

Improvement in Recombinant Protein Production in ppGpp-Deficient *Escherichia Coli*

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Abstract: Maintaining a metabolically productive state for recombinant *Escherichia coli* remains a central problem for a wide variety of growth-dependent biosynthesis. This problem becomes particularly acute under conditions of minimal cell growth such as fed-batch fermentations. In this, we investigated the possibility of manipulating the protein synthesis machinery of *E. coli* whereby synthesis of foreign proteins might be decoupled from cell growth. In particular, the effects of eliminating intracellular ppGpp on the synthesis of foreign proteins were studied in both batch and fed-batch operations. A significant increase in CAT production was observed from the ppGpp-deficient strain during both exponential and fed-batch phases. The increase in CAT production during exponential growth was accompanied by a simultaneous increase in CAT mRNA levels. Interestingly, CAT production was increased five-fold, while the level of CAT-specific mRNA increased only three-fold. Thus, eliminating intracellular ppGpp appears to have increase the production of recombinant protein by increasing not only the pool sizes of CAT mRNA but also possible alternations in the post-transcriptional processes. © 1997 John Wiley & Sons, Inc. *Biotechnol Bioeng* 53: 379–386, 1997.

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INTRODUCTION

Typically, production of recombinant proteins from *E. coli* requires a strong coupling between cell growth and production (Luli and Strohl, 1990; San et al., 1994). Production is severely curtailed under slow-growing conditions due to the intense competition between normal cell function maintenance and recombinant protein production for the limited available metabolic machinery. From a processing perspective, this problem becomes particularly acute under conditions of minimal cell growth such as fed-batch fermentations (San et al., 1994).

Recent investigations have revealed that the cellular content of many direct participants in protein synthesis (e.g., initiation factors, elongation factors, even RNA polymerase) are decreased when *E. coli* enters stationary phase as

the result of nutrient and energy starvation (Matin, 1991; Matin et al., 1989). In particular, the amount of active ribosome is drastically different between fast- and slow-growing cultures (Dennis and Bremer, 1973). Ribosomes are large ribonucleoproteins that are responsible for the translation of mRNAs, and each ribosome is composed of 52 distinct ribosomal proteins (r-protein) and three kinds of rRNA (Jinks-Robertson and Nomura, 1987). Expression of r-protein can vary by more than 500 times depending on the growth rate (Bremer and Dennis, 1987), and is precisely coordinated with the rate of rRNA synthesis (Gourse et al., 1985; Norris and Koch, 1972). Thus, the rate of rRNA synthesis determines the overall availability of ribosome.

Coordination of rRNA synthesis is a central and complex metabolic task. Under extremely fast-growing conditions, rRNA can constitute over half of the total cellular RNA (Jinks-Robertson and Nomura, 1987). Stringent response has been reported to be responsible for regulation of rRNA synthesis (Cashel and Rudd, 1987). The nucleotide ppGpp is the regulatory signal that appears to repress rRNA synthesis under starvation condition. When *E. coli* is subjected to carbon and amino acid starvation, an immediate consequence is the accumulation of unusual nucleotides, ppGpp and pppGpp. Synthesis of ppGpp is governed by at least two ways. The enzyme ppGpp synthetase I (PSI) encoded by the *relA* gene is responsible for ppGpp synthesis during the stringent response to amino acid deprivation (Block and Haseltine, 1974; Cashel and Gallant, 1969). When growth is slowed by the depletion of a primary carbon source, the stringent response is activated by a pathway that is independent of the *relA* gene (Hernandez and Bremer, 1991). A second enzyme, ppGpp synthetase II (PSII), encode by the *spoT* gene, is responsible to catalyze ppGpp synthesis (Gentry and Cashel, 1996; Hernandez and Bremer, 1991; Xiao et al., 1991).

The synthesis of rRNA is known to be inhibited by ppGpp (Ryals et al., 1982). *In vitro* experiments demonstrated that ppGpp has a direct inhibitory effect on *rrn* transcription (Glaser et al., 1983). Even though the exact mechanism of this control is not yet clear, numerous data suggest that ppGpp affects RNA polymerase selectivity, rendering it unable to initiate transcription at stable RNA promoters (Ham-

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ming et al., 1980; Travers et al., 1982). In addition, ppGpp itself is a potent inhibitor of protein synthesis by reducing the RNA chain growth rate, making mRNA limiting for translation during starvation (Svitil et al., 1993). The inability of starved cells to further synthesize recombinant proteins may also be due to this mechanism.

