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Measuring the relative promoter activity in *Escherichia coli* by a calorimetric method

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Abstract

A calorimetric method was used to monitor the relative promoter activity of the chromosome DNA fragments cloned from *Pseudomonas maltophilia* AT18 in *Escherichia coli*. The promoter probe vector, plasmid pKK232-8, was used to form the recombinants. Three recombinants were selected to study by calorimetric method and agar plate method. The relative strength of the promoter was represented by the resistance level to chloramphenicol. The results showed that the agar plate method could only provide the MIC of the antibiotic to the recombinants, but the calorimetric method not only provided a more precise MIC but also IC_{50} that quantitates the activity of these promoter fragments.

Keywords: Chloramphenicol acetyltransferase; Plasmid pKK232-8; Relative promoter activity; Calorimetric method

1. Introduction

In recombinant DNA work, many target genes can be expressed in engineered bacteria strains with a high efficiency. The efficiency of the transcription process strongly depends on the promoter, a specific DNA sequence recognized and bound by RNA polymerase in the gene transcription initiation process. Convenient, credible methods to measure the promoter activity are important.

Over the past 25 years, a number of vectors have been developed to quantitate transcription initiation on cloned DNA fragments. In 1978, Widera et al. monitored the tetracycline sensitivity of transformants as a measure of promoter activity [1]. Improved vectors then replaced tetracycline resistance by other reporter genes encoding products easy to assay e.g. *lacZ* [2–5], *trpA* [6], *galK* [7], *luc* [8,9], *cat* [10–14] or GFP [15–17].

The use of antibiotic resistance for promoter selection has proven difficult in several respects. The selection of weak or temporally regulated promoters is difficult. The need to use high concentrations of antibiotics when working with Pseudomonas limits the utility. The expense and limited sensitivity of available assay systems for quantitating promoter activity are major drawbacks [3]. The use of promoterless *lacZ* overcame the problems of the first-generation constructs. GFP provided a simple, rapid and sensitive tool for measuring relative promoter activity in intact *Escherichia coli* cells [15], but the toxicity of GFP and lack of enzymatic amplification limited its utility.

By monitoring the heat effect, calorimetric method can directly determine the biological activity of a bacterial system [18,19]. In the present work, a calorimetric method was used, combined with the antibiotic resistant assay, to monitor the relative promoter activity of the cloned chromosome DNA fragments. The promoterless chloramphenicol acetyltransferase (CAT) reporter gene was taken as the reportor gene for recombinant plasmids introduced into *E. coli* HB101.

2. Materials and methods

2.1. Strains and culture conditions

The bacterial strains, *P. maltophila* AT18, *E. coli* HB101, *E. coli* HB101/pKK232-8, *E. coli* HB101/pPAS2, *E. coli*

Abbreviations: *P. maltophilia*, *Pseudomonas maltophilia*; *E. coli*, *Escherichia coli*; Amp, Ampicillin; Cm, Chloramphenicol; CAT, Chloramphenicol acetyltransferase; GFP, Green fluorescent protein

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HB101/pPAS12, *E. coli* HB101/pPAS10 used in this study were grown in Luria-Bertani (LB) complex medium supplemented with antibiotics when necessary [20] or 1.5% agar for the solid medium.

2.2. General molecular biological techniques

General molecular biological techniques (plasmid extraction and detection, DNA fragment isolation, ligation, transformation and PCR.) were performed as described [20].

The clones were inoculated in selective LB liquid medium for detection and isolation of plasmid DNA. The selected recombinants plasmids were subjected to further identification by restriction endonuclease digestion and electrophoresis analysis (SDS-10%). To confirm that the Sal I fragments cloned in pKK232-8 were from *P. maltophila*, the fragments were labeled by DIG as a probe to hybridize with *P. maltophila* AT18 DNA and *E. coli* HB101 DNA using the Dig DNA Labeling and Detecting Kit from Boehringer Mannheim.

2.3. Calorimetric experiment

An LKB 2277 Bioactivity Monitor (Thermometric AB, Sweden) [21] was used to determine the metabolic heat output rate of the strains.

