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A pH-REGULATED PROMOTER FOR THE EXPRESSION OF RECOMBINANT PROTEINS IN *ESCHERICHIA COLI*

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SUMMARY

A series of plasmid vectors have been developed which allows control of recombinant gene expression by altering the pH of the growth medium. Expression is controlled by the regulatory region of the *cad*A gene of *E. coli*. Experiments using β-galactosidase as the expressed gene have resulted in an induced expression of up to 60-fold when the pH of the growth medium is lowered from pH 7 to 5.5. Expression can also be induced by switching from aerobic to anaerobic growth environment. The pH and anaerobic effects are additive boosting the expression level of β-galactosidase to a dramatic value of 200 fold. Finally, the pH-induction effect is fully reversible, a unique property which allows continuous control of gene expression using exiting pH monitoring and control equipment.

INTRODUCTION

Various expression systems have been developed for high level protein production. The most common and useful promoters include lac, tac, λp_L, trp, lpp and more recently the oxygen-responsive promoter (1-7). The most widely used promoters usually require induction by heat (λp_L) or the addition of inducers (IPTG or Indole acrylic acid) for high level expression. More detailed discussions on various aspects of expression systems can be found in references 8 and 9.

Of particular interest is a class of bacterial enzymes called amino acid decarboxylases. Their pH-dependent synthesis was reported in the mid-1940's (10). Maximal production of these enzymes up to a few percent of cell protein occurs when *E. coli* is grown anaerobically in low pH medium containing an excess of their respective amino acid substrates (11).

Although little work has been published on the regulation of the amino acid decarboxylases, the best studied is lysine decarboxylase. Its gene (*cad*A) has been mapped to 93 minutes on the *E. coli* chromosome (12). Its regulation is not well understood, but experiments indicate a complicated mechanism. *Cad*A is known to be regulated by the product of the *cad*R gene located at 46 minutes (12). Induced expression of *cad*A by low pH cannot occur unless high concentrations of lysine are present in the growth media. The pH

effect is not dependent on *cad*R as *cad*R$^-$ mutants exhibit pH-induced expression of *cad*A without requiring lysine (13).

We have isolated, sequenced and studied the regulatory region of the *cad*A gene (14,15) and fused it to the β-galactosidase gene. Several different plasmids have been made with variations of this basic construct. The first generation of these plasmids, pSM-10, is reported here. Some of the unusual expression characteristics of this plasmid in the S-2-aminoethyl-L-cysteine (SAEC)-resistant strain, GJT-001, are presented. The results reported here show that the pH/anaerobic inducible promoter system can serve as a convenient and inexpensive method for regulated and high-level expression of recombinant proteins.

MATERIALS AND METHODS

Bacterial Strains and Plasmids GJT-001 is a spontaneous SAEC-resistant mutant of MC4100 a Δlac strain (16). Selection of this mutant is described below. To construct the plasmid, pSM-10, a 3000 bp region from 93 minutes on the *E. coli* chromosome was isolated. This region contains the amino terminus of the *cad*A protein, lysine decarboxylase, as well as its pH-controlled promoter region. By operon fusion into the plasmid, pRS-550 (17), the *lac*Z-expressed plasmid, pSM-10 (Ap^R, Km^R, ColE1 ori, *lac*ZYA), was constructed.

Selection of *E. coli* mutant GJT-001 SAEC-resistant mutants of *E. coli* spontaneously form and are easily isolated due to the reduced lysine transport found in *cad*R$^-$ strains. When an *E. coli* culture is streaked onto rich, lysine-free agar supplemented with the toxic lysine analog, SAEC, only *cad*R$^-$ colonies will form (18). A 5 ml culture of MC4100 was grown at 37°C. in lysine-free Davis/Mingoli media (19) supplemented with amino acids (18,20), 2 g/l thiamine, 25 mg/l streptomycin and 2 g/l glucose. At 0.3 OD at 600 nm, 0.1 ml of culture was spread onto plates made from the above media with 30 g/l agar and 100 mg/l SAEC according to the procedure of Popkin and Maas (18). The SAEC-resistant colonies were assayed for pH-induced production of lysine decarboxylase as described. Strain GJT-001 had high pH-induction but did not require the presence of lysine substrate in the culture media.