Cells with null alleles in both the *relA* and *spoT* genes were found no longer to accumulate ppGpp upon amino acid and carbon starvation (Hernandez and Bremer, 1993; Tedin and Bremer, 1992; Xiao et al., 1991). As a result, ribosomes were 10–20% overproduced in the ppGpp-less strains at lower growth rates (Herman and Wegryzn, 1995; Sorensen et al., 1994; Tedin and Bremer, 1992). Overall, the synthesis rate of translational factors were found to be increased by 5–20 fold, while their expression was almost totally shut-off in wild type cells (Nystrom, 1994).

In this paper, our main goal is to study how production of recombinant protein is affected if the potential ppGpp inhibition is removed by eliminating intracellular ppGpp. Although previous results on ppGpp have clearly demonstrated the importance of this factor in determining the translational potential of *E. coli* during restricted growth, one key question which is critical for the strategy proposed here remains. Due to the complexities of the regulation of protein synthesis, it is possible that ppGpp would have little effect on recombinant protein synthesis. We have conducted experiments outlined below, which addressed this concern. These experiments also entail the first reported effects of ppGpp on recombinant protein synthesis in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli strains CF1648 (MG1655 (λ^- , F⁻)) and CF1693 (same as CF1648 except it is also *spoT* and *relA*) were used in this study (Hernandez and Bremer, 1993). Routine transformations were performed with *E. coli* DH1. Plasmid pKC6 (Chen et al., 1993) which expresses chloramphenicol acetyltransferase (CAT) under control of a *tac* promoter, was used as the CAT expression vector.

Media and Growth Conditions

LB medium (10 g/L Difco tryptone, 5 g/L Difco yeast extract, 10 g/L NaCl, 3 g/L K₂HPO₄ and 1 g/L KH₂PO₄, pH 7.0) and M9 medium (6 g/L Na₂HPO₄, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, 2.5 g/L NaCl, 3 mg/L CaCl₂, 0.2 mL of 1M MgSO₄·7H₂O and 1 mL of 20% Casamino acids) were used for both batch and fed-batch cultivations. For all experiments, media were supplemented with 0.2% glucose as the carbon source. Ampicillin was added at 50 mg/L for selection. For the induction of CAT production, 1 mM IPTG was added unless otherwise described. Shake flask experiments

were carried out at 275 rpm in a New Brunswick INNOVA 4000 incubator shaker at 37°C.

Fed-batch fermentations were carried out in a 5 L Bioflow 3000 (New Brunswick Scientific, Edison, NJ) bioreactor. The starting working volume was 4 L. The conditions of operation were temperature, 37°C; agitation, 400 RPM; pH, 7.0; initial air flow-rate, 1.5 L/min. pH was controlled by addition of NaOH and HCl. Foam was controlled by the addition of a 2% anti-foam A solution. The inoculum (1:50) was grown in 100 mL of the same medium in a 250 mL flask for approximately 16 h. The amount of inoculum added to the bioreactor was adjusted to give a starting OD of 0.1. The fed-batch feeding was initiated when the dissolved oxygen (DO) concentration rose from 15–20% to nearly 100%. This indicated that the cells were about to enter stationary phase. A solution of 20% glucose and 10% casamino acids was fed to the bioreactor at a feed-rate of 4 mL/h. After 26 hours post-inoculation, the feed-rate was changed to 8 mL/h and the air flow-rate to 3 L/min. Ampicillin was added continuously to maintain selection pressure throughout the feed-batch fermentation.

Chemicals, Reagents, and DNA Manipulations

All restriction endonucleases, modifying enzymes (T4 DNA polymerase, Klenow fragment, T4 DNA Ligase) and IPTG were purchased from New England BioLabs, Boehringer Mannheim Biochemicals, or Promega. ³²P-dCTP was purchased from New England Nuclear. All DNA manipulations were done according to standard methods (Sambrook et al., 1989). DNA fragments were eluted from agarose gels using a Sephaglas BandPrep Kit from Pharmacia. Rifampicin was purchased from Sigma Chemical.

