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MICROCALORIMETRIC STUDIES ON THE PROMOTER FUNCTION IN *E. COLI* TG_1 FROM *P. MALTOPHILIA* AT18 CHROMOSOME DNA

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Abstract

In this paper, a promoter-probe plasmid pKK232-8 was used as a vector, which functioned in *Escherichia coli* TG_1 host. The plasmid DNA fragments from *Pseudomonas maltophilia* AT18 chromosome DNA active as promoter in *Escherichia coli* TG_1, the promoter function was studied by means of microcalorimetry, the promoter is about 800 bp DNA, it can promote the chloramphenicol (Cm) gene in plasmid pKK232-8, the Cm resistance level is about 80 μg mL^{-1}, the promoter activity is high. It implicates that there are probably many promoters in *Pseudomonas maltophilia* AT18 chromosome. All these information is readily obtained by an LKB 2277-204 heat conduction microcalorimeter. Microcalorimetry is a quantitative, inexpensive, and versatile method for microbiological genetic research.

Keywords: *Escherichia coli* TG_1, microcalorimetry, plasmid pKK232-8, promoter function, *Pseudomonas maltophilia* AT18

Introduction

Pseudomonas maltophilia AT18 is a tyrosinase-producing bacterium isolated from soil [1]. It has been shown that tyrosinase is synthesized intracellularly and constitutively in *P. maltophilia* AT18 [2]. We have employed this property to synthesize melanin [3] and L-dopa [4] by means of an immobilized cell technique. We have also studied its characteristics of genetics, physiology and ecology, and its application.

The promoter probe vector pKK232-8, contained a promoterless chloramphenicol acetyltransferase (CAT) gene, was used to screen for promoter fragments from *Pseudomonas maltophilia* AT18 and determine the activity of the promoter by the resistance to chloramphenicol (Cm).

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The promoter function in *Escherichia coli* TG_1 from *Pseudomonas maltophilia* AT18 chromosome DNA was studied by means of microcalorimetry. It will provide information and basis for new gene expression systems. It is the first report on studying gene expression by the microcalorimetric technique and gives an important idea on other gene expression research.

Experimental

Materials and methods

The bacterial strains, *E. coli* TG_1, *E. coli* TG_1/pKK232-8 and *E. coli* TG_1/pPAS, used in this study are collected and preserved by the Microbiological Genetics Laboratory of Wuhan University. Plasmid pKK232-8 was used as a promoter-probe vector. LB medium, consisting of NaCl 10 g, tryptone 10 g, bactoyeast extract 5 g per liter, pH 7.2, was sterilized by autoclaving for 20 min at 120°C.

Calorimeter

LKB 2277 Bioactivity Monitor, a type of heat conduction microcalorimeter, was used to determine the metabolism of cells. It is designed to monitor continuously a wide variety of processes and complex systems over the temperature range of 20 to 80°C. There are three operating modes for LKB2277 Bioactivity Monitor: ampoule mode, flow-through mode (including stopped-flow mode) and flow-mix mode. The performance of this instrument and the details of its construction have been described previously [5].

Cloning of promoter-function fragments from chromosome DNA of Pseudomonas maltophilia AT18

The plasmid pKK232-8 is a very useful promoter-probe vector [6]. There are two resistance genes on this plasmid, ampicillin resistance gene (amp^r) and chloramphenicol resistance gene (cm^r), and the sequence of the promoter required for the initiation of transcript of a cm^r gene has been deleted and replaced by a polylinker-site. Chloramphenicol resistance can be regained when a fragment promoter activity is inserted in the correct polarity into this site.

The recombinant transformants having a promoter active fragment, with the site of 800 bp DNA, can be selected directly on plates having Amp and Cm, the recombined plasmid is called pPAS. There is a direct relationship between chloramphenicol resistance levels and promoter activity.

The cloning of a promoter-active fragment from *Pseudomonas maltophilia* AT18 chromosome DNA into *Escherichia coli* TG_1 has been described previously [7].

E. coli TG_1/pKK232-8 having the plasmid pKK232-8, and *E. coli* TG_1/pPAS having the recombined plasmid pPAS.

Preparation of the sample

In this type of experiment, the solution of antibiotics was prepared in sterilized distilled water and prepared freshly every time. In the beginning of the experiment *E. coli* was inoculated in the prepared LB culture medium, initially having $1 \cdot 10^6$ cells mL^{-1}, and the cells used were suspended in the LB culture medium, then the fresh antibiotics solution was added into the cell suspension.

