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MICROCALORIMETRIC STUDY OF *Bacillus thuringiensis* GROWTH WITH DIFFERENT PLASMID NUMBERS AND VARIOUS PROMOTERS

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A microcalorimetric technique was used to investigate the influence of plasmid copy number and various promoters on the green fluorescent protein expression in *Bacillus thuringiensis*. The power–time curves of the growth metabolism of *B. thuringiensis* have been determined by using an LKB-2277 bioactivity monitor at 28°C. For evaluation of the results, the maximum peak-heat output power (P_{max}), the generation time (t_G), the growth rate constants (k) in the log phase, the total heat effect (Q_{total}) for the growth metabolism of *Bacillus thuringiensis* were determined. The results firstly indicated that the more plasmid copy number per cell the more protein synthesization, and *promoter BtI-BtII* had a stronger impact on the gene expression than promoter *3A* investigated by the method of microcalorimetry.

Keywords: *engineering strain, green fluorescent protein, microcalorimetry*

Introduction

In some living system, the various metabolic events occurring within the cells are producing heat reactions. Therefore, by monitoring the heat effects with sufficient sensitive calorimeters, the metabolic processes of living cells can be studied. Microcalorimetric measurements may be made directly without introducing any chemical and mechanical perturbation into the system, thus not disturbing the 'normal activity' and metabolism of living system. Furthermore, microcalorimetry is a non-invasive and non-destructive manner and allows *in situ* biochemical analysis of the samples under investigation after the curve of a reaction is measured [1, 2]. Miles and Beezer demonstrated that microcalorimetric studies of bacterial growth reveal temporal details not observable by other techniques [3].

Bacillus thuringiensis is a gram-positive soil bacterium and it produces crystal proteins, which are pore-forming toxins. The whole growth cycle begins with spore activation then into the vegetative phase and during the vegetative phase it propagates by binary fission. When the last binary fission is finished it goes into the sporulation phase. According to the mathematic growth model the vegetative phase is divided into three phases: lag phase, exponential phase, stationary phase. In the sporulation phase large quantities of one or more insecticidal proteins are produced and accumulated in the form of parasporal crystals. These proteins make up the main components of *B. thuringiensis* agents that are active against several orders of economically important insects, and other invertebrates including nematodes. It is extensively exploited as a biological pesticide for the control of various *lepidopteran*, *dipteran*, and *coleopteran* insect pests. With its widely use in the environment whether the rudimental bacterium in the environment is safe is raised. In order to determine its biosafety in the environment, we utilize green fluorescent protein (GFP) as a protein marker to investigate its environmental distribution. In this work, we study the expression of green fluorescent protein with different plasmid origins and various promoters by microcalorimetry. Ruan reported that there was a dynamic relationship between expression of gene and heat flow rate [4]. Therefore, it should be a safe, convenient and versatile method for measuring the impact of plasmid copy number and various promoters on the gene expression in *B. thuringiensis*. Heat production in a cell suspension is measured by the thermopile of a LKB 2277 heat conduction microcalorimeter. By analyses of the thermogenic curves, we have studied the impact of plasmid number and various promoters on the GFP expression, and obtained considerable kinetic data.

Experimental

Bacteria strains

The bacteria strains that had different plasmid numbers per cell were BMB304GFP and BMB315GFP. The

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strains that had various promoters were BMB1206RB and BMB1206RA. All of the strains used in this work were constructed and preserved by our Lab.

Growth medium

LB medium consists of 0.5% NaCl, 1% bactotryptone, 0.5% Bactoyeast-extract, pH=7.2. The medium was sterilized by autoclaving for 20 min at 0.1 MPa.

Instrument

An LKB-2277 bioactivity monitor (Thermometric, Jarfalla, Sweden) was used. The operation of this instrument and the details of its construction have been described previously [5]. According to the bacterium growth all measurements were made at 28°C.