Protein and CAT Assays

Cells were harvested by centrifugation and disrupted by sonication. The soluble fraction was used for protein and CAT analysis. Total protein concentration was determined using a Bio-Rad Protein Assay kit. Total CAT content (g CAT) was measured by a CAT ELISA kit obtained from Boehringer Mannheim. The specific CAT content was expressed as g CAT/g total protein. CAT activity was assayed in parallel following the procedure described in Rodriguez and Tait (1983). Dilution of samples in TDTT buffer was required for samples with high activity. Kinetic measurements were performed with a Beckman spectrophotometer in a 37°C temperature-controlled sample chamber.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). For SDS-PAGE analysis, cell lysate was boiled for 5 min in gel loading buffer (10% glycerol, 5% 2-mercaptoethanol, 3.3% SDS and 0.5 M Tris, pH 6.8) and then electrophoresed on a 12.5% polyacrylamide gel.

RNA Isolation and Analysis

One mL sample from CF1693 (\pm 1 mM IPTG induction) and CF1648 (induced with 1 mM IPTG) cell cultures were col-

lected at various time intervals (pre- and post-induction). RNA was isolated from each sample (Sambrook et al., 1989), then quantified at 260 nm. Six μg aliquots of total RNA from each sample were glyoxylated (Maniatis et al., 1982), then electrophoresed through 1.3% agarose gels. RNA molecular weight standards (0.24–9.5 Kb RNA Ladder, Gibco-BRL) were included. RNA was transferred to nitrocellulose filters for hybridization analyses (Maniatis et al., 1982).

^{32}P -riboprobes were utilized for detection of chloramphenicol acetyltransferase (CAT) mRNAs. A CAT-containing DNA fragment (Chloramphenicol Acetyltransferase GenBlock[®], Pharmacia) was ligated into the *Hind*III site of (pT7T3 19U, Pharmacia) to yield pT3CAT. ^{32}P -labelled riboprobe was prepared from *Pst*I-linearized pT3CAT as described by Rao et al. (1994). Hybridization was performed overnight at 65°C (Maniatis et al., 1982). The stringency used during the final washings of the blot was 68°C; 0.1 \times SSC. Autoradiographs were scanned using a LKB Ultrascan scanning laser densitometer for quantification of the CAT mRNA. Counts thus obtained were normalized with total RNA in order to obtain a measure of specific gene activity as described before (Chen et al., 1995).

RESULTS AND DISCUSSION

Effect of ppGpp on Recombinant Protein Synthesis

The first set of experiments was designed to explore whether ppGpp plays an important role in recombinant protein synthesis. Comparison of CAT production profiles between ppGpp-less and wild-type strains would provide us this information. *E. coli* strain CF1693, which is totally devoid of

ppGpp, and a wild-type control strain CF1648 (MG1655) were employed in these initial studies. Plasmid pKC6, which expresses chloramphenicol acetyltransferase (CAT) under control of a *tac* promoter, was introduced into these strains. The resulting strains were grown in 250 mL shake flasks containing 50 mL of LB medium with 0.2% glucose, and the levels of CAT production were monitored throughout the batch cultivation. These results are depicted in Figure 1. Before induction, CAT production was very low in both strains due to tight regulation of the *tac* promoter. However, upon IPTG addition, the CAT level increased gradually for strain CF1648, while a rapid increase in CAT production was observed for strain CF1693. The amount of CAT eventually leveled off after four hours of induction, and remained relatively constant thereafter. The final CAT level was about 5-fold higher in strain CF1693. These findings are important for several reasons. It demonstrates that ppGpp has a profound effect on recombinant protein synthesis. Secondly, it also illustrates that eliminating ppGpp has a beneficial effect on recombinant protein production even during exponential growth. This observation is unexpected since most reports on ppGpp have indicated differences in protein synthesis only during slow-growing conditions (Nystrom, 1994; Sorensen et al., 1994; Svitil et al., 1993).