Calorimetric experiment procedure

The flow-cell was cleaned and sterilized as follows: (1) sterilized distilled water was pumped through the system for 30 min at a flow rate of 40 mL h^{-1}; (2) a 0.1 mol L^{-1} solution of HCl was pumped through the system for 30 min at a flow rate of 40 mL h^{-1}; (3) a 75% alcohol solution was pumped through the system for 30 min at a flow rate of 25 mL h^{-1}; (4) a solution of 0.1 mol L^{-1} NaOH was pumped through the system for 30 min at a flow rate of 40 mL h^{-1}; (5) sterilized distilled water was again pumped through the system for 30 min at a flow rate of 40 mL h^{-1}.

Once the system was cleaned and sterilized, sterilized distilled water was pumped through the system at a flow rate of 10 mL h^{-1} to run the baseline. After a stable baseline had been obtained, the cell suspension, having *E. coli* and antibiotics, was pumped into the flow-cell (0.6 mL) by the aid of an LKB-2132 pump at a flow rate of 50 mL h^{-1}. When the flow cell was full, the pump was stopped, and the monitor recorded the thermogenic curves of the growth of *E. coli* continuously at 37°C.

When the pen of the chart recorder returned to the baseline and stabilized, *E. coli* growth ended. The thermogenic curves of *E. coli* TG_1, *E. coli* TG_1/pKK232-8 and *E. coli* TG_1/pPAS growth at 37°C have very good reproducibility and correlationship. If necessary, further calibration should be carried out after a stable baseline has been obtained.

Results

Calculation of the growth rate constant of E. coli

In the log phase of growth, the cell growth is exponential [5]. If the cell number is n_0 at time 0, and n_t at time t, then

$$n_t = n_0 \exp(kt) \tag{1}$$

k is the growth rate constant. If the power output of each cell is w, then

$$n_t w = n_0 w \exp(kt) \tag{2}$$

$P_0 = n_0 w$ and $P_t = n_t w$, giving

$$P_t = P_0 \exp(kt) \text{ or } \ln P_t = \ln P_0 + kt \tag{3}$$

The growth thermogenic curves of the log phase correspond to Eq. (3). So, making use of the data $\ln P_t$ and t taken from the curves to fit a linear equation, one can ob-

tain the growth rate constant (k). The rate constants (k) are shown in Table 1. All of the experimental results have a very good reproducibility and correlationship.

Table 1 Data of the experimental results

E. coli	Antibiotics	C/ μg mL^{-1}	k/ min^{-1}	P_m/ μW	t_p/ min	I/%	IC_{50}/ μg mL^{-1}
TG_1	–	0	0.03932	45.0	175	–	
	Amp	0.5	0.03630	46.5	160	7.7	9.2
		2.0	0.03158	45.0	190	19.7	
		3.0	0.02541	31.5	210	32.8	
		5.0	0.02318	8.0	175	41.0	
		12.0	0.01717	3.5	145	56.3	
	Cm	0.2	0.03863	43.0	160	1.8	1.04
		0.5	0.03075	38.0	198	21.8	
		1.0	0.02045	22.0	285	48.0	
		2.0	0.01131	10.0	430	71.2	
TG_1/ pKK232-8	–	0	0.03269	39.0	182	–	
	Amp	10	0.03683	43.5	150	–1.1	–
		60	0.03001	45.0	155	8.2	
		100	0.02988	45.0	175	8.6	
		400	0.03043	47.0	191	6.9	
		700	0.03208	46.5	178	1.9	
		1000	0.02836	49.5	188	13.2	
		1500	0.03302	47.5	160	–1.0	
	Cm	0.2	0.02927	40.5	162	10.5	0.80
		0.5	0.02502	38.0	164	23.5	
		1.0	0.01565	29.0	270	52.1	
		1.5	0.01490	18.2	312	54.4	
TG_1/pPAS	–	0	0.04505	32.0	141	–	
	Amp	100	0.03806	35.0	160	15.5	–
		200	0.03601	34.5	145	20.1	
		400	0.03680	35.0	150	18.3	
		800	0.03847	34.0	140	14.6	
		1200	0.03958	34.5	145	12.1	
	Cm	2.5	0.03280	29.5	175	27.2	15.0
		5.0	0.02797	24.5	200	37.9	
		10.0	0.02401	18.5	210	46.7	
		20.0	0.02203	17.0	245	51.1	
		40.0	0.01434	14.0	320	68.2	
		80.0	0.01151	5.8	530	74.5	
		120.0	0	–	–	100	

