

## BACILLUS THURINGIENSIS ISOLATED FROM THE PHILIPPINES

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Seventeen isolates of crystalliferous sporeformers were obtained from soil and silkworm litter samples collected in various regions of the Philippines. Fifteen isolates belonged to the following *Bacillus thuringiensis* subspecies: *kurstaki* (serotype 3a:3b), *sotto* (or *subtoxicus*) (serotype 6), *aizawai* (serotype 7), *morrisoni* (serotype 8a:8p), *tolworthi* (serotype 9), and *darmstadiensis* (serotype 10). Two isolates were nonmotile and morphologically similar to Fowler's bacillus. Five isolates belonging to subspecies *kurstaki*, *sotto* (or *dendrolimus*), and *darmstadiensis* were toxic to the silkworm, *Bombyx mori*. One isolate of subsp. *morrisoni* was highly toxic to mosquito larvae, *Aedes aegypti*, but nontoxic to the silkworm.

Owing to the adverse effects of chemical pesticides on human, domestic animals, plants, and wild life, the importance and necessity of microbial control of insect pests have been recognized and emphasized in recent years. *Bacillus thuringiensis* is the most promising agent for microbial control and has been mass-produced as microbial insecticide in several countries.

*B. thuringiensis* is effective against more than 137 insect species belonging to the orders Lepidoptera, Hymenoptera, Diptera, and Coleoptera (Heimpel, 1967). Several workers (Goldberg and Margalit, 1977; Hall et al., 1977; de Barjac, 1978; Undeen and Nagel, 1978) have reported the potential of this bacterium in controlling medically important insects such as mosquito and blackfly larvae.

A worldwide distribution of naturally occurring *B. thuringiensis* has been demonstrated by many workers. However, little is known about the distribution of this bacterium in Southeast Asian countries. This paper describes the isolation and serological identification of *B. thuringiensis* isolated in the Philippines. Qualitative toxicity tests of the crystalliferous spore-former were also carried out against larvae of the mosquito, *Aedes aegypti*, and the silkworm, *Bombyx mori*.

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## MATERIALS AND METHODS

### Isolation of *B. thuringiensis*

Sixty-two soil and two silkworm litter samples were collected in 1978-1980 from different parts of the country (Table 1). The soil samples were taken from fields with growing crops and the silkworm litters were collected from the silkworm-rearing room of the Department of Entomology, University of the Philippines at Los Baños.

*B. thuringiensis* was isolated according to the method of Aizawa et al. (1961). One g of the sample was suspended in 10 ml of sterile distilled water and shaken vigorously for five min. After allowing the suspension to stand for ten min., the upper layer of the suspended sample was transferred to a test tube and heated at 65°C for thirty min. Tenfold serial dilutions of the heated suspension in sterile distilled water or physiological saline (0.85% NaCl) were plated on nutrient agar (pH 7.4). After incubation for two days at 28°C, *Bacillus* colonies were counted and recorded. Isolation of crystalliferous sporeforming bacteria was done by examination under a phase-contrast microscope after five to seven days of incubation. Twenty-five to 30 colonies were examined in each sample.

### Preparation of Antisera

Seventeen reference subspecies of *B. thuringiensis* belonging to 12 serotypes (de Barjac and Bonnefoi, 1973) were supplied by Dr. H. de Barjac, Institut Pasteur, Paris. The bacteria were grown on nutrient agar (pH 7.4) at 27-28°C for 18-24 hr. Vegetative cells were harvested by centrifugation at 3,000 rpm for 15 min and suspended in physiological saline. The bacterial suspension was mixed with an equal volume of saline containing two percent formaldehyde. After incubation at 37°C for one hr, dead cells were sedimented by centrifugation and resuspended in saline. Rabbits were immunized with formalin-killed vegetative cells by intravenous injections four times at four-day intervals and bled eight days after the last injection. Antisera were heated at 56°C for 30 min prior to use.

For the preparation of *H* antiserum, the *OH* antiserum was saturated with the homologous heat-stable *O* antigen. The *O* antigen was prepared by heating vegetative cells at 100°C for two hr. Monospecific antisera to 3b, 4b, 4c, 8b, and 8c. *H* subfactors were prepared by cross-saturation of *H* antisera.