The cultures were firstly incubated in the LB complex medium, initially with 1×10^6 cells ml^{-1}. The freshly prepared antibiotics solutions were added into the cell suspension. The stop-flow method was chose. After the flow-cell was cleaned and sterilized according to [22], the cell suspension containing *E. coli* and antibiotics was pumped into the flow-cell by an LKB-2132 pump at a flow rate of 50 ml h^{-1}. When the flow-measuring cell was full, the pump was stopped, and the monitor system recorded the thermogenic curves of the growth of *E. coli* continuously at 37 °C.

3. Results and discussion

3.1. Screening transformants and detecting their resistant level by agar plate method

Transformants were obtained from LB agar plates containing 100 μg/ml ampicillin (Amp) and 10 μg/ml chloramphenicol (Cm). Three transformants (denoted pPAS2, pPAS12 and pPAS10) representing strong, moderate and weak strength, respectively, were selected for further study. Electrophoresis analysis showed that these contained inserted fragments of different size (result not shown). Their characteristics and the resistance levels to chloramphenicol by the agar plate method are shown in Table 1.

3.2. Dig DNA labeling and detecting

Dig DNA labeling and detecting kit assay showed that the recombinant plasmids pPAS2, pPAS12 and pPAS10 carried the chromosome DNA fragment from *P. maltophila* AT18 (data not shown).

Table 1
The promoter size and resistance level to chloramphenicol detected by the LB agar plate method

Transformants	pPAS2	pPAS12	pPAS10
Promoter fragment size (bp)	1100	1200	600
Cm resistance level (μg/ml)	900	500	40

3.3. Studying the relative promoter activity by calorimetric method

3.3.1. Calculation of the growth rate constant, Inhibitory ratio and half inhibitory concentration from the metabolic power–time curves

The metabolic power–time curves of different strains of *E. coli* growing in LB medium containing different concentrations of Cm or Amp are shown in Fig. 1.

As described [22], in the logarithmic growth phase, the heat output of bacterium growth is exponential:

$$P_t = P_0 \exp(kt) \quad \text{or} \quad \ln P_t = P_0 + kt \tag{1}$$

where k represents the growth rate constant, P_0 and P_t, the heat power of the bacteria at 0 and t, respectively. The rate constants (k) derived by fitting Eq. (1) to the data are shown in Table 2.

High concentrations of antibiotics inhibit the growth of *E. coli*, and the growth rate constant decreases. The inhibitory ratio (I/%) can be defined as:

$$I = \left[\frac{k_0 - k_c}{k_0}\right] \times 100\% \tag{2}$$

where k_0 is the rate constant of the control and k_c is the rate constant for *E. coli* growth inhibited by antibiotic with a concentration of c. When I is 50%, IC_{50} is the half-inhibitory concentration. Because the relative promoter activity of the plasmid directly determines the resistance level to chloramphenicol the IC_{50} can be used to represent the relative strength of the promoter fragments contained in the recombinants. The Data for k, P_m, t_p (the maximum heat power and the corresponding time), I and IC_{50} are shown in Table 2.

3.3.2. Analysis of the growth of the different strains

3.3.2.1. Growth of the different strains without antibiotic. When the plasmids with different promoter fragments were introduced into the host strain (E. *coli* HB101), the metabolic power–time curves changed slightly in Fig. 2. The changes in k, P_m, t_p and Q_{log} (the heat output in the log phase obtained by integrating the power–time curves of the log phase) are shown in Table 3. Therefore, the introduction of the different plasmids did not influence the growth of the host strain. The heat outputs of the strains with the plasmids are higher than the host, but the agreement of the Q_{log} values of the different recombinants indicated that the cloned DNA fragments did not change the basic function of the plasmid in the host strain.

3.3.2.2. Growth of the different strains under the antibiotic. As shown in Fig. 1a and b and Table 2, for the host strain *E. coli* HB101, the growth rate constant (k) and the maximum

