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The Toxicity of *Bacillus thuringiensis* Strains h3 from Oceans and Ly30 from Insect Larvae Against Diamondback Moth(*Lepidoptera*), Culex pipiens pallens and Musca domestica(*Diptera*), Blattella germanica(*Coleoptera*)

Wei Hua, Yuan Sheng, Zhang Wen

(School of Life Sciences, Nanjing Normal University, Jiangsu Key Laboratory for Microbes and Functional Genomics, Jiangsu Engineering and Technology Research Center for Industrialization of Microbial Resources, Nanjing 210023, China)

Abstract: In order to find and identify more targets species of pests for toxic insecticidal *Bacillus thuringiensis* (*Bt*) strains, a toxicity survey was carried out of two strains h3 and Ly30 against diamondback moth (*Lepidoptera*), Culex pipiens pallens and Musca domestica (*Diptera*), Blattella germanica (*Coleoptera*), which were respectively isolated from Lianyungang oceans with excellent characteristic of high salt tolerance, and from insect Componotus iaponicu Mayr larvae, respectively. Bioassay showed that h3 and Ly30 have efficient insecticidal activity against diamondback moth (*Lepidoptera*), Culex pipiens pallens and Musca domestica (*Diptera*) by 99% - 100% mortality at 3 d - 5 d, while only have 30% mortality at 5 d against coleoptera pests. It provided strong evidence for the insecticidal role of autotrophic *Bt* strains h3 and Ly30 against pests populations of lepidoptera and diptera and coleoptera, and aslo evidence for the application in the fields of agriculture and forest and public health. Furthermore, high salt tolerance of h3 could provide great benefit not only in expanding the application fields of biological insecticides, but also increasing the applicable object, and enhancing the efficacy of *Bt*.

Key words: *Bacillus thuringiensis*, *Coleoptera*, *Lepidoptera*, *Diptera*, insecticidal activity

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海洋来源的苏云金芽孢杆菌 h3 菌株和昆虫幼虫来源的 Ly30 对鳞翅目小菜蛾、双翅目家蝇和淡色库蚊、鞘翅目德国小蠊的毒性

魏　华, 袁　生, 张　文

(南京师范大学生命科学学院, 江苏省微生物与功能基因组学重点实验室, 江苏省微生物资源产业化工程技术研究中心, 江苏 南京 210023)

[**摘要**]　为发现和验证杀虫剂苏云金杆菌(*Bt*)菌株对目标种类的害虫的毒性, 本文用两株菌种(h3 和 Ly30)对小菜蛾(鳞翅目)、淡色库蚊和家蝇(双翅目)和德国小蠊(鞘翅目)几种害虫进行了毒力检测. 其中, 菌株 h3 来源于连云港海水, 具有高盐耐受的优点; 菌株 Ly30 分离自连云港市云台山赤松林区自然死亡的膜翅目昆虫日本弓背蚁幼虫体内. 杀虫活性实验显示, 菌株 h3 和 Ly30 具有高效杀虫活力, 对小菜蛾(鳞翅目)、淡色库蚊和家蝇(双翅目)3 d~5 d 致死率可达 99%~100%, 对德国小蠊(鞘翅目)5 d 致死率仅达 30%. *Bt* 菌株 h3 和 Ly30 对鳞翅目和双翅目害虫的强力杀虫活性及对鞘翅目害虫的毒力, 不仅为其在农业、林业领域及公共卫生等方面的应用提供了理论支持. 同时也拓展了 *Bt* 菌株毒杀害虫种类的研究. 此外, 具有高盐耐受优势的菌株 h3 不仅扩大了 *Bt* 生物杀虫剂的应用领域, 也有利于增加适用对象, 提高杀虫功效.

[**关键词**]　苏云金芽孢杆菌, 鞘翅目, 鳞翅目, 双翅目, 杀虫活性

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Corresponding author: Yuan Sheng, professor, majored in regulation of microbial growth and metabolism. E-mail: yuansheng@njnu.edu.cn

The increased urbanization of a growing global population makes imperative the development of sustainable integrated pest management strategies for urban pest control, which are closely associated with the health and wellbeing of humans and domesticated animals. Because of inevitable hazards of chemical insecticides, which are toxic to animals and may result in environmental contamination, destruction of non-target organisms and the development of resistant pests, biological insecticides are safe alternatives for several decades due to environment friendly, among which, *Bacillus thuringiensis* (*Bt*) is used most successfully, which is a gram-positive sporeforming bacterium that synthesizes a discrete intracellular glycoprotein crystal during sporulation[1-2], and some toxin genes from *Bt* have been engineered into crops to kill target herbivores[3].