Microcalorimetric measurements

The metabolic thermogenic curves of *B. thuringiensis* were determined using the ampoule method. When the system was cleaned and sterilized and the baseline had been stabilized, solutions of *B. thuringiensis* were put into stainless steel ampoule, containing 5 mL LB growth medium. Hook the lifter on the ampoules, and then lower them to the thermal equilibration position [6]. The ampoules were kept at this position for 20 min for pre-heating. Then the ampoules were lowered to the measurement position. Meanwhile, the LKB-2210 recorder recorded the thermogenic curves of *B. thuringiensis* growth continuously.

Results and discussion

The heat flow rate change of recombinants BMB304GFP BMB315GFP which had different plasmid copy numbers in LB medium

There are two stages in the growth process of *B. thuringiensis*: the vegetative period and the spore-forming period. As previously described, the growth process of *B. thuringiensis* is matched with the thermogenic growth curve. In the power-time curves, the first peak represents the vegetative growth and the second is for the spore-forming [7].

In the log phase of growth, if the cell number is n_0 at time 0, and n_t at time t, then

$$n_t=n_0\exp(kt) \quad (1)$$

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

$$n_tP_w=n_0P_w\exp(kt) \quad (2)$$

If the heat output power is P_0 at time 0, and P_t at time t, then

$$P_t=P_0\exp(kt)$$

or

$$\ln P_t=\ln P_0+kt \quad (3)$$

The calorimetric curves of the exponential phase correspond to Eq. (3). So, making use of the data $\ln P_t$ and t from the curves, we can obtain the thermokinetic parameters.

The average copy number of BMB304GFP and BMB315GFP is 4 and 15 per cell respectively. To discover whether the expression of green fluorescence protein is dependent on the plasmid copy number, we investigated the strains' growth thermogenic curves. The growth thermogenic curves of *B. thuringiensis* BMB304GFP and BMB315GFP in LB medium at 28°C are shown in Fig. 1.

The thermokinetic parameters of these strains are shown in Table 1. All of the experimental results have a very good reproducibility and consistency. Comparing their generation time (t_G), we can clearly know that BMB304GFP grows faster than BMB315GFP in the log phase. That is to say the plasmid number has a significant effect on the log phase growth. The less plasmid number per cell the faster grows. From the table we know the linearity of Eq. (3) for BMB315GFP is poorer than that for BMB304GFP. We have repeated the experiment at least for three times the results are the same. What's more there is no other difference between the two strains besides the plasmid number per cell so we concluded the difference should be caused by the plasmid.

From data in Table 1, we also observed that the total heat flow rate Q_{total} of strain BMB304GFP was higher than that of BMB315GFP. We can know from the data of this work the more plasmid number, the

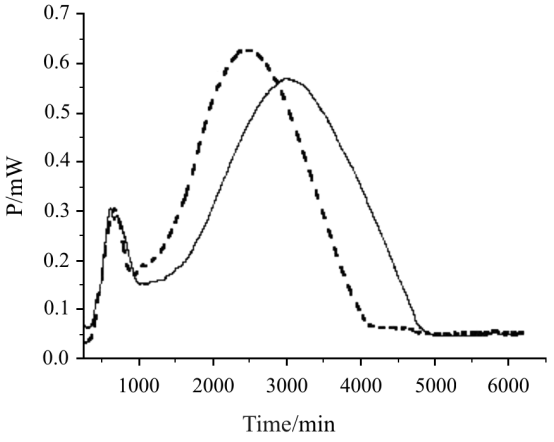

Fig. 1 Power-time curves of *Bacillus thuringiensis* with different plasmid copy number; — – BMB304GFP and - - - – BMB315GFP

Table 1 Thermokinetic parameters of *Bacillus thuringiensis* with different copy number

Strains	log phase					Sporulation phase		Q_{total}/J	Number of plasmid copy
	k/min^{-1}	t_G/min	R	P_{max}/mW	t_{max}/min	P_{max}/mW	t_{max}/min		
BMB304GFP	0.0131	52.91	0.9997	0.2766	728	0.5755	2333	83.617	4
BMB315GFP	0.0119	58.25	0.996	0.2503	718	0.575	2515	81.466	15

less heat output. It indicated that the synthesis of the plasmid during the growth also needs energy. As first reported the gene expression consumed energy and it resulted the more heat output and the less protein synthesis [4]. Increasing the plasmid copy number per cell can enhance the protein yield was also identifed by biological method [8].

