Journal of Thermal Analysis and Calorimetry, Vol. 79 (2005) 29–32

MICROCALORIMETRIC INVESTIGATION OF THE GROWTH OF THE ENGINEERING *BACILLUS THURINGIENSIS* WITH DIFFERENT PLASMID NUMBERS AND VARIOUS PROMOTERS

L. Xiaoyan[1], *L. Yi*[2], *S. Ming*[1], *L. Peng*[2], *Z. Juncheng*[2], *L. Lin*[1], *Q. Songsheng*[2] *and Y. Ziniu*[1*]

[1]State Key Laboratory of Agricultural Microbiology of China, Huazhong Agricultural University, Wuhan 430070, P. R. China
[2]Department of Chemistry, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, P. R. China

A microcalorimetric technique based on the bacterial heat output was applied to evaluate the influence of plasmid copy number and various promoters on the green fluorescent protein expression in *Bacillus thuringiensis*. The thermogenic curves of the aerobic metabolism of *B. thuringiensis* strains were determined by using an LKB-2277 bioactivity monitor at 28°C. The analysis of the thermogenic curves indicated for the first time that the more plasmid copy number per cell the more protein synthetization. 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. The thermogenic curve of growing cells could be plotted out by continuous measurements of the heat effects of the growing cells with a calorimeter. The obtained thermogenic curve could reflect time-dependent changes in growth pattern of the cells tested. 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]. Now it has been widely used in the biological field because its high sensitivity and quite informative in both qualitative and quantitative aspects [4].

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. 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 [5]. 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*.

Experimental

Bacteria strains

The bacteria strains that had different plasmid number per cell were BMB304GFP and BMB315GFP. The strains that had various promoters were BMB1206RB and BMB1206RA. All of the strains used in this work were constructed and preserved by our Lab [6].

Growth medium

LB (Luria–Bertani) 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.

* Author for correspondence: yz41@public.wh.hb.cn

1388–6150/$20.00

Akadémiai Kiadó, Budapest, Hungary
Springer, Dordrecht, The Netherlands

Instrument

An LKB-2277 Bioactivity monitor (Thermometric, Jarfalla, Sweden) was used. The best proliferation temperature of the strain is 28°C, so the microcalorimeter was thermostated at 28°C. The voltage signal was recorded by means of an LKB-2210 recorder (1000 mV range). The baseline stability was 0.2 μW/24 h. The operation of this instrument and the details of its construction have been described previously [7].

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 [8]. 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 [9].

Results and discussion

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

The average copy number of BMB304GFP and BMB315 is 4 and 15 per cell respectively. To discover whether the expression of green fluorescence protein was 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. 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, the first peak represents the vegetative growth and the second is for the spore-forming [10].

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

$$n_t P_w = n_0 P_w \exp(kt) \quad (2)$$

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

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

If the generation time of log phase is t_G, then

$$t_G = \ln 2 / k \quad (4)$$

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.

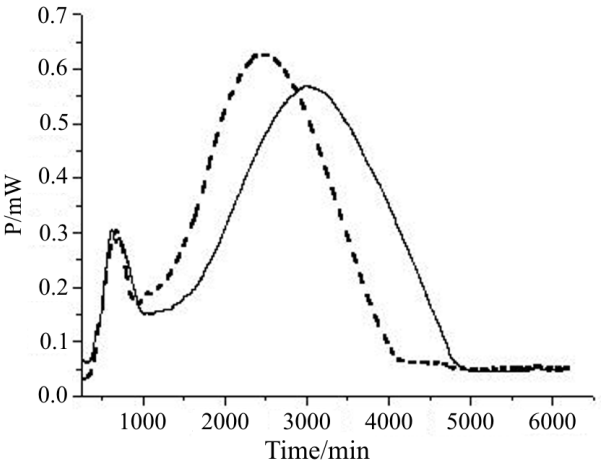

Fig. 1 Power-time curves of *Bacillus thuringiensis* with different plasmid copy number; — – BMB304GFP and - - - – BMB315GFP

The thermokinetic parameters of these strains are shown in Table 1. All of the experimental results had a very good reproducibility and consistency. Comparing their generation time (t_G), we clearly know that BMB304GFP grown faster than BMB315GFP in the log phase, which was also identified by calculating their growth time with biological method. That is, the plasmid number had a significant effect on the log growth phase. The less plasmid numbers per cell the faster it grown.

From data in Table 1, we observed that the total heat flow rate Q_{total} of strain BMB304GFP was higher than that of BMB315GFP. The more plasmid number, the 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 less heat output and the more protein synthesis [5]. Increasing the plasmid copy number per cell can enhance the protein yield was also identifed by other biological method [11].

Comparing the R-value the linearity of equation 3 for BMB315GFP was poorer than that for BMB304GFP. In order to study the reason, we repeated the experiment at least for three times but the

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

Strains	log phase					Sporulation phase		Q_{total}/J	No. of plasmid copy
	k/min	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	0.9960	0.2503	718	0.5750	2515	81.466	15

results were the same. There was no other difference between the two strains besides the plasmid number per cell, so we concluded the difference should be caused by the plasimd.