There has been growing interest in finding more efficient and safer biological control *Bt* agents against hazardous insects. However, no *Bt* strains with toxic activity against Dictyoptera such as cockroach have been isolated. These *Bt*. strains are a potential reservoir for new toxin genes that might be useful for the control of different insect pests. The difference *Bt* strains from difference sources, which maybe kill different insects species, may have developed different mechanisms or specific toxins proteins[4-5].

In order to find and identify more targets species for toxic insecticidal *Bt*. strains, a toxicity survey was carried out. In this study, we used two natural isolated *Bt* strains h3[6], which is respectively isolated from Lianyungang oceans with excellent characteristic of high salt tolerance, and Ly30[7-9] isolated from insect Camponotus iaponicu Mayr larvae to examine its bioefficacy against four insect pests, diamondback moth, housefly (*Musca domestica*), mosquitoes (*Culex pipiens pallens*) and cockroach (*Blattella germanica*). This study is useful to evaluate the potential of *Bt* h3 and Ly30 as a natural biocontrol.

1 Materials and Methods

1.1 *Bacillus thuringiensis* strains and insect

Bacillus thuringiensis h3, is the first *Bacillus thuringiensis* strain isolated in 2007 from Lianyungang sea area and conserved at Key Laboratory of Microbiology and Functional Genomics, School of Life Sciences, Nanjing Normal University.

B. thuringiensis Ly30, was used as the control strain. It was isolated in 1995 from natural death Hymenoptera-Componotus iaponicu Mayr larvae in the pine forest at the Jiangsu Lianyungang Education Institute in Yuntai Mountain, Lianyungang city, Jiangsu province.

Larvae and adults of the diamondback moth (*Lepidoptera*) were provided by the Jiangsu Academy of Agricultural Sciences. Larvae and adults of the insects-Culex pipiens pallens and Musca domestica (*Diptera*), Blattella germanica (*Coleoptera*) were provided by Jiangsu Province CDC. Tested pests were diamondback moth of first instar larva, Culex pipiens pallens of 3rd instar larva, Musca domestica of 3 day old adults after eclosion with half male and half female, Blattella germanica of two week old adults with half male and half female. All insects were reared at (25±1) ℃, and (65±5)% relative humidity, with a 16:8 h light:dark photoperiod.

1.2 Collection of *Bt* spore-crystal mixture

To obtain a *Bt* spore-crystal mixture, all strains were incubated in culture broth containing trypone 1%, yeast extract 0.5%, NaCl 1% (pH 7.0), at 30 ℃, with rotation rate 240 r/min for 3 d. The concentration of viable spores of *Bt* fermentation broth was estimated by spectrophotometric determination at 600 nm absorbance with a spectrophotometer.

1.3 Laboratory toxicity assays against diamondback moth (*Lepidoptera*)[10]

In this study, toxicity of *Bt* h3 and Ly30 against lepidoptera pests were tested and compared in the laboratory using diamondback moth as experiment insect and cabbage (*Brassica oleracea* L.) and bok choy (*Brassica campestris* L. ssp.) as host plant, by two application methods of leaf dipping and spraying separately, with commercial chemical pesticides of emamectin benzoate EC and water as control. Fermentation broth of *Bt* were divided into three groups of high, middle, low concentrations as a diagnostic dose, with being diluted 5 times, 50 times and 500 times.

Commercial chemical pesticides of emamectin benzoate EC(1%) was used diluted from 10 000 to 160 000 times to a diagnostic dose.

1.3.1　Leaf dipping method

Cabbage(*Brassica oleracea* L.) leaves without chemical pollution were cut into 60 mm diameter round with a punch, and immersed for 30 s in *Bt* sample of different concentrations, and dried naturally, and then put in the dishes of 60 mm diameter (containing agar of 12 g/L) facing the lower berth. Each concentration was repeated 3 times, with commercial pesticide(1% Emamectin benzoate EC) and distilled water as control. Finally, pests first instar diamondback moth larva were transferred to the handle leaves with 15 heads each dishes. The dishes were covered and inverted on incubator with 24 ℃, 17∶7 h(L∶D), 40%-50% relative humidity(RH). After 3 d, the number of alive and dead pests were observed and counted for toxicity assays. Data were analyzed to measure reduction in the pest larvae numbers in comparison with untreated controls.