The heat flow rate of recombinant strain BMB1206RB and BMB1206RA which had various promoter

We also investigated the gene expression with various promoter by microcalorimetry. The thermogenic curves of BMB1206RA and BMB1206RB were shown in Fig. 2. The thermokinetic parameters of *B. thuringiensis* with various promoter were listed in Table 2. All of the biological characteristics of BMB1206RA are the same to that of BMB1206RB except the promoter of the green fluorescent protein gene. As we have known that the *promoterBtI-BtII* only drives the gene expression in the sporulation phase while the *promoter3A* can drive the gene expression both in the log phase and sporulation phase [9]. But it is unclear which promoter has a stronger impact on gene expression.

The data in Table 2 shows the heat output of strain with *promoterBtI-BtII* is less than that of the strain with *promoter3A*. It suggests the two promoters have different drive impact on the gene expression. According to the rule of the more protein synthetization the more energy consumed and the less heat output, it indicated the *promoterBtI-BtII* had stronger impact on the gene expression than that of *promoter3A*. So we can deduce that although the *promoter3A* has a longer time to drive the gene expression than *promoterBtI-BtII*, its drive effect is lower than *promoterBtI-BtII* .

According to their generation time in the log phase there is a significant difference. The t_G of BMB1206RA is longer than that of BMB1205RB. As firstly reported that the *promoter3A* can drive the gene expression in the log phase so we can deduce that the protein expression in the log phase delayed the strain's growth.

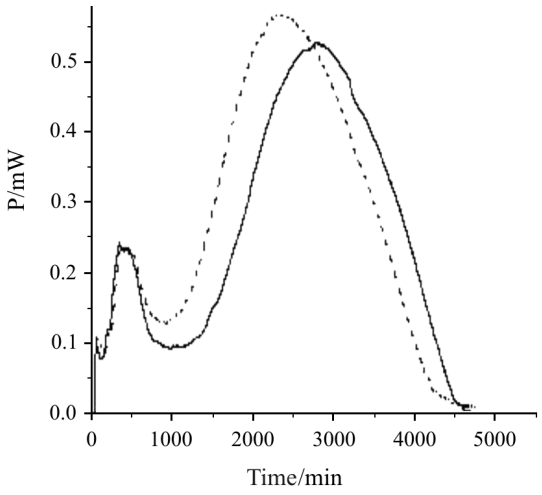

Fig. 2 Power-time curves of *Bacillus thuringiensis* with various promoters; — –BMB1206RBG and - - - –BMB1206RAG

Conclusions

In this research, we firstly investigated the effect of plasmid copy number and promoter on the green fluorescent protein expression in *B. thuringiensis* by microcalorimetry. The results directly confirm that the plasmid copy number is a direct proportion to the protein yield. The more plasmid copy number the more protein yield. What's more the more plasmid number has an effect on the strains growth in the log phase. From the thermogenic data, we also deduce that the driving impact of *promoterBtI-BtII* is stronger than that of *promoter3A*. Therefore, the microcalorimetric technique can examine the plasmid copy number and promoter's effect on the protein expression and the strains' growth. The microcalorimetric techniques are more direct and convenient than biological methods.

Table 2 Thermokinetic parameters of *Bacillus thuringiensis* with various promoters

Strains	log phase					Sporulation phase		Q_{total}/J	Promoter
	k/min^{-1}	t_G/min	R	P_{max}/mW	t_{max}/min	P_{max}/mW	t_{max}/min		
BMB1206RB	0.0185	37.42	0.9913	0.233	413	0.5282	2809	89.1419	*proBtI-BtII*
BMB1206RA	0.0164	42.39	0.9954	0.2243	413	0.5571	2372	95.3596	*pro3A*

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