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Microcalorimetric Studies on Influence of Sm^{3+}, Dy^{3+} on Growth and Sporulation of *Bacillus thuringiensis*

ZHAO, RU-Ming[a,b](赵儒铭) Liu, Yi*,[a](刘义) YANG, Chang-Ying[a,b](杨昌英)
XIE, Zhi-Xiong[c](谢志雄) SHEN, Ping[c](沈萍) QU, Song-Sheng[a](屈松生)

[a] *Department of Chemistry, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, China*
[b] *China Three Gorges University, Yichang, Hubei 443002, China*
[c] *College of Life Sciences, Wuhan University, Wuhan, Hubei 430072, China*

By using an LKB-2277 Bioactivity Monitor and cycle-flow method, the thermogenic curves of aerobic growth for *Bacillus thuringiensis cry II* strain at 28 ℃ have been obtained. The metabolic thermogenic curves of *Bt cry II* contain two distinct parts: the first part reflects the changes of bacterial growth phase and the second part corresponds to sporulation phase. From these thermogenic curves in the absence or presence of Sm^{3+}, Dy^{3+} ions, the thermokinetic parameters such as the growth rate constants k, the interval time τ_I, the maximum power $P_{MAX}1$ and heat-output Q_{LOG} for log phase, the maximum power $P_{MAX}2$ and heat-output Q_{STAT} for stationary phase, the heat-output Q_{SPOR} for sporulation phase and total heat effects Q_T were calculated. Sm^{3+} and Dy^{3+} ions have promoting action on the growth of *Bt cry II* in their lower concentration range, on the other hand, they have inhibitory action on the sporulation of *Bt* in their higher concentration range. It has also been found that the effects of Sm^{3+} and Dy^{3+} ions on *Bt* during the sporulation phase were far greater than those during the bacterial growth phase. It was concluded that the application of *Bt* for controlling insecticide could not be affected by the presence of the rare-earth elements in the environmental ecosystem.

Keywords microcalorimetry, Sm^{3+}, Dy^{3+}, thermokinetics

Introduction

Bacillus thuringiensis (Bt) is a gram-positive, spore-forming bacterium, present in soil, water, and on plant surface. It produces characteristic proteins during sporulation which, when are ingested, are highly toxic to susceptible insects.[1] The use of biopesticides based on some *Bt* strains has escalated in recent years due to their advantages over traditional chemical insecticides. Since global population grows rapidly, the environment deteriorates and ecosystem is being damaged, *Bt* microbial pesticides are being rapidly developed and playing a much larger role in pest control.

It was well known that the Chinese mainland abounds in rare-earth resources. In the recent years, the rare-earth compounds in soluble form are dispersed annually over agriculture land as fertilizer components to increase crop yield.[2] In such cases, growing attention has been payed to the rare-earth compounds because of their possible effects on the environment and other microorganisms. It was reported that the contents of the rare earths in aquatic body and the concentration of the rare-earth for spoiling were 10^{-6}—10^{-3} μg/mL and 100—250 μg/mL, respectively. At this moment, we have no information on the metabolism of *Bt* in the presence of the rare earth elements. In order to obtain precise and quantitative information about the action of rare earth elements on the growth and sporulation of *Bt*, we chose the rare-elements, such as samarium (Sm) and dysprosium (Dy), to investigate their effect on the growth and sporulation of *Bt*.

Microcalorimetry can directly determine the "biological activity" of a living system and provide a continuous measurement of the heat production,[3] thereby it has a great advantage over many conventional bioassay procedures.