Because *relA*⁻ mutants have unusually high level of amino acid mis-incorporation (Sorensen et al., 1994), in order to ensure that the CAT protein levels measured from the ELISA kit were not the artifacts of inactive or truncated CAT fragments, CAT activity measurements were also performed for selected samples. The resulting CAT activities were in good agreement with the ELISA results, indicating that most of the proteins were indeed active (data not shown). Moreover, selected samples were analyzed by SDS-PAGE as shown in Figure 2. It can be seen clearly that

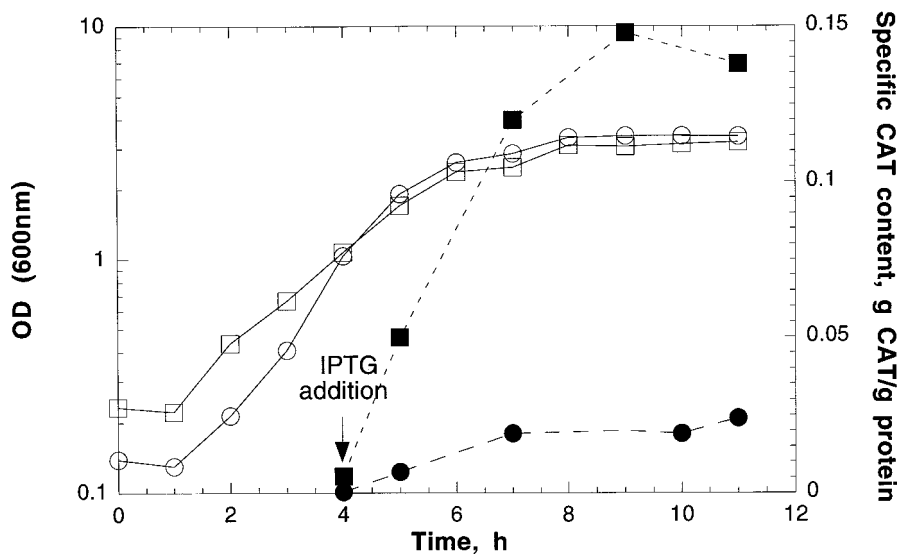


Figure 1. Cell growth (Open symbol) and CAT production (closed symbol) from strains CF1648 (○) and CF1693 (□) carrying plasmid pKC6. Cells were grown in LB medium with 0.2% glucose.

1 2 3 4 5

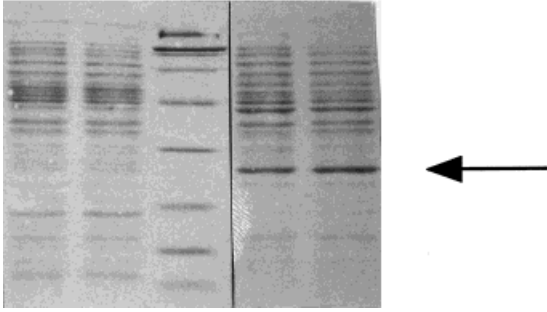


Figure 2. SDS-PAGE of total proteins from strains CF1648:pKC6 (Lane 1 and 2) and CF1693:pKC6 (Lane 4 and 5). Samples were taken at 2 and 4 h post-induction. The molecular weight markers in Lane 3 are as follows: 6.5 kDa, 14.4 kDa, 21.5 kDa, 31 kDa, 45 kDa, 66.3 kDa, 97 kDa, 116 kDa, and 200 kDa. CAT protein bands are indicated by the arrow.

the intensity of the protein bands corresponding to CAT was higher in CF1693, and no other major degraded CAT products were observed. At least under our experimental conditions, amino acid mis-incorporation does not appear to be affecting the production of active CAT products.

A similar enhancement in CAT production was also observed when cells were grown in slower-growing M9 medium. As depicted in Figure 3, there were essentially no differences in cell growth between the two strains. However, the specific CAT level was about 2 times higher in the ppGpp-deficient strain. In contrast to results from LB medium, the major differences in CAT production were observed only when cells shifted into the slow-growing regime with essentially no difference in CAT production during exponential growth. These results suggest that ppGpp affects CAT production differently under different growth conditions, and possibly by different mechanisms.