Enzyme Assays Cell extract preparation for the lysine decarboxylase assay is described by Auger (21) and is summarized as follows. Samples of cell culture were harvested and centrifuged at 9000 x g for 10 min at 4 degrees C. After washing in 20 ml of 0.15 M NaCl the cells were repelletted. The pellet was resuspended in 1/100 volume of lysine decarboxylase buffer (1mM dithiothreitol, 1mM EDTA (pH 7.2), 0.1mM pyridoxal-5'-phosphate). The suspension was sonicated at 0°C in 4 15-second bursts. The assay for lysine decarboxylase followed the procedure described by Phan, et al (22). For the β-galactosidase assays the cell extracts were prepared according to the method of Miller (23) except that the harvested cells were resuspended twice in 10 ml "A" buffer and the chloroform/SDS method of cell lysis was employed. After the reaction the tubes were centrifuged at 1000 x g for 10 minutes. The centrifugation step eliminates the need for cell debris correction. Specific activity is expressed as Miller units; 1 Miller unit = 1000 $\Delta A_{420}/min \cdot ml \cdot OD_{600}$ (23). All assays were done in triplicates.

Batch Fermentation Conditions Fermentations were carried out in a 1 liter Virtis Omniculture reactor using 1 liter of Luria Broth supplemented with 2 g/l glucose at 37°C. Sterile air sparging at 2 l/min was maintained throughout except when anaerobic step changes were required. Under those conditions nitrogen gas was substituted. pH was continually measured by a combination electrode (Ingold) and controlled by injection of 1 M NaOH using a controller (Chemcadet, Cole Palmer). pH step changes were initiated by rapid injection of concentrated HCl, reaching the desired pH within 15 seconds. Cell density was monitored spectroscopically at 600 nm after appropriate dilution with 0.15 M NaCl.

RESULTS AND DISCUSSIONS

Characterization of the SAEC-resistant mutant, GJT-001

In order to circumvent the requirement of lysine in the medium for proper induction by pH, an SAEC-resistant mutant which exhibits the same phenotype behavior as *Cad*R$^-$, GJT-001, was isolated as described. Cultures of the *cad*R$^+$ parent strain, MC4100, and GJT-001 were grown in shake flasks of Luria Broth in 40 mM MES or MOPS for buffered pH growth at 5.5 and 7.0, respectively. A comparison of the pH dependence of lysine decarboxylase activity for the two strains, with and without lysine, in late exponential phase is shown in Table 1. In contrast with the parent strain, MC-4100, the expression of lysine decarboxylase in GJT-001 is independent of external lysine concentration.

Strain		Induction	Specific Lysine Decarboxylase Activity (unit/mg protein)	
			pH = 5.5	pH = 7.0
*cad*R$^+$	MC-4100	None	90	not detectable
	MC-4100	0.4% Lys	550	6
*cad*R$^-$	GJT-001	None	1200	3
	GJT-001	0.4% Lys	910	8

Table 1. Lysine Decarboxylase Activity of strains MC-4100 and GJT-001. Strains were grown aerobically at pH 5.5 and pH 7.0 as described in Experimental Protocol

Since pSM-10 utilizes the same regulatory region of the *cad*A gene to control β-galactosidase, transformation of strain GJT-001 with pSM-10 allows study of the pH dependence of β-galactosidase expression without the need for externally added lysine.

Effect of pH on β-galactosidase Expression

A series of batch fermentations of the GJT(pSM-10) system were conducted with different pH step changes. After inoculation of the fermenter, the cultures were allowed to grow at pH 7.0 until an OD of approximately 0.2 was reached. The pH was then lowered to a series of values from 6.5 to 5.5. The results are shown on Figure 1.

Upon initiation of the pH step change, the level of β-galactosidase specific activity (i.e., on a per unit soluble protein basis) increases with cell growth. Of particular importance is the trend that higher levels of β-galactosidase specific activity are observed with lower pH down to pH 5.5. Thus, a pH step change to 5.5 appears to be optimum.

The above set of experiments demonstrates that the pH-regulated promoter associated with the *cad*A gene has been transferred to pSM-10 and controls the expression of β-galactosidase. That the pH regulation of foreign gene expression is the basis for the observed results was confirmed by measuring the activity of another plasmid encoded enzyme, β-lactamase. Within the same pH range the specific activity of β-lactamase was the same at all pH values (data not shown). This indicates that the copy number of pSM-10 is not significantly affected by pH and that the increase in β-galactosidase activity with pH is due to variation in gene transcription.

