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Fine Regulation of *c*I857-Controlled Gene Expression in Continuous Culture of Recombinant *Escherichia coli* by Temperature

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The expression at different temperatures of the *lacZ* gene, which is controlled by the lambda p_L and p_R tandem promoters and the *c*I857 temperature-sensitive repressor, was studied in *Escherichia coli* continuous cultures. At temperatures between 30 and 42°C, β-galactosidase activity behaved according to an exponential equation. By inducing a culture at a temperature within this range, predefined, nearly constant submaximal levels of gene expression and recombinant product yield can be obtained.

The design of expression vectors based on the strong p_L (or p_R) lambda promoters used in combination with the *c*I857^{ts} repressor was described some years ago (4). *Escherichia coli* recombinant cultures carrying such vectors are usually induced by a temperature shift from 30 to 42°C. In this situation, RecA-independent, temperature-mediated inactivation of the *c*I857 repressor allows transcription from the p_L promoter. This is very convenient for scaled-up production of heterologous proteins in bioreactors (5), because no external genotoxic inducers, such as nalidixic acid or mitomycin, have to be added (13). However, some problems, such as toxicity for the host, cell wall alterations, formation of inclusion bodies, or incorrect folding of recombinant proteins, have been frequently reported during the use of this procedure (17). At least some of these problems are caused by the high temperature at which cells have to be grown (6, 8). To overcome these problems, induction of this system at low temperatures by alkaline pH shift has also been investigated (15).

Thermal induction is not an all or nothing process. When cultures are induced at temperatures up to 42°C, lower product levels are obtained after induction (10, 14) and cell viability and even productivity are improved in longer processes (7, 10, 16). This offers the possibility of control of gene expression by temperature at levels lower than the maximum level promoted by the system. This procedure could be used to produce recombinant products of pharmacological interest (e.g., mammalian or viral proteins) which are commonly toxic for *E. coli* host cells. Such modulation is hardly expected to be realized by using chemically induced expression systems, in which it is extremely difficult (if not impossible) to define the actual concentration of an active inducer at suboptimal doses, especially in continuous processes. In this study, we examined the performance of gene expression in continuous recombinant *E. coli* cultures carrying a lambda *c*I857-based plasmid system at different temperatures in the range from 30 to 42°C. We selected a very powerful expression vector containing the p_R and p_L strong promoters placed in tandem and the *lacZ* gene encoding the homologous, nontoxic *E. coli* β-galactosidase as an indicator of gene expression.

The strain employed in all of the experiments was *E. coli* K-12 strain MC1061 [*hsdR mcrB araD139* Δ(*araABC-leu*)*7679* Δ*lacX74 galU galK rpsL thi*] carrying plasmid pJLACZ. Plasmid pJLACZ, derived from pJLA602 (19), contains the *cI857* repressor gene and the *E. coli lacZ* gene downstream from promoters p_L and p_R (2). All of the experiments were performed in a Braun-Biotech MD bioreactor controlled by a Micro-MFCS software package (version 3.01). The working volume was 1.5 liters, and CAM9 (11) supplemented with 0.2% glucose and 100 μg of ampicillin per ml was used as the culture medium. The pH was kept at 7.0, the partial O_2 pressure was kept at 75%, and both the air flow and stirring were controlled in cascade. The batch phase was incubated at 28°C after inoculation with a 1:100 inoculum, and the continuous phase started when significant oscillations in the stirring and air flow rates indicated that the stationary phase was beginning. The dilution rate was 0.1 h^{-1}. After about 20 h of continuous culture at 28°C, the temperature was increased sequentially in intervals, and the β-galactosidase activity in outlet samples was analyzed as described previously (12) at the end of each interval. The temperature was changed only once after constant values for stirring and air flow rates had been established after the previous shift. The fermentation period at each temperature was usually 4 to 5 h. The plasmid DNA content and the percentage of plasmid-free cells were determined by standard methods (2).

Table 1 shows the β-galactosidase activities obtained in two fermentations in which several temperatures in the range from 28 to 42°C were examined. No gene expression was detected at temperatures below 32°C, although as determined by a Western blot (immunoblot) analysis of cell extracts (1), a very slight band corresponding to the molecular mass of the enzyme was perceptible even at 28°C (data not shown). These results revealed a lack of linearity between temperature and product yield and proved that at temperatures up to 36°C, there was very inefficient denaturation of the lambda *c*I857 repressor. The very consistent values for β-galactosidase activity obtained in the two experiments suggest that the efficiency of gene transcription was not affected by the history of the culture and that the

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TABLE 1. Parameters in continuous cultures sequentially induced at different temperatures