Effect of ppGpp on Recombinant Protein Synthesis During Fed-Batch Fermentations

Wild-type cells accumulate ppGpp particularly under slow-growing conditions and hence the greatest difference in the amount of ppGpp in cultures of CF1648 and CF1693 would be during slow-growing conditions. Fed-batch fermentations provide a means to create an extended period of slow culture growth to compare the effect of ppGpp deficiency on the production of CAT during extended slow growth.

Cultures of CF1648 and CF1693 carrying pKC6 were grown in a bioreactor as described in Materials and Methods. 0.5 mM of IPTG was added when the culture reached an OD_{600} of about 1.4. Cultures of CF1648/pKC6 were induced 2.5 hours post-inoculation and cultures of CF1693/pKC6 were induced 4.17 hours post-inoculation.

From Figure 4, we see that addition of IPTG induced the production of CAT in both cultures. At the end of exponential phase (~5 hours for CF1648 and ~9 hours for CF1693), cultures of CF1693 accumulated about 4.5-fold higher CAT per total cellular protein compared to CF1648. Thus, expected ppGpp deficiency in late exponential phase results in higher recombinant protein production similar to that observed in batch cultures grown in shake flasks.

Drastic differences in amount of CAT between the two cultures was observed after the initiation of slow feeding of nutrients. At the end of exponential phase, a solution of 20% glucose and 10% casamino-acids was fed to the cultures at a rate of 4 mL/h, when the dissolved oxygen (DO) concentration rose to nearly 100%. The amount of CAT per total protein decreased continuously in cultures of CF1648, even though the total cellular protein increased after feeding was initiated. On the other hand, cultures of CF1693 continued to accumulate CAT after feeding. For example, the amount of CAT per total protein was nearly 70-fold higher in cultures of CF1693 17 hours post-inoculation. Overall, about

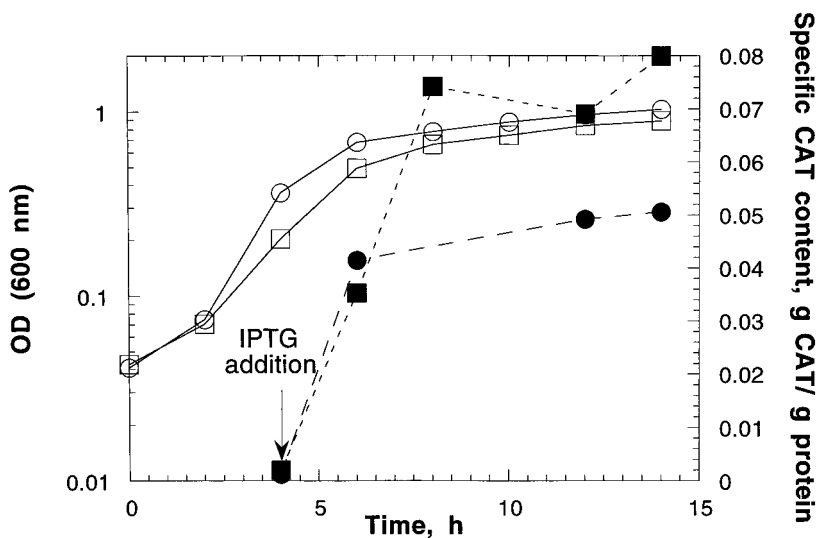


Figure 3. Cell growth (Open symbol) and CAT production (closed symbol) from strains CF1648 (○) and CF1693 (□) carrying plasmid pKC6. Cells were grown in M9 medium with 0.2% glucose.