### Serology

The *H* antigen test for identification was done by slide agglutination

**Table 1. Source and locality of samples for the isolation of *Bacillus thuringiensis*.**

LOCALITY	NO. OF SAMPLES EXAMINED	SOURCE
Batangas	3	Soil
Bicol Region	11	Soil
Bulacan	2	Soil
Cebu	3	Soil
IRRI <sup>a</sup>	18	Soil
Laguna	3	Soil
Nueva Ecija	2	Soil
UPLB (College) <sup>b</sup>	1	Soil
UPLB (C.E.S.) <sup>c</sup>	17	Soil
UPLB (C.F.) <sup>d</sup>	2	Soil
UPLB (D.E.) <sup>e</sup>	2	Silkworm litters

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using well-motile bacteria selected by using Craigie's tube. One drop of flagellated suspension was mixed with one drop of the *H* antiserum against reference *B. thuringiensis* or monospecific antiserum on a slide glass. Agglutination was observed after two to three min.

### *Toxicity Test*

The bacteria were grown on nutrient agar slants (pH 7.4) for seven to 10 days at 27-28°C. One loopful of the sporulated culture was suspended in one ml of distilled water. Mulberry leaves were smeared with 0.3 ml of suspension and air dried at room temperature. Five 3rd instar silkworm larvae were fed on the smeared mulberry leaves and reared for 48 hr at 25°C.

In the toxicity test using *A. aegypti*, 20 four-day-old larvae were introduced into a test tube (2.5 x 20 cm) containing 40 ml seasonal water with two loopfuls of the sporulated culture. They were reared at 25°C for 48 hr without feeding. Corresponding controls without bacterial culture were included in the tests.

## RESULTS

### *B. thuringiensis* serotypes

*B. thuringiensis* and crystalliferous sporeformers morphologically similar to Fowler's bacillus were isolated from 12 soil samples and two silkworm litter out of the 64 samples examined (Table 2). Among the samples where crystalliferous sporeforming bacteria were isolated, four soil samples contained more than  $10^4$  sporeformers per one g of soil sample and more than  $10^5$  sporeformers per one g were detected in three samples.

The crystalliferous sporeforming bacteria were identified using *H* agglutination test (Table 3). Fifteen isolates proved to belong to *B. thuringiensis* subsp. *kurstaki* (serotype 3a:3b), subsp. *sotto* (or *dendrolimus*) (serotype 4a:4b), subsp. *entomocidus* (or *subtoxicus*) (serotype 6), subsp. *aizawai* (serotype 7), subsp. *morrisoni* (serotype 8a:8b), subsp. *tolworthi* (serotype 9), and subsp. *darmstadiensis* (serotype 10). Two isolates were nonmotile and morphologically similar to Fowler's bacillus (Hannay, 1961) whose inclusions firmly attached to the spores. Subsp. *kurstaki* and subsp. *tolworthi* were predominantly isolated. Based on the present study, *B. thuringiensis* appears well-distributed in the areas sampled particularly at Central Experiment Station, University of the Philippines at Los Baños.

### Toxicity of *B. thuringiensis* isolates and related bacteria

The toxicity of the isolates was examined qualitatively. Most of the isolates produced rhomboidal or diamond-shaped parasporal inclusions

Table 2. Isolation of *Bacillus thuringiensis* and related bacteria in the Philippines.

Locality	Accession	Source	No. of Spore-Formers/1 g of sample	No. of <i>B. thuringiensis</i> /No. of spore-former examined
UPLB (C.F.) <sup>a</sup>	79-PG-5-01 79-PG-5-02	Soil	$6.5 \times 10^5$	2/30
IRRI <sup>b</sup>	79-PG-6-03	Soil	$6.9 \times 10^5$	1/28
IRRI	79-PG-38-04	Soil	$7.6 \times 10^3$	1/22
UPLB (C.E.S.) <sup>c</sup>	79-PG-43-05	Soil	$3.5 \times 10^3$	1/27
UPLB (C.E.S.)	79-PG-52-06	Soil	$4.7 \times 10^3$	1/19
UPLB (C.E.S.)	79-PG-55-07	Soil	$3.3 \times 10^3$	1/30
UPLB (C.E.S.)	79-PG-56-08 79-PG-56-09	Soil	$4.6 \times 10^3$	2/30
UPLB (C.E.S.)	79-PG-60-10	Soil	$1.6 \times 10^3$	1/25
UPLB (C.E.S.)	79-PG-64-11	Soil	$2.6 \times 10^3$	1/21
UPLB (D.E.) <sup>d</sup>	80-PG-2-01	Silkworm litter	$1.1 \times 10^4$	1/30
UPLB (D.E.)	80-PG-10-02	Silkworm litter	$2.0 \times 10^4$	1/30
Guadalupe, Cebu City	80-PG-17-03	Soil	$2.2 \times 10^4$	1/30
	80-PG-19-04 80-PG-19-05	Soil	$1.6 \times 10^5$	2/30
UPLB (College) <sup>e</sup>	81-PG-2-01	Soil	$8.7 \times 10^4$	1/21

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Table 3. Serological identification of *Bacillus thuringiensis* and related bacteria.