Expt	Temp (°C)	Biomass (OD_{600})[a]	Enzymatic activity (U/ml/OD)	No. of viable cells (10^9 CFU/ml)	% of plasmid-free cells
A	28	1.45	ND[b]	3.6	ND
	30	1.47	ND	4.7	15.3
	32	1.41	46	3.5	ND
	34	1.34	65	4.9	12.8
	36	1.32	132	3.3	ND
	38	1.29	565	2.9	ND
	40	1.26	3,177	2.7	12.3
	42	1.22	9,603	2.3	ND
B	28	1.63	ND	2.3	ND
	37	1.75	270	4.6	ND
	38	1.84	538	3.3	8.2
	39	1.68	768	3.1	ND
	40	1.70	3,037	2.8	ND
	40.5	1.76	3,693	1.9	ND
	41	1.80	4,688	2.0	4.5
	41.5	1.83	5,189	1.6	ND
	42	1.92	7,327	0.8	ND

[a] OD_{600}, optical density at 600 nm.
[b] ND, not detected.

amount of inactivated *c*I857 molecules in the intracellular pool had a definite value for a given temperature. No plasmid loss was observed during either fermentation.

Using data from Table 1, we studied the relationship between temperature and enzymatic activity by plotting the natural logarithm of activity versus temperature, and we obtained evidence of a linear relationship. The resulting exponential equation, which had a regression coefficient of 0.98, was as follows: $y = 19.71\ e^{0.58(x - 32)}$, where y is the enzymatic activity in enzymatic units (U) per milliliter per OD and x is the temperature in degrees Celsius. The value 32 in the exponent was obtained from our experimental data as a reference, since lower temperatures did not promote detectable gene expression. The resulting constant value, 19.71 U/ml/OD, could be interpreted as the minimum working detection level of our assay.

To check whether intermediate expression levels could provide a constant recombinant protein yield during longer bioprocesses, three different inducing temperatures (38, 40, and 42°C) were used for three independent continuous cultures, which were grown for more than 4 residence times after induction (Fig. 1). At 38 and 40°C, β-galactosidase activity increased during the first 20 h and then remained nearly constant at values of around 1,100 and 6,000 U/ml/OD, respectively. At 42°C, lower levels than expected were observed only transiently. This was probably related to both dramatic cell death, which occurred a few hours after induction (the viable cell concentration decreased from 6×10^9 CFU/ml at 24 h to 8.5×10^6 CFU/ml at 72 h), and plasmid segregation. This suggests that even the overexpression of an homologous gene at such high levels is detrimental for the host and that the plasmid-free cells are quickly selected in the culture. All attempts to carry out a continuous fermentation at this temperature were unsuccessful, because a steady state was never reached. The best results were obtained when a steady state was previously established at 40°C and the culture was then induced at 42°C, resulting in enzymatic activity of 11,361 U/ml/OD. When this enzymatic activity value was used as a representative value for product concentration at 42°C, the product concentrations at 38 and

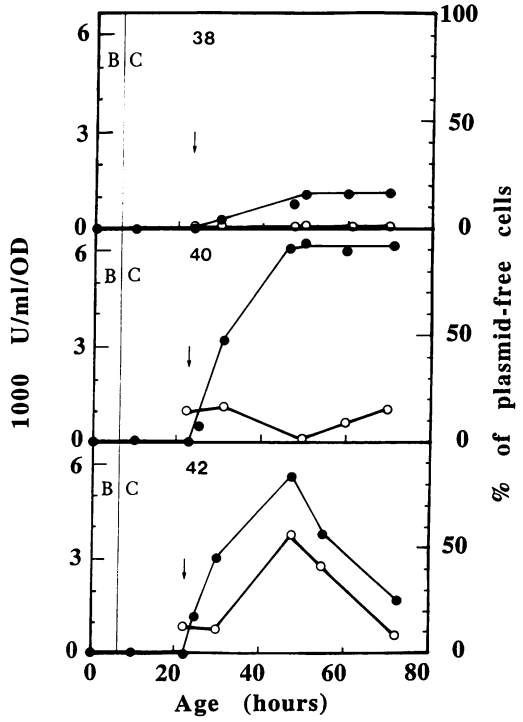

FIG. 1. Levels of β-galactosidase activity (●) and plasmid-free cells (○) in continuous cultures of MC1061/pJLACZ induced at 38, 40, and 42°C. The vertical line divides the batch (B) and continuous (C) phases, and the arrows indicate when the temperature shift occurred.

40°C were 9.7 and 52.8%, respectively, of this hypothetical maximal yield. These values are comparable to the relative expression levels predicted from the equation given above (9.8 and 31.4%).

Table 1 shows that 42°C is the most efficient induction temperature among the temperatures that we tested for *c*I857 repressor inactivation. This finding is consistent with data from classical experiments performed with heat-inducible lambda bacteriophages (9) and with mRNA transcription data (3). However, intracellular accumulation of recombinant proteins is toxic for cells in a concentration-dependent fashion. For this reason and because of other factors, such as protein solubility (18, 20), new expression protocols adapted to temperatures below 42°C are very convenient. The constancy of temperature as an inducing agent (compared with chemical inducing agents) and the fine regulation at submaximal levels offered by heat-inducible *c*I857-repressed lambda promoters provide an interesting way to produce cytotoxic proteins, if an optimal, specific value of product concentration with regard to toxicity is previously defined.

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