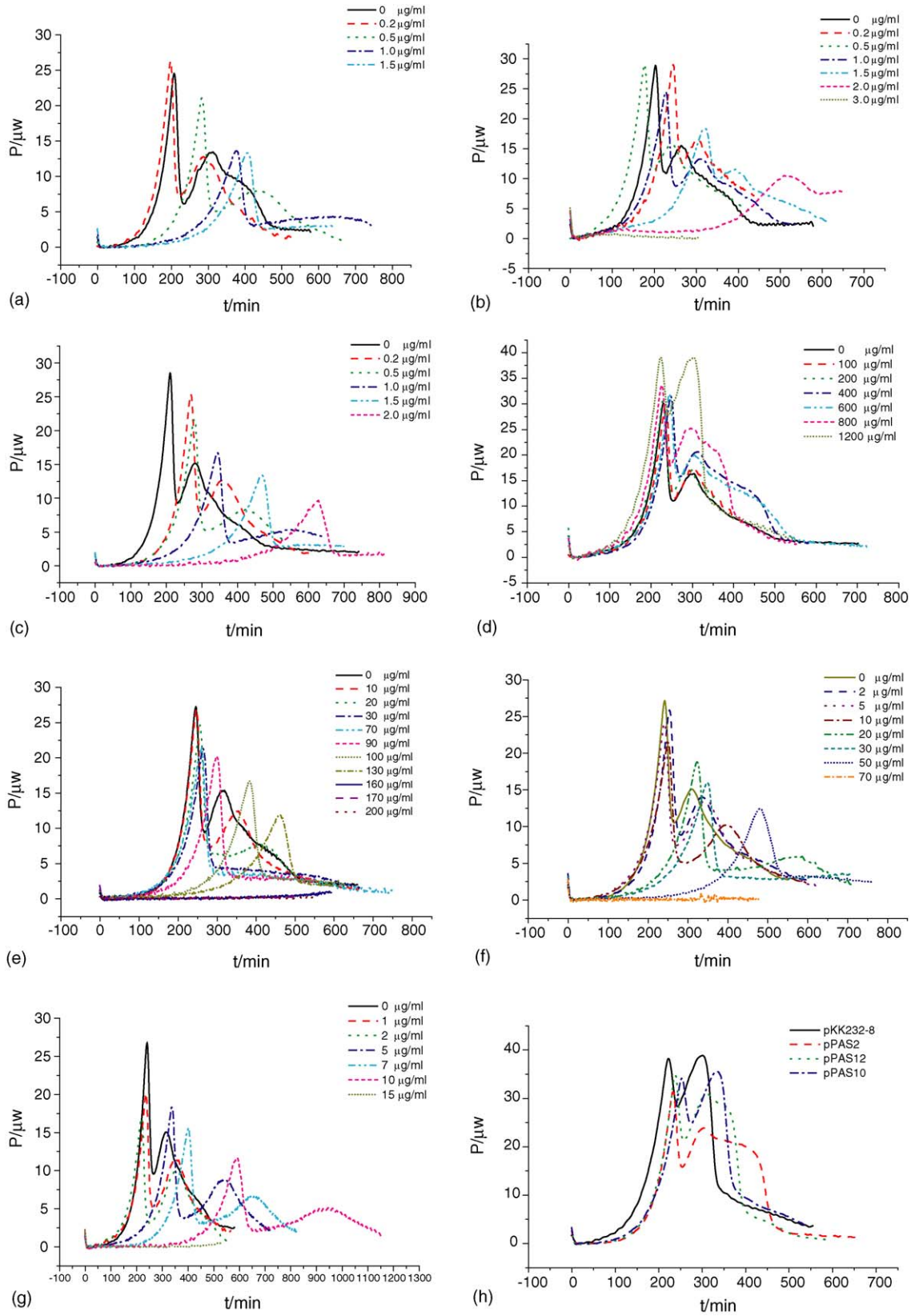

Fig. 1. The metabolic power–time curves obtained from the microcalorimetric method of *E. coli* transformants growing in LB medium containing various concentrations of chloramphenicol or ampicillin. (a) *E. coli* HB101 + Cm (C_{Cm} is 0, 0.2, 0.5, 1.0 and 1.5 μg ml^{-1}); (b) *E. coli* HB101 + Amp (C_{Cm} is 0, 0.2, 0.5, 1.0, 1.5, 2.0 and 3.0 μg ml^{-1}); (c) *E. coli* HB101/pKK232-8 + Cm (C_{Cm} is 0, 0.2, 0.5, 1.0, 1.5 and 2.0 μg ml^{-1}); (d) *E. coli* HB101/pKK232-8 + Amp (C_{Amp} is 0, 100, 200, 400, 600, 800 and 1200 μg ml^{-1}); (e) *E. coli* HB101/pPAS2 + Cm (C_{Cm} is 0, 2, 10, 20, 30, 50, 70, 90, 100, 130, 160, 170 and 200 μg ml^{-1}); (f) *E. coli* HB101/pPAS12 + Cm (C_{Cm} is 0, 2, 5, 10, 20, 30, 50 and 70 μg ml^{-1}); (g) *E. coli* HB101/pPAS10 + Cm (C_{Cm} is 0, 1, 2, 5, 7, 10, and 15 μg ml^{-1}); (h) *E. coli* HB101/pKK232-8, *E. coli* HB101/pPAS2, pPAS12, pPAS10 + Amp (1200 μg ml^{-1}).