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

As we have known there are two kinds promoter in *B. thuringiensis*, one is spore-dependent promoter, such as the promoter *BtI-BtII* that only drives the gene expression in the sporulation phase, the other is spore-independent promoter, such as the promoter *3A* that drives the gene expression both in the log phase and sporulation phase [12]. But it is unclear which promoter has a stronger impact on gene expression. In this study, we investigated the drive impact of the two promoters by microcalorimetry but not the traditional protein quantification method.

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.

The data in Table 2 shows the heat output of strain with promoter *BtI-BtII* is less than that of the strain with promoter *3A*. That is, the protein yield of BMB1206RB was more than that of BMB1206RA. It indicated the promoter *BtI-BtII* had stronger impact on the gene expression than promoter *3A*. So we deduced that although the promoter *3A* had a longer time to drive the gene expression than promoter *BtI-BtII*, its drive effect was lower than promoter *BtI-BtII* .

According to their generation time in the log phase there was a significant difference. The t_G of BMB1206RA was longer than that of BMB1205RB. As firstly reported that the promoter *3A* can drive the gene expression in the log phase so it suggested that the protein expression in the log phase delayed the strain's growth.

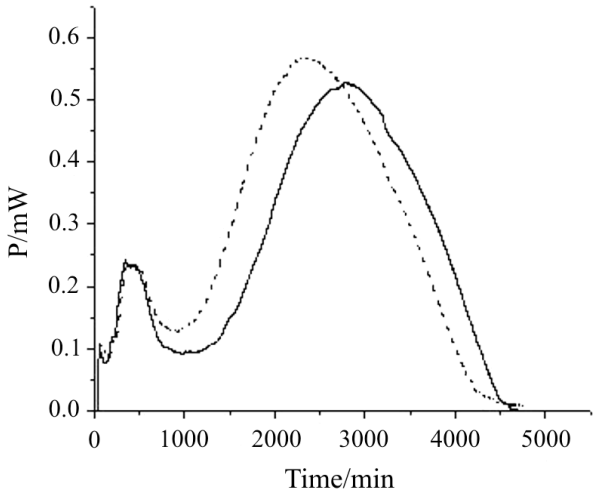

Fig. 2 Power-time curves of *Bacillus thuringiensis* with various promoters; — – BMB1206RGB and - - - – BMB1206RAG

Conclusions

In this study, a microcalorimetric technique based on the bacterial heat output was applied to evaluate the effect of plasmid copy number and various promoters on the green fluorescent protein expression in *B. thuringiensis* for the first time. The results directly confirmed that the plasmid copy number was a direct proportion to the protein yield. The more plasmid copy number the more protein yield. What's more the more plasmid number had an effect on the strains growth in the log phase. With the more plasmid the slower it grows. Analysis of the thermogenic data, we deduced that the driving impact of promoter *BtI-BtII* was stronger than that of promoter *3A*.

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	0.2330	413	0.5282	2809	89.1419	pro *BtI-BtII*
BMB1206RA	0.0164	42.39	0.9954	0.2243	413	0.5571	2372	95.3596	pro *3A*

Acknowledgements

This work was supported in part by the Teaching and Research Award Program for Outstanding Young Professors in High Education Institute, MOE, P. R. China, the National Natural Science Foundation of China (No.20373051, 30170032), Youth 'Chenguang' Scientific Project of Wuhan, the Open Foundation of Key Laboratory of Agro-Microbiology of the Chinese Agricultural Ministry and by National “863” project (No.2001AA212301).

References

1 M. L. H. Gruwel, C. Alves and J. N. Schrader, Am. J. Physiol., 268 (1995) H351.
2 O. Culic, M. L. H. Gruwel and J. Schrader, Am. J. Physiol., 273 (1997) C205.
3 R. J. Miles, A. E. Beezer and D. H. Lee, Microbiol., 45 (1986) 7.
4 Y. Liu, X. Li, S.S. Qu and Shen Ping, J. Biochem. Biophys. Methods, 45 (2000) 231.
5 L. Ruan, Y. Liu, Z. Gao and P. Shen, J. Therm. Anal. Cal., 70 (2002) 521.
6 X. Y. Lin, Huazhong Agriculture University, Wuhan, China: M.S. Thesis (2003) 33.
7 J. Suurkuusk and I. Wadsø. Chem. Scr., 20 (1992) 155.
8 Y. Yang, Y. Liu, J. Zhu and P. Shen, J. Therm. Anal. Cal., 75 (2004) 293.
9 Y. P. Huang, Y. Liu, P. Shen and S. S. Qu, J. Therm. Anal. Cal., 74 (2003) 163.
10 X. Y. Lin, Y. Liu, M. Sun and Z. T. Gao, Acta Chim. Sinica, 59 (2001) 769.
11 M. Itaya, M. Syed, S. Haheduzzaman, K. Matsui, A. Omori and T. Tsuji, Biosci. Biotechnol. Biochem., 65 (2001) 579.
12 H. W. Park, B. X. Ge and L. S. Baner, Appl. Environ. Microbiol., 64 (1998) 3932.

Received: August 8, 2004
In revised form: August 16, 2004