factors influencing DNA synthesis than chromosomal replication. In fact, it is well-known that plasmid copy number and stability are influenced by several intrinsic and extrinsic factors (Kumar et al., 1991).

To define the actual doses of ammonium causing this event, we used M9 culture medium without any complex supplement to study *lacZ* expression and cell growth. In this case, a reduction in gene expression levels was

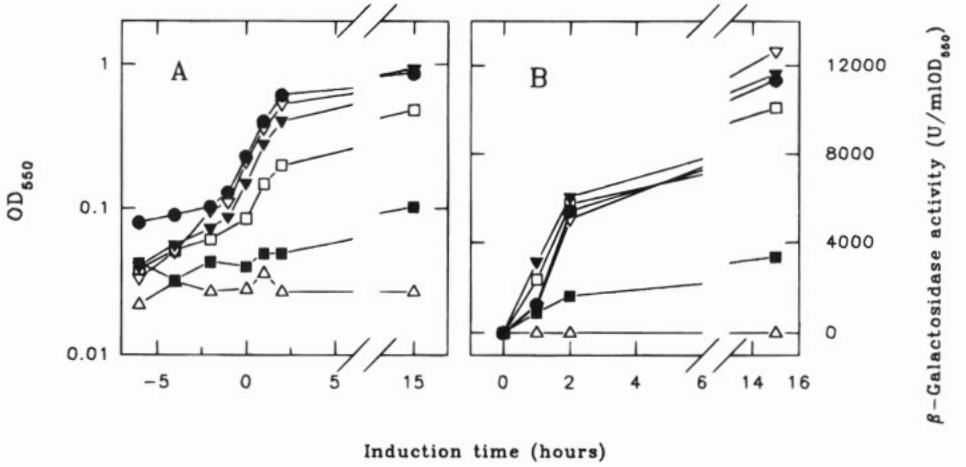

Figure 4. Optical density (A) and β-galactosidase activity (B) in MC1061/pJCO46 cultures growing in M9 medium supplemented with the standard ammonium chloride concentration of 1 g/L (●) and special concentrations of 2 (▽), 5 (▼), 10 (□), 20 (■), and 40 g/L (△).

clearly observed at 20 g/L simultaneous with a decrease in biomass gain (Figure 4). Again, low doses of supplemented ammonium chloride resulted in moderate increases in gene expression levels. Since the maximal dose stimulating gene expression (5 g/L) in M9 is higher than the equivalent in LB (0.5 g/L), we can assume that the reduced ammonium from the complex medium and that added as a supplement represent cumulative effects.

Altogether, the results presented here prove that plasmid DNA content and, subsequently, gene expression can be dramatically influenced by ammonium concentration at doses that have an undetectable influence on cell growth. Apart from the practical implications that this observation could have for media formulation, the strong dependence that β-galactosidase concentration has on plasmid copy number indicates that translation is not an important bottleneck in recombinant protein production, at least in the thermoinducible system and under the small-scale batch conditions described here. This is in agreement with previous results on the influence of temperature-mediated inactivation of the CI857^{ts} repressor on gene expression (Villaverde et al., 1993) and suggests that mRNA, and not protein synthesis, is in fact a critical step limiting yield. Presumably, the translation machinery could become insufficient in large-scale processes in which the improved cell growth conditions provided by bioreactors enhance the transcription rate.

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