Table 2
The calorimetric results on the growth of *E. coli* in LB containing antibiotics

System	Concentration (mg/l)	k (min^{-1})	R	P_m (μw)	t_p (min)	I/%	IC_{50} (μg/ml)
HB101 + Cm	0	0.0292	0.999	24.5	210	0	1.53
	0.2	0.0288	0.999	26.2	200	1.37	
	0.5	0.0235	0.999	21.1	285	19.52	
	1.0	0.0151	0.999	13.6	378	48.29	
	1.5	0.0152	0.999	13.3	409	47.95	
	2.0	0.00743	0.958	–	–	74.55	
HB101 + Amp	0	0.0293	0.999	28.9	204	0	1.82
	0.2	0.0239	0.999	29.0	246	18.43	
	0.5	0.0250	0.999	29.1	178	14.68	
	1.0	0.0231	0.999	24.4	229	21.16	
	1.5	0.0185	0.999	18.4	321	36.86	
	2.0	0.0122	0.998	10.5	510	58.36	
	3.0	0	0			100	
HB101/pkk232-8 + Cm	0	0.0296	0.999	28.5	212	0	1.34
	0.2	0.0268	0.999	25.3	271	9.46	
	0.5	0.0229	0.999	21.7	280	22.64	
	1.0	0.0173	0.999	16.7	346	41.55	
	1.5	0.0136	0.999	13.4	470	54.05	
	2.0	0.0114	0.995	9.6	629	61.49	
HB101/pkk232-8 + Amp	0	0.0293	0.999	30.1	231	0	
	100	0.0274	0.999	30.1	237	6.48	–
	200	0.0270	0.999	30.7	230	7.85	–
	400	0.0262	0.999	31.5	249	10.58	–
	600	0.0244	0.999	31.9	245	16.72	–
	800	0.0250	0.999	33.7	227	14.68	–
	1200	0.0233	0.999	39.1	225	20.48	–
HB101/pPAS2 + Cm	0	0.0296	0.999	27.2	247	0	122.2
	10	0.0288	0.999	26.8	248	2.70	
	20	0.0259	0.999	24.6	257	12.50	
	30	0.0222	0.999	21.3	266.	25.00	
	70	0.0214	0.999	21.4	262	27.7	
	90	0.0216	0.999	20.2	301	27.03	
	100	0.0186	0.999	16.7	384	37.16	
	130	0.0134	0.999	11.8	459	54.73	
	160	0.00874	0.932	–	–	70.47	
	170	0.00631	0.673	–	–	78.68	
	200	0	0	–	–	100	
HB101/pPAS2 + Amp	1200	0.0310	0.999	31.3	235	−4.73	–
HB101/pPAS12 + Cm	0	0.0288	0.999	27.1	243	0	49.7
	2	0.0253	0.999	25.8	255	12.15	
	5	0.0235	0.999	24.0	243	18.4	
	10	0.0212	0.999	21.4	252	26.39	
	20	0.0184	0.999	18.8	324	36.11	
	30	0.0162	0.999	16.0	349	43.75	
	50	0.0142	0.999	12.4	483	50.69	
	70	0	–	–	–	100	
HB101/pPAS12 + Amp	1200	0.0297	0.999	34.8	240	−16.02	–
HB101/pPAS10 + Cm	0	0.0279	0.999	26.7	243	0	10.0
	1	0.0246	0.999	20.2	238	11.83	
	2	0.0238	0.999	16.9	221	14.7	
	5	0.0180	0.999	18.3	339	35.48	
	7	0.0184	0.999	15.6	404	34.05	
	10	0.014	0.998	11.7	594	49.82	
	15	0.00998	0.777	–	–	64.23	
HB101/pPAS10 + Amp	1200	0.0229	0.999	34.1	255	17.92	–