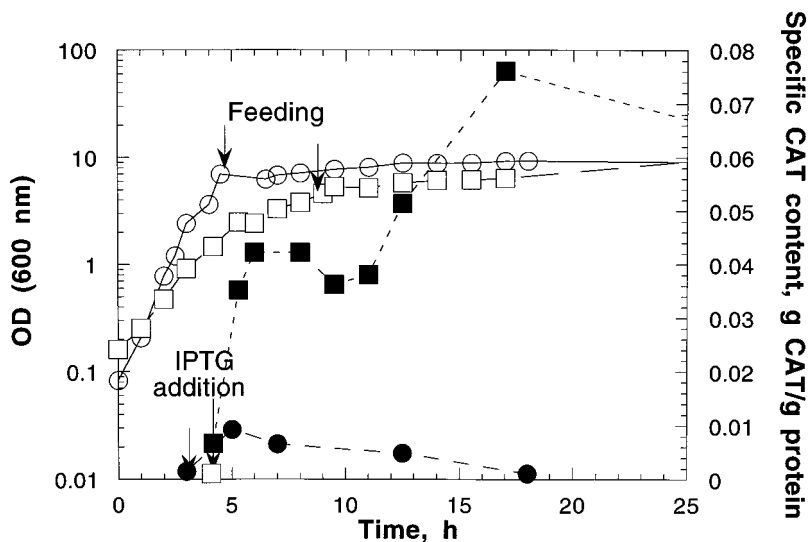


Figure 4. Comparison of cell growth (Open symbol) and CAT production (closed symbol) from strains CF1648 (○) and CF1693 (□) carrying plasmid pKC6 in fed-batch fermentation. Fed-batch fermentations were carried out in a 5 L Bioflo 3000 bioreactor. The starting working volume was 4 L. The conditions of operation were temperature, 37°C; agitation, 400 RPM; pH, 7.0; initial air flow-rate, 1.5 L/min. The fed-batch feeding was initiated when the dissolved oxygen (DO) concentration rose from 15–20% to nearly 100%. A solution of 20% glucose and 10% casamino acids was fed to the bioreactor at a feed-rate of 4 mL/h. After 26 hours post-inoculation, the feed-rate was changed to 8 mL/h and the air flow-rate to 3 L/min. Ampicillin was added continuously to maintain selection pressure throughout the fed-batch fermentation.

20 times as much CAT was produced in the ppGpp-deficient strain during the fed-batch phase.

Comparison of CAT mRNA Level

In wild-type cells, a low level of ppGpp is normally detected during exponential growth (Hernandez and Bremer, 1993), and very little difference in cellular mechanism is expected between strains CF1693 and CF1648. It is therefore uncertain what mechanism is responsible for the increase in CAT production during exponential growth in LB medium. However, in addition to an improved translational apparatus, the rate of mRNA synthesis has also been shown to be drastically higher in ppGpp-less strains, particularly during rapid cell growth (Hernandez and Bremer, 1993; Kingston and Chamberlin, 1981). This could be a possible explanation for our observed results. To investigate this possibility, we have conducted experiments to compare the level of CAT mRNA upon induction. Samples were taken as early as 4 min. post-induction up to 6 h post-induction. As depicted in Figure 5, the level of CAT mRNA was at least 2-fold higher in the ppGpp-deficient strain CF1693, with the most significant difference during the initial 2 hours post-induction. This difference in CAT mRNA levels gradually decreased with time, and the levels were essentially the same between the two strains when cells shifted into stationary growth (~6 h post-induction).

For ppGpp-deficient cells growing exponentially in LB medium, the improvement in CAT production appears to be partially due to an increase in transcript levels. Since little difference was observed between the two strains in CAT-specific mRNA levels during stationary growth (see Figure

5), the observed increase in CAT production in stationary phase cultures of CF1693 does not appear to be attributable to a difference in transcript levels, suggesting that other post-transcriptional mechanisms, such as proteolysis (Harcum and Bentley, 1993) or translation (Hernandez and Bremer, 1993; Nystrom, 1994; Sorensen et al., 1994), may be responsible for the enhancement.

In order to investigate whether differential proteolytic activities might be at least partially responsible for the observed increase in CAT expression in CF1693 versus CF1648, CAT activities were monitored in late log-phase cells in the presence of rifampicin. Cultures of CF1648:pKC6 and CF1693:pKC6 were induced with 1 mM IPTG and incubated until OD₆₀₀ approximately 1.0. 4 mg of rifampicin was then added every 30 minutes to the cultures to terminate translation of new polypeptide chains. Samples were collected every 30 minutes for 2.5 hours and their CAT activities were quantified. No significant decline in CAT activity could be detected in the wild-type strain, while a small decline in activity was observed in strain CF1693 (Fig. 6). These results indicated that proteolysis does not appear to play an important role for the increase in CAT production in the ppGpp-less strain.