1.3.2　Spray method

The pests first instar diamondback moth larva were accessed to potted bok choy(*Brassica campestris* L. ssp.) extensively, whose number were counted after the quantity is stable. Then the *Bt* sample of different concentrations were sprayed on the plants by spray bottle with 200 mL each treatment including 3 parallel samples. Other steps are the same as 2.3.1.

1.4　Laboratory toxicity assays against Culex pipiens pallens(*Diptera*)

The mosquito(*Culex pipiens pallens*) 3rd instar larvae were cultured with fermentation broth mixture of *Bt* sample, with water and blank culture medium as control[11]. After 3 days and 5 days and longer time, the number and the growth effect of pests were observed and counted for toxicity assays, including the total number, the alive or dead number. Feed trials were run at the same time in separate boxes of the same laboratory, and all treatments were repeated three times. Data were analyzed to measure reduction in the pests larvae numbers in comparison with untreated controls.

1.5　Laboratory toxicity assays against Musca domestica(*Diptera*) and Blattella germanica(*Coleoptera*)

The tested houseflies(*Musca domestica*) were of 3 day old adults after eclosion with half male and half female. The tested cockroaches(*Blattella germanica*) were of two week old adults with half male and half female. The fermentation broth mixture of *Bt* sample were used as drinking water source to fed flies and cockroaches, with water and blank culture medium as control[12].

After 3 days and 5 days and longer time, the number and the growth effect of cockroaches were observed and counted for toxicity assays, including the total number, the alive or dead number. Feed trials were run at the same time in separate boxes of the same laboratory, and all treatments were repeated three times. Data were analyzed to measure reduction in the pest larvae numbers in comparison with untreated controls.

Besides, in order to reduce the error to calculate the actual consumption of flies and cockroaches, the volume of drinking water source was measured regularly, and the natural volatile errors were observed with the same volume of water as control. The water error of fly and cockroache body carrying when drinking can only be man-made estimated.

1.6　Statistical analysis

The number of target pests in all treatment groups were observed and counted and meaned from three parallel groups for toxicity assays, including the total number, the alive and dead number, and according to which, the mortality data were obtained with formula calculation.

$$\text{Mortality}(\%)=\frac{\text{thetotalnumberofallpests(heads)}}{\text{thenumberofdeadpests(heads)}}\times 100\%.$$

Finally, the mortality data for the laboratory assays was corrected against control mortality. The data were analyzed using repeated-measures analysis of variance(ANOVA). The least significant difference(LSD) method was used to separate and compare means within the treatments.

2 Results and Discussion

2.1 Collection of *Bt* fermentation broth

Fermentation broth of *Bt* strains h3 and Ly30 were collected and the concentration of their viable spores were estimated by spectrophotometric determination at 600 nm absorbance, which was 11.31±0.13 and 9.77±0.11 with culture medium without bacteria inoculation as control. The crystals and spores of fermentation broth were diluted and resuspended in deionized water to a diagnostic dose. These preparations were stored in small aliquots at 20 ℃ for use in the insecticidal effect assays, including the Lepidoptera of Cabbage moth, Diptera of Culex pipiens pallens and Musca domestica, Coleoptera of Blattella germanica.

2.2 Toxicity assays against diamondback moth(*Lepidoptera*)

2.2.1 Toxicity assays of leaf dipping method against diamondback moth(*Lepidoptera*)

With leaf dipping method, cabbage(*Brassica oleracea* L.) leaves were immersed in *Bt* sample and naturally dried, then pests were accessed and the toxicity assays were carried out.

Table 1 Bioefficacy of h3 and Ly30 against diamondback moth(*Lepidoptera*) by leaf dipping method

Insecticide tested	Dilution ratio	Number of alive insects (head)	Number of dead insects (head)	Total number of insects	Mortality/%
h3	1∶5	0	53	53	100.00
	1∶50	0	58	58	100.00
	1∶500	1	58	59	98.31
Ly30	1∶5	0	55	55	100.00
	1∶50	0	57	57	100.00
	1∶500	9	43	52	82.69
1% Emamectin benzoate EC	1∶10 000	4	55	59	93.22
	1∶40 000	11	40	51	78.43
	1∶160 000	17	44	61	72.13
Water	0	38	5	43	11.63

As shown in table 1 and Fig. 1, h3 and Ly30 were both have high insecticidal against diamondback moth larvae at all groups of different concentrations, whose toxicity were in related to the concentration. At high and middle dose, mortality value of h3 and Ly30 were both 100%, even higher than control Emamectin benzoate EC groups. Surprisingly, at the low dose, the 98% mortality value of h3 was 16% higher than that of Ly30, and 36% higher than that of the control group with application of Emamectin benzoate EC.