In this work, we explored the model, *i.e.*, nutrient concentrations are usually much lower in nature than in the usual laboratory media, frequently 100 to 1000 times lower.[4] So, we chose the concentration range being lower than 250 μg/mL. The application of microcalorimetric method for evaluating the effect of different concentration of ions on the metabolic responses under stimulation with Sm^{3+} and Dy^{3+} ions is being reported. The thermogenic curves were measured using an LKB-2277 Bioactivity Monitor. In addition, an *in situ* observation by using Schaeffer-Fulton method for en-

* E-mail: prof.liuyi@263.net; liuyi@chem.whu.edu.cn
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dospore staining and phase microscope was used to follow the process of metabolism of *Bt.* We found that there are obvious differences among their metabolic thermogenic curves and kinetics, on the other hand, there is marked similarity between the thermogenic curves produced by Sm^{3+} and Dy^{3+} ions. Comparing the metabolic thermogenic curves, we obtained a lot of interesting results. All the experimental results are very important and significant to the study of metabolism of *Bt* and the bioeffect of rare-earth element.

Experimental

Equipment

An LKB-2277 BioActivity Monitor (Thermometric AB, Sweden) was used, and the performance of this apparatus and the details of its construction has been described previously.[5]

Materials

All chemicals used, such as Sm_2O_3, Dy_2O_3, NaCl and HNO_3, were of analytical grade. Bactotyptone and bactoyeast-extract were from Sigma. Ampicillin was from Oxoid. All solutions were prepared by doubly distilled water.

Bacillus thuringiensis cry II was kindly provided by the Lab. of *Bacillus thuringiensis* Molecular Biology, State Key Laboratory of Agri-microbiology, Huazhong Agriculture University, Wuhan 430072, China.

Bt was grown on LB medium culture, which consists of 10 g of NaCl, 10 g of bactotyptone, and 5 g of bactoyeast-extract per liter, pH＝7.2—7.4. The medium was sterilized by autoclaving for 30 min at the pressure of 0.1 MPa.

The Sm_2O_3 and Dy_2O_3 were roasted, and weighted, then changed into $Sm(NO_3)_3$ and $Dy(NO_3)_3$ with HNO_3, respectively. They were made into solution with sterile deionized water respectively.

Experimental determination

A schematic representation of the experimental apparatus is shown in Figure 1.[6]

First, the flow cell was completely cleaned and sterilized. Then, 25 mL of liquid LB medium was added to the cycle-flow system (flow rate 20 mL/h). The amplifier of the monitor was set at 1000 μW. The experimental procedures were performed twice.

Staining method

Using Schaeffer-Fulton endospore staining method to stain, for details of the staining method see reference.[7]

Malachite green, the primary stain, was applied to a heat-fixed smear and heated to steaming for about 5 min. The heat helped the stain to penetrate the endospore wall. Then washing was carried out for about 30 s with water to remove the malachite green stain from all of the cell parts except the endospores. Next, safranin, a counterstain, was applied to the smear to stain portions of the cell other than endospore.

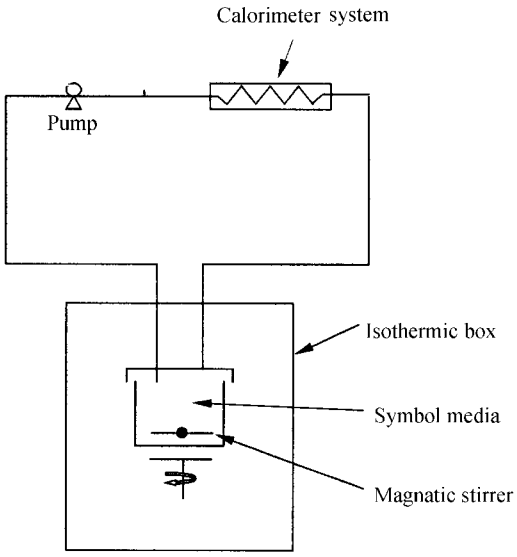

Figure 1 Schematic diagram of apparatus.