The possible enhancement during the slow-growing regime is better indicated in the fed-batch experiment in which the ppGpp-less strain accumulated a much higher level of CAT during the slow-growing fed-batch phase, where there was no significant difference in CAT mRNA levels (data not shown). The specific CAT accumulation rate was about 20-fold higher in the ppGpp-less strain. Along the same line, no difference in CAT production was observed in M9 medium until the stationary phase, because

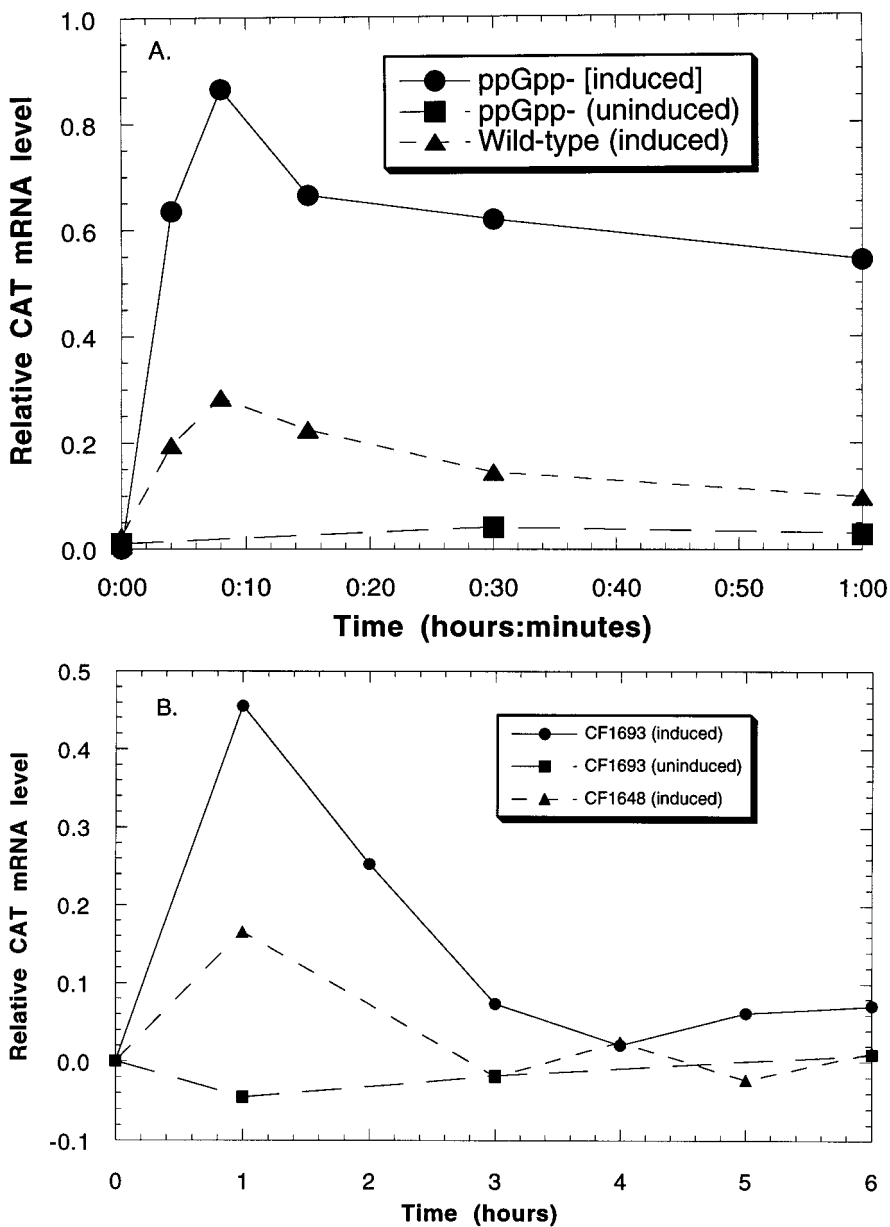


Figure 5. Comparison of CAT mRNA levels between strains CF1648 and CF1693. (A) The initial response during the first hr post-induction. (B) Response up to 6 h post-induction.

the CAT-specific transcript levels were increased in ppGpp-less strain only when cells were growing rapidly (as in LB medium), but not in slower growing M9 medium. Thus, eliminating intracellular ppGpp appears to have two effects on the production of CAT—an increase in transcript levels during exponential growth and enhancements in post-transcriptional processes during restricted growth.