Effect of Anaerobiosis on β-galactosidase Expression

A similar set of batch fermentations were performed to demonstrate the effect of anaerobiosis and the results are shown in Figure 2. When a culture had grown to an OD of 0.2 at pH 7.0, the culture was maintained at this pH but reactor sparging was instantaneously changed from air to nitrogen. Under the sudden change to anaerobic conditions the culture was immediately induced; the specific β-galactosidase activity increases to 30 times higher than the prior aerobic growth. The rate of increase parallels that

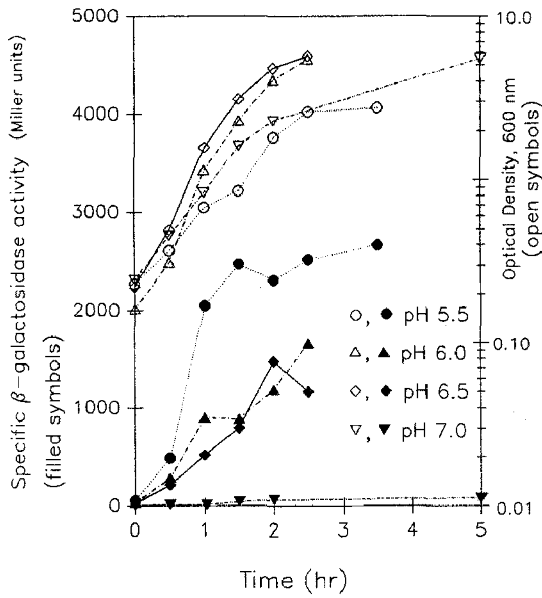

Fig.1 The effect of pH on β−galactosidase expression

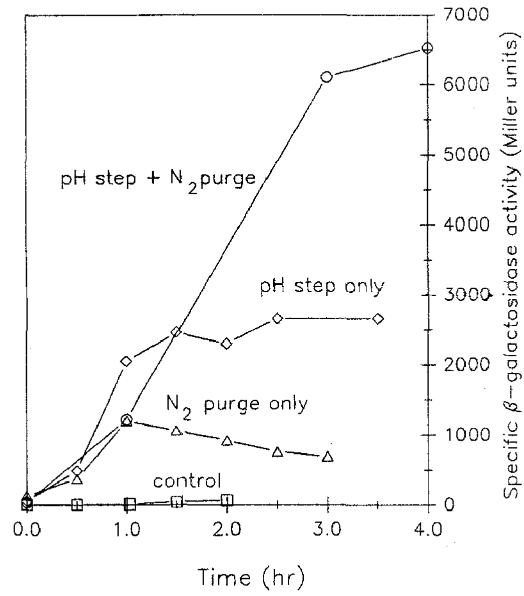

Fig.2 The effect of pH and anaerobic condition on β−galactosidase expression

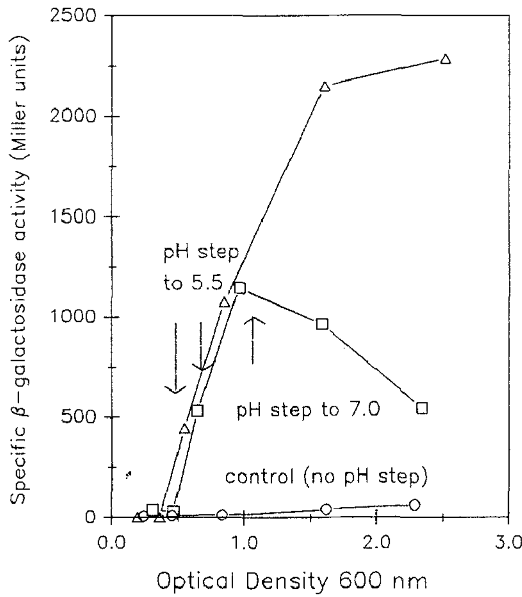

Fig.3 The effect of pH step changes on β−galactosidase expression (specific activity)

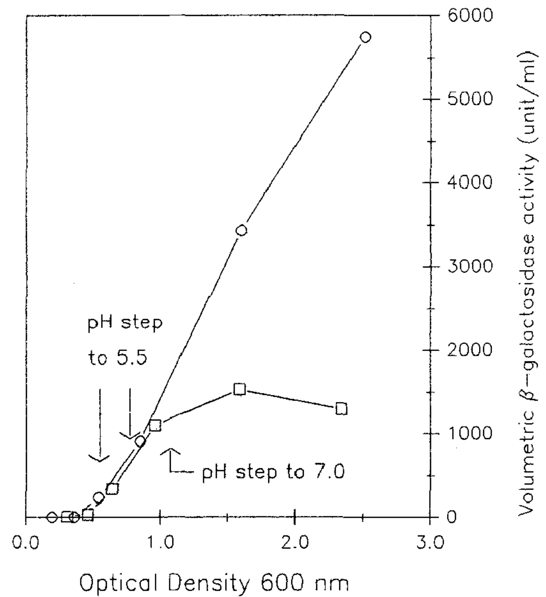

Fig.4 The effect of pH step changes on β−galactosidase expression (volumetric activity)