Result and discussion

Thermogenic curves of *Bt*

Figure 2 shows the complete thermogenic curve for the metabolism of *Bacillus thuringiensis*. At the same time, parallel studies were carried out in which we used Schaeffer-Fulton endospore staining method and phase microscope to examine the complete process for *Bt*. By comparing the thermogenic curve with those obtained from the parallel studies, we can see that the whole cultivation process can be divided into two parts: growth part (Part ABCDE), sporulation part (Part EFG). The first is the characteristic part of bacterial growth metabolism for *Bt*. Good consistency was obtained in both the experiments. The average time for this phase is about 10 h, commencing from the starting point A in the thermogenic curves. Part EFG corresponds to the sporulation phase for *Bt*. The two phases were separated by the line DE, on which no heat was observed. For investigation, the time corresponding to line DE was defined as the interval time τ_I. In detail, from Figure 2, we see the interval time τ_I is about 4.5 h. From microbiological observation for *Bt*, we can affirm that the second phase could produce a large amount of endospores. However, it is difficult to give a reasonable explanation for the peak in the calorimetric signal during this phase. The growth curve obtained by microcalorimeric method is consistent with that obtained by the plating counting.

Thermogenic curves of *Bt* in the presence of Sm^{3+} and Dy^{3+} ions

In order to investigate the biological effects of Sm^{3+} and Dy^{3+} on *Bt cry II*, we added $Sm(NO_3)_3$ or $Dy(NO_3)_3$ solution into the suspension of *Bacillus thuringiensis*, and then recorded the metabolic thermogenic curves, respectively. The metabolic thermogenic curves

of *Bt* in the presence of $Sm(NO_3)_3$ are shown in Figure 3.

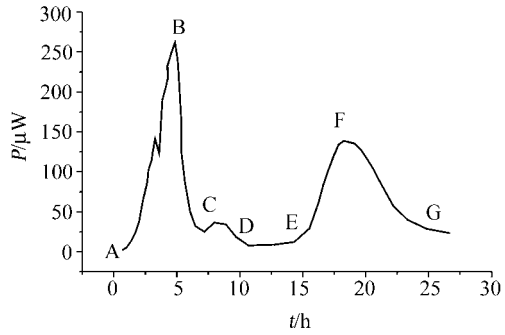

Figure 2 The metabolic thermogenic curve of *Bacillus thuringiensis*.

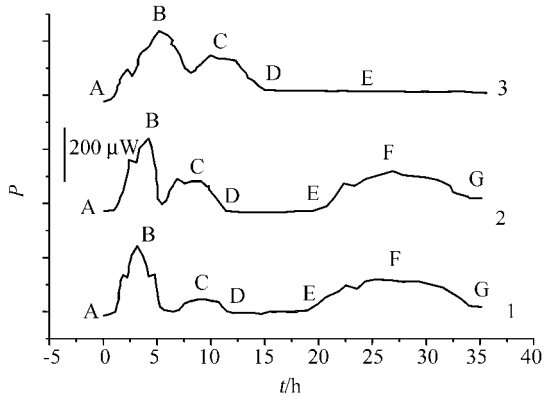

Figure 3 The metabolic thermogenic curves of *Bacillus thuringiensis* affected by Sm^{3+} ion of 50 μg/mL (1), 150 μg/mL (2) and 250 μg/mL (3).

It was found that the shapes of the thermogenic curves with different concentrations of Sm^{3+} (<250 μg/mL) are very similar. Analysis of these curves reveals the two distinct parts: the first one (ABCDE) corresponds to bacterial growth, and the second one (EFG) to sporulation. The average time for the first part ABCDE is longer than about 10 h, commencing from the starting point A in the thermogenic curves. When Sm^{3+} and Dy^{3+} were added, the heat out-put power for the first parts were increased immediately. The heat out-put power was larger than that in the absence of Sm^{3+}. Also, from these thermogenic curves and data, we can see that the time for the line DE become longer with increasing concentration of Sm^{3+} and Dy^{3+} ion. When the concentration of Sm^{3+} reach 250 μg/mL, Part EFG disappeared during the experimental time, showing that Sm^{3+} and Dy^{3+} inhibit the formation of endospores. The microscopic observation shows that no endospores were formed. Therefore, Sm^{3+} ion has promoting action on the bacterial growth of *Bt cry II*, on the contrary, Sm^{3+} ion has inhibitory action on the sporulation of *Bt*.