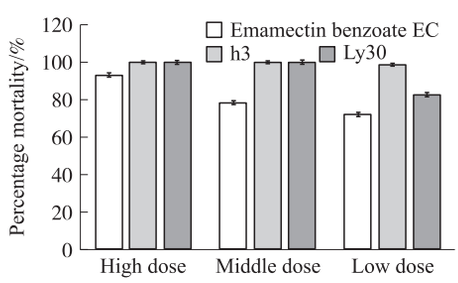

Fig. 1 Larval mortality when exposed to a diagnostic dose of *Bt* strains h3 and Ly30 with Emamectin benzoate EC as control by leaf dipping method

It confirmed that the *Bt* strains h3 and Ly30 both have high toxicity against diamondback moth(*Lepidoptera*). In addition, *Bt* h3 showed a more advantage than the other insecticide at low concentrations especially, which is a potential biocontrol agent.

2.2.2 Toxicity assays of spray method against diamondback moth(*Lepidoptera*)

With spray method, pests were accessed on the bok choy(*Brassica campestris* L. ssp.) leaves, and the *Bt* samples were directly sprayed on insect body, and then the toxicity assays were carried out.

It was showed in table 2 and Fig. 2 that when pests were treated for 3 d, the *Bt* groups of high dose showed decrease rate of 73.53% and 84.21%, which was lower than the control emamectin benzoate EC groups by mortality of 89.72%. When pests were treated for 5 d, all groups of different dose showed mortality rate of from 90% to 100%, similar as that of control emamectin benzoate EC groups, while that of the water group was negative highly.

It confirmed that the *Bt* strains h3 and Ly30 both have high toxicity against diamondback moth(*Lepidoptera*)by spray method at all groups of different concentrations,whose toxicity were in relation to the treatment time especially, and to the concentration either. In addition,*Bt* strain h3 and Ly30 both are very potential strains with high toxicity against lepidoptera pests to have a very good development prospects in the agriculture and forestry fields.

Table 2　Bioefficacy of h3 and Ly30 against diamondback moth(Lepidoptera)by spray method

Insecticide tested	Dilution ratio	Total number of insects	Treatment 3d		Treatment 3d	
			Number of alive insects(head)	Decrease rate/%	Number of alive insects(head)	Decrease rate/%
h3	1:5	136	36	73.53	1.00	99.26
	1:50	172	74	56.98	15.00	91.28
	1:500	164	73	55.49	19.00	88.41
Ly30	1:5	171	27	84.21	2.00	98.83
	1:50	170	55	67.65	13.00	92.35
	1:500	259	119	54.05	21.00	91.89
1% Emamectin benzoate EC	1:10 000	399	41	89.72	3.00	99.25
Water	0	234	305	-30.34	412.00	-76.07

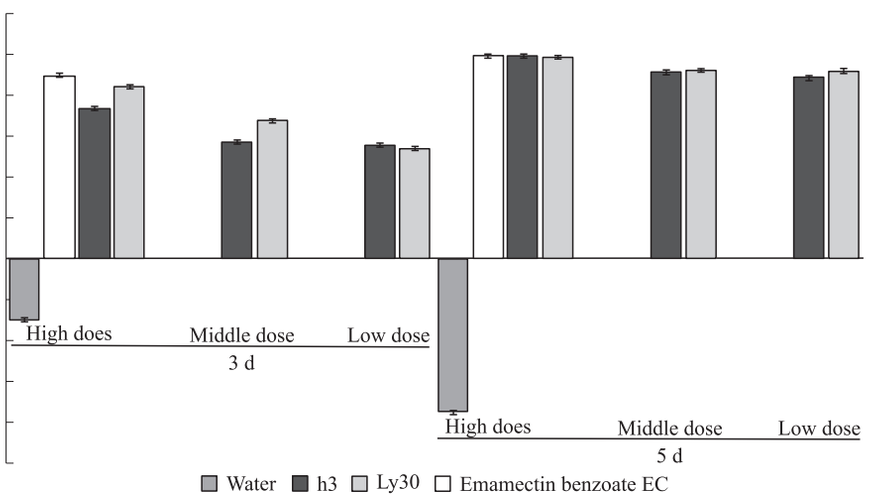

Fig. 2　Percentages of larval mortality when exposed to a diagnostic dose of *Bt* strains h3 and Ly30 with emamectin benzoate EC as control by spray method

2.3　Toxicity assays against Culex pipiens pallens and Musca domestica(*Diptera*)

Culex pipiens pallens and Musca domestica are both diptera, which are serious pests threatening human health. *B. thuringiensis* is a bioinsecticide used for larval mosquito and housefly control and it represents a safe alternative to chemical insecticides. So,we tested the toxicity of new *Bt* strains h3 and Ly30 against diptera pests Culex pipiens pallens,and the *Bt* fermentation broth of original concentration were used.