Inhibitory ratio and half inhibitory concentration

High concentrations of antibiotics will inhibit *E. coli* growth, and the growth rate constant will decrease. So, the inhibitory ratio (I) can be defined as:

$$I=[(k_0-k_C)/k_0]100\% \quad (4)$$

where k_0 is the rate constant of the control, and k_c is the rate constant for *E. coli* growth inhibited by an antibiotics with a concentration of *C*.

When the inhibitory ratio (*I*) is 50%, the corresponding half-inhibitory concentration of the antibiotics can be represented as IC_{50}, which can be regarded as the inhibiting concentration causing a 50% decrease of the *E. coli* growth rate constant. Data for *I* are shown in Table 1.

The growth of E. coli TG1

E. coli TG_1 is a plasmid-free strain. As shown in Table 1, when the concentration of ampicillin and chloramphenicol increased, the growth rate constant of *E. coli* TG_1 decreased, and the maximum heat power of log growth phase, P_m, decreased. *E. coli* TG_1 is sensitive to ampicillin (amp) and chloramphenicol (cm), IC_{50} for ampicillin is 9.2 μg mL^{-1} and for chloramphenicol, IC_{50} is 1.04 μg mL^{-1}, that is to say *E. coli* TG_1 is amp^s and cm^s. Because *E. coli* TG_1 is host cell, it does not carry ampicillin resistance gene and chloramphenicol resistance gene, so *E. coli* TG_1 is sensitive to ampicillin and chloramphenicol.

The growth of E. coli TG1/pKK232-8

E. coli TG_1/pKK232-8 having the plasmid pKK232-8, there are two resistance genes on this plasmid, ampicillin resistance gene (amp^r) and chloramphenicol resistance gene (cm^r), and the sequence of the promoter required for the initiation of transcript of a cm^r gene has been deleted and replaced by a polylinker-site. So, *E. coli* TG_1/pKK232-8 is amp^r and cm^s. As shown in Table 1, results indicate that *E. coli* TG_1/pKK232-8 is resistant to ampicillin, even when ampicillin reaches 1500 μg mL^{-1}, *E. coli* TG_1/pKK232-8 still grows very well, but IC_{50} for chloramphenicol is 0.8 μg mL^{-1}, it is still sensitive to chloramphenicol, and the resistant level to chloramphenicol has not been raised. Because, the sequence of the promoter required for the initiation of transcript of a cm^r gene has been deleted and replaced by a polylinker-site, the cm^r gene does not expressed.

The growth of E. coli TG1/pPAS

E. coli TG_1/pPAS carries the recombinant plasmid pPAS, a promoter-active fragment has been inserted in the correct polarity into the polylinker-site of plasmid pKK232-8 [7]. The experimental results, as shown in Table 1, indicate that *E. coli* TG_1/pPAS is not only resistant to ampicillin, but also resistant to chloramphenicol. So, *E. coli* TG_1/pPAS is amp^r and cm^r. When the concentration of ampicillin reaches 1200 μg mL^{-1}, and chloramphenicol reaches 80 μg mL^{-1}, *E. coli* TG_1/pPAS can still grow. C_{50} for chloramphenicol is 15 μg mL^{-1}. But when the concentration of chloramphenicol reaches 120 μg mL^{-1}, *E. coli* TG_1/pPAS cannot grow. The experimental results show that the resistant level to chloramphenicol for *E. coli* TG_1/pPAS

has been raised, the cm^r gene in the plasmid pKK232-8 has expressed. Because the promoter-active DNA fragment, coming from *Pseudomonas maltophilia* AT18 chromosome DNA, has been inserted in the correct polarity into the polylinker-site of plasmid pKK232-8, the cm^r gene has expressed, so the resistant level to chloramphenicol for *E. coli* TG_1/pPAS has been raised. The experimental results show that the 800 bp DNA from *Pseudomonas maltophilia* AT18 chromosome can promote the cm^r gene expression, it has the gene promoter function in *Escherichia coli* TG_1.