Comparing the curves of *Bt* growth in the presence of Sm^{3+} ion with those in the presence of Dy^{3+} ions, it was found that both of the curves are very similar to each other, that is, the first phase becomes longer as the concentration of Sm^{3+} and Dy^{3+} ion increased, but the second phase becomes shorter.

The relationship between *k* and *c*

The analysis of the power-time curves in Figures 1 and 2 shows an exponential increase of the heat out-put during the log growth phases. The growth rate constants *k* for *Bt* can be fitted by means of the thermokinetic equation:

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

or $$\ln P_t = \ln P_0 + kt$$

The calculated values for *k* are given in Table 1. These rate constants *k* are inversely proportional to the concentrations of Sm^{3+} and Dy^{3+}.

Table 1 The growth constants *k* (min^{-1}) for *Bt* in the presence of Sm^{3+} or Dy^{3+}

c/(μg•mL^{-1})	*k* (Sm^{3+})	*k* (Dy^{3+})
0	0.02519	0.02519
50	0.02726	0.02664
100	0.02789	0.02722
150	0.03053	0.02703
200	0.02908	0.02562
250	0.02825	0.02502

From these data, it can be found that the value of growth rate constant for Sm^{3+} is greater than that for Dy^{3+}. These *k* values are increased with increasing of these ions concentration. This observation is in agreement with the laws discovered from the rare earths, such as the association between amino acids and the rare earths.

Relation between *P* and *c*

From the thermogenic curves of *Bt* under the different concentrations of Sm^{3+} and Dy^{3+}, we calculated the maximal heat out-put power for the different phases. The data are listed in Table 2.

Table 2 The peak-height (μW) and *c* in the presence of Sm^{3+} or Dy^{3+}

c/(μg•mL^{-1})	Sm^{3+}		Dy^{3+}	
	P_1	P_2	P_1	P_2
0	139	266	139	266
50	145	264	140	261
100	151	260	138	259
150	146	258	150	256
200	148	251	145	249
250	145	0	135	0

The interval time τ_I and c

As mentioned above, the interval time τ_I between the two parts was changed with the concentrations of added Sm^{3+} or Dy^{3+}. From Figures 2 and 3, we calculated the values of interval time under different concentrations of Sm^{3+} or Dy^{3+}. These data are listed in Table 3. From these data, we may draw the following conclusions, *i.e.*, the higher the concentrations of Sm^{3+} or Dy^{3+}, the longer the interval time for *Bt*. Comparing the data in Table 3, we also found that the values of interval time for Sm^{3+} are shorter than those for Dy^{3+}.

Table 3 The τ_I(min) under different concentrations of Sm^{3+} or Dy^{3+}

c/(μg•mL^{-1})	Sm^{3+}	Dy^{3+}
0	270	270
50	415	446
100	489	551
150	581	645
200	923	1053

The relation between heat-output Q and c

From the above thermogenic curves, we calculated the heat-output (Q_{LOG}, Q_{STAT} and Q_{SPOR}) at different phases, and total heat effects (Q_T), the results being listed in Table 4.

Table 4 The heat-output (Q_{LOG}, Q_{STAT}, Q_{SPOR}) and total heat (Q_T)

c/	Sm^{3+}				Dy^{3+}			
(μg•mL^{-1})	Q_{LOG}	Q_{STAT}	Q_{SPOR}	Q_T	Q_{LOG}	Q_{STAT}	Q_{SPOR}	Q_T
0	0.327	0.748	0.08	1.155	0.327	0.748	0.08	1.155
50	0.315	0.769	0.222	1.306	0.340	0.762	0.190	1.292
100	0.324	0.798	0.631	1.753	0.320	0.760	0.311	1.391
150	0.272	0.836	0.843	1.951	0.281	0.765	0.690	1.736
200	0.297	0.864	0.125	1.286	0.286	0.771	0.114	1.171
250	0.279	0.847	0	1.126	0.26	0.768	0	1.028

From the above data, we can obtain the Q—c equations: (0—150 μg/mL)