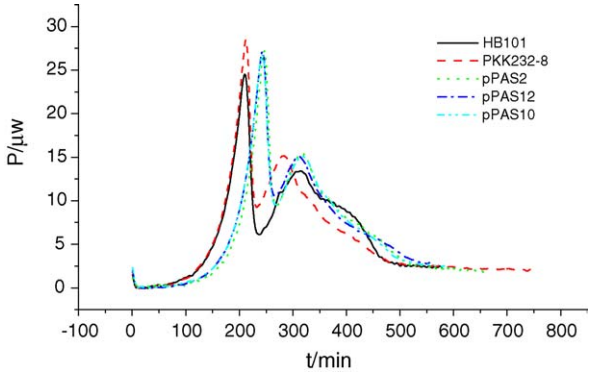

Fig. 2. The controls of the different strains, *E. coli* HB101, *E. coli* HB101/pKK232-8, *E. coli* HB101/pKK232-8/pPAS2 pPAS12 and pPAS10.

heat power of logarithmic growth phase (P_m) decreased rapidly with increasing chloramphenicol concentration and the corresponding time (t_P) became longer. The IC_{50} is 1.82 μg/ml for ampicillin, and 1.53 μg/ml for chloramphenicol. Therefore the gene type of *E. coli* HB101 is Amp^s and Cm^s.

E. coli HB101/pKK232-8 carries the plasmid pKK232-8, which contains two resistance genes: ampicillin resistance gene (Amp^r) and promoterless chloramphenicol resistance gene (Cm^s). Accordingly, the value of *I* for it is just 20.48% when the concentration of ampicillin reaches 1200 μg/ml. However, with the increasing of chloramphenicol concentration, the growth rate constant (*k*) of *E. coli* HB101/pKK232-8 decreased rapidly. The IC_{50} for chloramphenicol is 1.34 μg/ml. The results showed in Fig. 1c and d and Table 2.

From Fig. 1e–g and Table 2, the three transformants have different resistance to Cm. With increasing antibiotic concentration the metabolic power–time curves were gradually depressed. As shown in Fig. 1h, when the concentration of Amp reached 1200 μg/ml all of these transformants grow very well, indicating that all of them obtained strong ampicillin resistance. The minimal inhibition concentrations (MIC) for the Cm are 200, 70 and 15 μg/ml, respectively, and the IC_{50} are 122.2, 49.7, 10.0 μg/ml, respectively, which indicate that the chloramphenicol resistance (Cm^r) gene in the plasmid pKK232-8 has been expressed in these recombinants at different levels. The strength sequence of these three promoter fragments is pPAS2 > pPAS12 > pPAS10. The result coincides with that from the agar plate method. But the strengths of the promoters obtained by the two methods are different, which were compared in Table 4. The calorimetric method gives more accurate results for the activity of the promoter fragments by using IC_{50} to denote the relative promoter activity.

Table 3
Calorimetric results for the growth of *E. coli* in LB

Strains of *E. coli*	k (min^{-1})	P_m (μW)	t_p (min)	Q_{log} (J)
HB101	0.0292	24.5	210	0.0547
HB101/pKK232-8	0.0296	28.5	212	0.0630
HB101/pPAS2	0.0296	27.2	247	0.0626
HB101/pPAS12	0.0288	27.1	243	0.0643
HB101/pPAS10	0.0279	26.7	243	0.0634

Table 4
Comparison of the results obtained from the agar plate method and calorimetric method

Strains of *E. coli*	Resistance level (Cm) by agar plate method (μg/ml)	Resistance level (Cm) (MIC) by calorimetric method (μg/ml)	IC_{50} for calorimetric method (μg/ml)
HB101	<5	2–3	1.53
HB101/pKK232-8	<5	2–3	1.34
HB101/pPAS2	900	200	122.7
HB101/pPAS12	500	70	49.7
HB101/pPAS10	40	15	10.0

4. Conclusions

The results show that the agar plate method and the calorimetric method give the same strength sequence for the three promoter fragments. The different results on the resistance level by the two methods are caused by the different growth conditions. By using IC_{50} to represent the promoter activity, more accurate results for the activity of the promoter fragments were obtained. The present work shows that the calorimetric method can be conveniently used to evaluate the promoter activity of promoter fragments, with very good reproducibility and sensitivity. The growth rate constant (*k*) of the log phase can be obtained in just a few hours, which makes the highthrough screening of the promoter possible.

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