As shown in Fig. 3A, mortality data of the laboratory bioassays with the *Bt* h3 and Ly30 against Culex

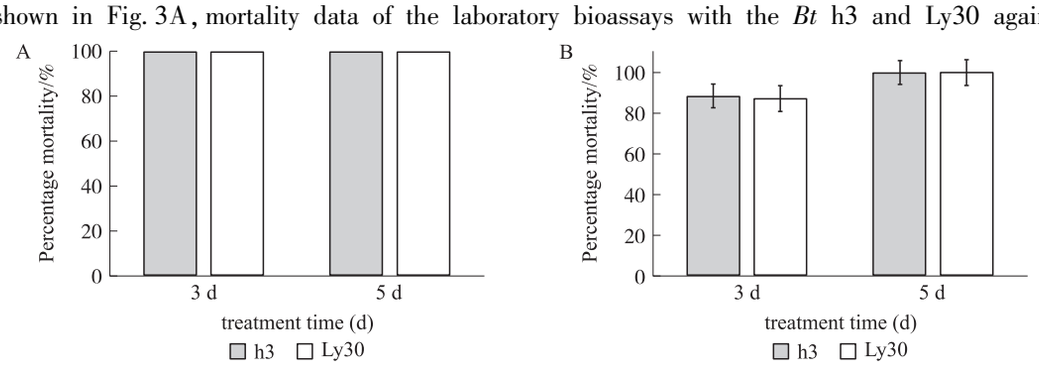

Fig. 3　Percentage mortality of Culex pipiens pallens(A)and Musca domestica(B) larvae(*Diptera*) treated respectively with two *Bt* strains of h3 and Ly30 for 3 d and 5 d

pipiens pallens were both reached 100% at 3 d and 5 d. The data of Musca domestica had reached 100% when 5 d, while more than 90% at 3d.

It indicated that *Bt* h3 and Ly30 have high toxicity against diptera pests of Culex pipiens pallens and Musca domestica, which have a very good development prospects in fields of public environment and medical and health and so on.

2.4 Toxicity assays against Blattella germanica (*Coleoptera*)

Blattella germanica belongs to *Coleoptera*, which is another serious pest. There is little research about *Bt* toxicity assays against Blattella germanica (*Coleoptera*). So, h3 and Ly30 were applied totest its bioefficacy against Blattella germanica.

Mortality data of the laboratory bioassays with the *Bt* h3 against Blattella germanica (*Coleoptera*) were 0% at 3 d, and reached to 30% at 5 d, while that of Ly30 were always 0% at 3 d and 5 d(table 3). These results indicated that As compared with Ly30, *Bt* strain h3 showed a potential toxicity against Blattella germanica (*Coleoptera*). On one hand, it has a very good development prospects in the new field of public health. On the other hand, it probably has some special functional toxicity crystalline protein, which is different from the other *Bt* strains.

Table 3 Percentage mortality of Blattella germanica (*Coleoptera*) larvae treated respectively with two *B. thuringiensis* strains of h3 and Ly30 for 3 d and 5 d

Bt strains	Percentage mortality/%	
	3 d	5 d
h3	0	30.0±3
Ly30	0	0

3 Conclusion

Bioassay showed that the toxicity of two *Bt* strains h3 and Ly30 against lepidoptera and diptera pests was efficiently with satisfactory results of about 99%-100% mortality at 3 d-5 d, and little toxicity against coleoptera pests of only 30% at 5 d. It provided strong evidence for the insecticidal role of autotrophic *Bt* strains h3 and Ly30 against pests populations of lepidoptera and diptera and coleoptera, and aslo evidence for the application in the fields of agriculture and forest and public health. Furthermore, high salt tolerance of h3 could provide great benefit not only in expanding the application fields of biological insecticides, but also increasing the applicable object, and enhancing the efficacy of *Bt*. The two biological insecticide *Bt* strains h3 and Ly30 can become an invaluable tool as an intermediate and be widespreadly used for future urban integrated pest management.

4 Acknowledgements

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