Sm^{3+}:

$$\ln Q_T = 0.13014 + 0.00373c \qquad R = 0.9819$$

$$\ln(Q_{STAT} + Q_{SPOR}) = -0.20359 + 0.00497c \qquad R = 0.9877$$

Dy^{3+}:

$$\ln Q_T = 0.12603 + 0.00259c \qquad R = 0.97299$$

$$\ln(Q_{STAT} + Q_{SPOR}) = -0.21991 + 0.00362c \qquad R = 0.97309$$

The experimental results show that the total heat effects and heat-outputs at stationary and decline phases increased with the increasing of Sm^{3+} and Dy^{3+} concentrations, respectively. The total heat effects correlated linearly with the concentration, and so do the sums of heat outputs at stationary and decline phases. The conclusion can be drawn that Sm^{3+} and Dy^{3+} have stimulatory effects on *Bt cry II*, and Sm^{3+} has the greater biological effects than Dy^{3+}.

Discussion

Recent studies[8] show that the rare-earth ions can participate in or interrupt the metabolism of cells at the subcellular levels, furthermore they can activate or inhibit the extracellular enzyme systems. Our results can be explained on the basis of the fact that Sm^{3+} and Dy^{3+} have the similar chemical and physical properties as Ca^{2+}. *Bt* can produce intracellular crystals of toxic glycoproteins when it sporulates. It was well known that Ca^{2+} plays an important role in sporulation of *Bt*. Since Sm^{3+} and Dy^{3+} have the similar ionic radius as Ca^{2+}, they can replace Ca^{2+} to participate in the sporulation phase. This is the reason why the addition of Sm^{3+} can cause the delaying of the interval time. Indeed, when the concentration of Sm^{3+} and Dy^{3+} reaches 250 μg/mL, the heat signal for the sporulation phase has not been observed in the experimental period.

According to the above results, it was found that the effects of Sm^{3+} and Dy^{3+} on *Bt* during the sporulation phase were far greater than those during the bacterial phase. Considering the life of *Bt* and the real concentration of Sm^{3+} in the environmental ecosystem, we could draw the conclusion that under the natural conditions, Sm^{3+} and Dy^{3+} have some slight stimulatory on the growth of *Bt*, on the contrast, they have almost no effect on the sporulation of *Bt*. Therefore, the application of *Bt* for controlling insecticide could not be affected by the presence of the rare-earth elements in the environmental ecosystem, which implying that the extensive applications of rare-earth micro-fertility in agriculture may be safe.

In conclusion, our results will be of important and significant in theory and practice to study both the metabolism of *Bt* and the biological effects of rare earth elements. Thus we believe that microcalorimetric method can be used in almost every aspect of environmental sciences, and can obtain a lot of information that other methods can not do. We can also study further the kinetics and thermodynamics of environmental sciences, all of which are very significant for environmental sciences.

References

1 Yu, Z. N.; Sun, M.; Liu, Z. D.; Dai, J. Y.; Chen, Y. H.; Yu, L.; Luo, X. X. *Chin. J. Biol. Control*. **1996**, *12*, 85.

2 Ni, J. Z. *Bioinorganic Chemistry of Rare-Earth Elements*, Science Press, Beijing, **1995**. (in Chinese)
3 Guo, B. S. *Chin. Rare Earths* **1999**, *20*, 64. (in Chinese)
4 Liu, Y; Deng, F. J.; Zhao, R. M.; Qu, S. S. *Chemosphere* **2000**, *40*, 851.
5 Brock, T. D.; Madigan, M. T. *Biology of Microorganisms*, 10th ed., Prentice-hall, Inc., New York, **2003**.
6 Xie, C. L.; Tang, H. K.; Song, Z. H.; Qu, S. S. *Thermochem. Acta* **1988**, *123*, 33.
7 Shen, P.; Fan, X. R.; Li, G. W. *Laboratory Experiments in Microbiology*, 3rd ed., Higher Education Press, Beijing, **1999** (in Chinese).
8 Yuan, Y. J.; Hu, G. W.; Wang, C. G.; Jing, Y.; Zhou, Y. Q.; Shen, P. W. *J. Rare Earths* **1998**, *16*(4), 7.

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