CONCLUSIONS

We have investigated the effects of eliminating ppGpp on recombinant protein synthesis in both batch and fed-batch cultivations. Results from batch cultivations indicated a 5-fold increase in CAT production in the ppGpp-less strain

when grown in LB medium and a 2-fold increase when grown in M9 medium. When the ppGpp-less strain was grown in LB medium, the rapid increase in CAT production during exponential growth was accompanied by a 2-fold increase in CAT mRNA level. This increase in transcript levels may in part explain the difference in CAT production during exponential growth. Many other cellular processes, such as plasmid replication, translation, and proteolysis, may also be responsible for this enhancement. However, plasmid copy effect is not expected to be significant. As reported by Herman and Wegrzyn (1995) the plasmid copy number of ColE1-based replicon was not affected significantly by different ppGpp levels. Furthermore, no major difference in translation or proteolysis is expected during

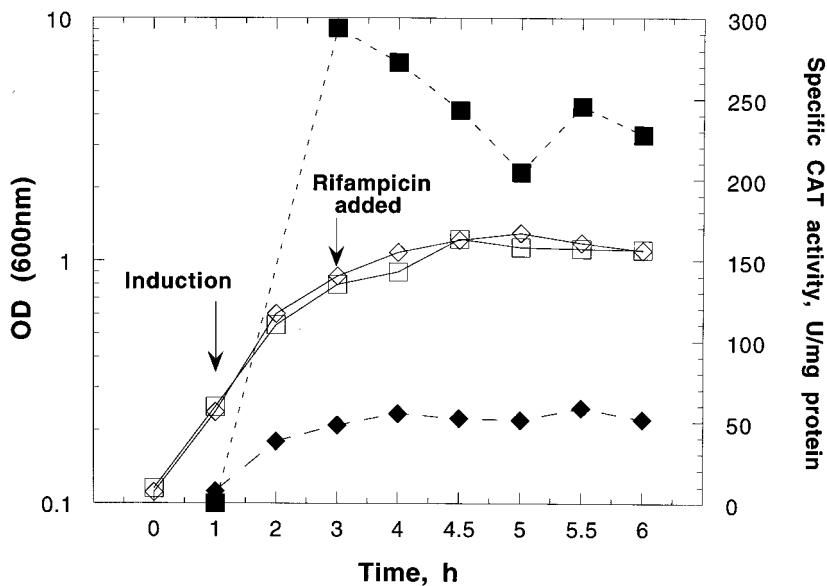


Figure 6. Effect of rifampicin treatment on CAT expression in CF1648:pKC6 and CF1693:pKC6 cells. Cells were induced with IPTG (to 1 mM), then with rifampicin as described in materials and methods. Cell growth rate [\diamond -CF1648:pKC6, \square -CF1693:pKC6] and CAT activity [\blacklozenge -CF1648:pKC6, \blacksquare -CF1693:pKC6] are shown.

exponential growth since even wild-type cells do not synthesize an appreciable level of ppGpp.

In contrast, when cells were grown in M9 medium an increase in CAT production was observed in the ppGpp-less strain only during the slow-growing regime, where no difference in CAT transcript levels were detected. It is likely that a different mechanism in the post-transcriptional machinery, such as proteolysis or translation, may be responsible.

Similar enhancements in CAT production were observed in fed-batch fermentations. CAT accumulation was about 20-fold higher in the ppGpp-less strain during the fed-batch phase. This is indicative of the importance of intracellular ppGpp on recombinant protein synthesis especially during slow-growing fed-batch phase. We are currently investigating the possible effects of varying intracellular ppGpp concentrations on recombinant protein synthesis during both exponential growth and restricted growth.

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