The relationship between P_m and C

When the concentration of chloramphenicol increased, the growth of *E. coli* was inhibited, the maximum heat power (P_m) decreased. If we make a linear regression of P_m *vs*. *C*, we can obtain the linear relationship between P_m and *C*.

TG_1: P_m=45.55–18.7*C*, *R*= –0.9766 (0.2–2.0 μg mL^{-1})

TG_1/pKK232-8: P_m=45.33–17.5*C*, *R*= –0.9903 (0.2–2.0 μg mL^{-1})

TG_1/pPAS: P_m=32.20–1.38*C*, *R*= –0.9942 (0–10 μg mL^{-1})

P_m=20.67–0.18*C*, *R*= –0.9969 (10–80 μg mL^{-1})

The relationship between t_p and C

When the growth of *E. coli* was inhibited, the peak time t_p of log growth phase increased. We can also make a linear regression of t_p *vs*. *C*, and obtain the linear relationship between t_p and *C*.

TG_1: t_p=127.8+151.8*C*, *R*=0.9992 (0.2–2.0 μg mL^{-1})

TG_1/pKK232-8: t_p=124.1+128.6*C*, *R*=0.9686 (0.2–2.0 μg mL^{-1})

TG_1/pPAS: t_p=157.9+4.54*C*, *R*=0.9942 (0–80 μg mL^{-1})

Discussion

Transcription is a multiple reaction occurring at promoter area on the template DNA strands and is the first step of gene expression is also a key step, in eukaryotes and prokaryotes (including eubacteria and archaebacteria). The studies on promoter and structure, function, control of RNA polymerase are developing in depth, especially on eubacteria [8]. Our experiments show that the 800 bp DNA fragment from chromosome of *Pseudomonas maltophilia* AT18 has promoter activity in *E. coli* TG_1.

From the data in Table 1, it shows that promoter-active DNA fragment, coming from *Pseudomonas maltophilia* AT18 chromosome, has been inserted in the correct polarity into the polylinker-site of plasmid pKK232-8, and the cm^r gene has been expressed. All experimental results indicate that good results can be obtained by the ap-

plication of microcalorimetric method to microbiological genetics research. And it gives reliable proof for further development of microcalorimetric research of microbiological genetics.

The thermogenic power curves of bacterial metabolism completely describe growth metabolism processes. Under the same conditions, the thermogenic curves of every kind of bacterium all have good reproducibility and outstanding characteristics. When experimental factors were changed, the metabolic process would be influenced, the changes of bacterial metabolic thermogenic curves were also remarkable. Microcalorimetry has been useful in measuring the effects of various substances and culture conditions on the metabolism of the organism [5]. Microcalorimetric studies of bacterial growth reveal temporary details not observable by other techniques. Microcalorimetry can represent growth and metabolic characteristics and reflect variance of physiological and biochemical characteristics.

The microcalorimetric method is efficient for culture collections, bioactivity screening, taxonomy and optimal media components. Now, the detector system is very sensitive, the detection limit is 0.15 μW and baseline stability (over a period of 24 h) is 0.2 μW. Enough accuracy can be obtained only by needing a milligram or a microgram of the sample. It was difficult to do that in the past. The microcalorimetric technique can give and only thermodynamic information, but also a lot of kinetic information of cell growth. The microcalorimetric method has many unique features when it is applied for the detection of biosystems. By monitoring the heat effect with a sufficiently sensitive calorimeter, the metabolic process of living cells can be studied by a direct microcalorimetric method. The calorimetry can directly determine the biological activity of a living system and provide a continuous measurement of heat production, thereby give much information both in qualitative and quantitative ways, and do not disturb the normal activity of biosystems. Although the microcalorimetric method lacks specific property, the research samples have their unique characteristics. So, it can give new results by this method. In some sense, the microcalorimetric method does not only provide thermochemical data, but it has also become a new promising research tool. We believe that microcalorimetry is a useful tool for studying microbiological genetics because it is accurate, objective, simple to perform, reproducible and broadly applicable.

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