ACCESSION NO. <sup>a</sup>	H SEROTYPE	SUBSPECIES
79-PG-5-01	3a:3b	<i>kurstaki</i>
79-PG-5-02	3a:3b	<i>kurstaki</i>
79-PG-6-03	3a:3b	<i>kurstaki</i>
79-PG-38-04	10	<i>darmstadiensis</i>
79-PG-43-05	3a:3b	<i>kurstaki</i>
79-PG-52-06	9	<i>tolworthi</i>
79-PG-55-07	9	<i>tolworthi</i>
79-PG-56-08	9	<i>tolworthi</i>
79-PG-56-09	9	<i>tolworthi</i>
79-PG-60-10	7	<i>aizawai</i>
79-PG-64-11	7	<i>aizawai</i>
80-PG-2-01	9	<i>tolworthi</i>
80-PG-10-02	6	<i>entomocidus</i> (or <i>subtoxicus</i> )
80-PG-17-03	8a:8b	<i>morrisoni</i>
80-PG-19-04 <sup>b</sup>	— <sup>c</sup>	
80-PG-19-05	4a:4b	<i>sotto</i> (or <i>dendrolimus</i> )
81 PG-2-01 <sup>b</sup>	— <sup>c</sup>	

<sup>a</sup>The place collected and source of isolate; See Table II.

<sup>b</sup>Crystalliferous sporeformer morphologically similar to Fowler's bacillus.

<sup>c</sup>Nonmotile and untypable.

except one isolate of serotype 8a:8b with spherical or irregular-shaped inclusions. Some isolates belonging to subsp. *kurstaki*, subsp. *sotto* ( or *dendrolimus*), and subsp. *darmstadiensis* were toxic to the silkworm, *B. mori*, while others [subsp. *entomocidus* (or *subtoxicus*) subsp. *morrisoni*, and subsp. *tolworthi*] exhibited no toxicity. The isolate 80-PG-17-03 which produced spherical or irregular-shaped parasporal inclusions was the only isolate toxic to the larvae of *A. aegypti* but was nontoxic to the silkworm.

### DISCUSSION

Many investigation have been done on the isolation of *B. thuringiensis* in various parts of the world. In the Philippines, this paper is the first attempt to isolate and identify *B. thuringiensis* although Gabriel (1968, 1970) and Villacarlos and Gabriel (1974) started earlier surveys on different entomogenous microorganisms associated with insects of agricultural and medical importance. Gabriel (1968, 1970) reported the introduction of commercial preparations of *B. thuringiensis* subsp. *thuringiensis* (serotype 1) and subsequent use in the Philippines. However, this particular subspecies was not isolated from the sample collected in this study.

Aizawa et al. (1961) and Ohba et al. (1979) reported that in some cases more than  $10^5$  *B. thuringiensis* spores were contained in one g of the dust collected from the silkworm rearing room. No such a sample containing comparable number of spores was detected in the present study.

The isolation and serological identification of *B. thuringiensis* from silkworm litters have been done extensively in Japan (Ohba and Aizawa, 1978, 1979; Ohba et al. 1979). DeLucca et al. (1979) and Ohba et al. (1981) reported new serotypes of this bacterium based on the isolates from soil samples. In the present study, we could detect *B. thuringiensis* in 11 out of 62 soil samples. These results indicate that the soil is a good source for the isolation of *B. thuringiensis* as well as the silkworm litter.

The toxicity of *B. thuringiensis* strains varies considerably in a given insect species (Aizawa et al., 1975; de Barjac, 1978; Padua et al., 1980). Some isolates in the present study were toxic to the silkworm but not to mosquito larvae and vice versa, and some were nontoxic to both insect species. Crystalliferous strains of *B. thuringiensis* having highly preferential toxicity (de Barjac, 1978; Ohba and Aizawa, 1979; Padua et al., 1980) and no toxicity (Ohba and Aizawa, 1978; Ohba et al., 1979; Padua et al., 1980) have been reported. Although the toxicity tests have been done qualitatively, it is possible that our isolates which showed no toxicity to both insects tested have selective toxicity to other groups of insects. Hence, the screening of various *B. thuringiensis* strains to other insect species is important in utilizing this microorganisms for microbial control.

The isolation of seven serotypes and strains similar to Fowler's bacillus provides a clue to the rich flora of *B. thuringiensis* in the Philippines. Investigation of the preferential toxicity of the *B. thuringiensis* serotype 8a:8b isolate to mosquito larvae is now in progress. A continuing survey on this bacterium and related forms is being considered as a part of future works.

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