of the pH 7.0 to 5.5 step change for one cell generation. However, the growth rate of the cells decreased markedly and the expression of β-galactosidase decreased with increasing optical density. In fact, the cells ceased to grow after an OD of 2.3 was reached.

When a combined pH step change to 5.5 and anaerobiosis was performed under identical starting conditions, an enormous increase in β-galactosidase expression was observed. By the time an OD of 1.6 was reached (4 generations) the activity had increased to 200 times the basal value. The separate effects of pH and anaerobiosis are additive. However, just as the anaerobiosis effect alone resulted in a cessation of growth after 4 generations, this effect was also observed when combined with a pH step. In fact, growth stops shortly after maximum β-galactosidase activity is attained.

The Reversibility of Expression

Another batch fermentation experiment was performed to examine the reversibility of induction responses. Two cultures were grown to an OD of approximately 0.4 at pH 7.0 after which a pH step change to 5.5 was applied to both. One culture was maintained at pH 5.5 after the step change. The second culture, however, was allowed to grow at pH 5.5 for approximately one cell generation, up to an OD of 0.9. Then a second step change was imposed bringing the reactor pH back to 7.0. That is, the second culture was given a "square wave" pH change. The results are summarized in Figure 3.

As expected, during the initial pH step to 5.5, the cultures immediately exhibited increased β-galactosidase activity, matching each other reasonably well. The first culture, having been maintained at pH 5.5 step change, doubles its specific activity during the next generation of growth to an OD of 2.4. The second culture, which underwent the "square wave" pH change, however, exhibited a marked decrease in β-galactosidase specific activity. Another way to present to the same data set is by using the volumetric activity (Fig. 4). The volumetric β-galactosidase activity for the culture with induction maintained (i.e. constant pH 5.5) increased monotonically with optical density. However, the volumetric activity for the culture switching back to pH 7 stayed at about the same level after the change. This result indicates that the synthesis of the enzyme has stopped after the pH switched back to 7.

Discussions

The pH and anaerobic properties exhibited by the promoter of the *cad*A gene are clearly demonstrated by the plasmid construct, pSM-10. Step changes of pH and dissolved oxygen substantially increase the expression of β-galactosidase. The effects are additive and when both are employed, induce expression of foreign gene product to up to 200 times the basal level.

The variable degree of induction by pH within the range of 7.0 and 5.5 makes possible a degree of expression control not easily accomplishable with the lac and λP_L operons. By precise monitoring of induction pH, a "fine tuning" of foreign gene expression and growth rate to optimum levels is possible. Instrumentation for such control already exists.

More importantly, perhaps, is the reversibility of the pH induction effect. The pH control exhibited by the *cad*A promoter is fully reversible using simple addition of acid or base. This reversibility property together with the extreme responsiveness to pH changes (at least 60 fold within 1.5 unit pH change) opens a new dimension from a process aspect. That is, induction of gene expression is no longer restricted to the most commonly employed on-off type but can be of any profile. It may be fruitful to fine-tune the induction profile such that gene expression matches that of cellular activities.

Finally, a specific SAEC-resistant *E. coli* strain, GJT-001, was used for these experiments. This was necessary to remove the requirement for high concentrations of lysine in the growth media to permit pH/anaerobic induction. The experimental protocol of Popkin

and Maas (18) to isolate *cad*R⁻ strains is simple and straightforward such that appropriate strains can be screened for this property.

Work is currently underway in further characterizing the *cad*A promoter system with regard to nutrient requirements, time of induction, growth rate, and temperature. Special emphasis is to be given to fed-batch and continuous fermentations. Experiments to analyze the biochemical mechanism of pH and anaerobic induction and to improve the pH induction system by constructing optimized vectors and host strains are in progress. Also, similar work on other pH induced amino acid decarboxylases, such as arginine decarboxylase, have begun. Many interesting avenues of research are possible with this promoter system which is otherwise virtually unknown.

Acknowledgements

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