Distribution of *Bacillus thuringiensis* Serotypes in Ehime Prefecture, Japan

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Silkworm litters from 98 sericultural farms in Ehime Prefecture, Japan, were examined and 146 crystalliferous sporeformers were isolated from 18 farms. These isolates proved to belong to *Bacillus thuringiensis* serotypes 3a, 4a:4b, 4a:4c, 5a:5c, 7, 8, and 10. Serotypes 4a:4b, 4a:4c, 7, and 10 were distributed predominantly. Most of the isolates produced typical rhomboidal parasporal inclusions and showed toxicity to the silkworm larvae, *Bombyx mori*. On the contrary, an isolate belonging to serotype 5a:5c and 3 isolates belonging to serotype 10 produced unusual parasporal inclusions. They did not show any toxicity to the silkworm larvae.

INTRODUCTION

Since the discovery of "Sotto-Bacillen" by Ishiwata (1901), extensive investigations on *Bacillus thuringiensis* have been done in Japan for the control of the silkworm disease. The distribution of *B. thuringiensis* in sericultural farms has been studied as one of the major epizootiological problems (Chigasaki, 1919; Aizawa et al., 1961; Ishikawa et al., 1964; Ono, 1968) and serological surveys on *B. thuringiensis* and related bacteria isolated from sericultural farms and soils have been done by agglutination tests (Aoki and Chigasaki, 1916a, b, 1919; Aizawa et al., 1975; Ohba and Aizawa, 1978). One of the best sources of the isolation of *B. thuringiensis* in Japan is the litter of sericultural farms. This phenomenon should be investigated from the viewpoint of microbial ecology. The present paper reports the distribution of *B. thuringiensis* serotypes in sericultural farms of Ehime Prefecture, Shikoku (one of the main islands of Japan).

MATERIALS AND METHODS

Isolation of *B. thuringiensis*. A total of 104 silkworm litter samples examined in this investigation was collected in 1973 and 1974 from the following 98 sericultural farms of Ehime Prefecture: Saijo-shi, 3; Iyo-shi, 1; Iyomishima-shi, 4; Ozu-shi, 4; Uma-gun, 2; Syusyo-gun, 5; Ochi-gun, 5; Kita-gun, 2; Kitauwa-gun, 13; Minamiuwa-gun, 3; Kamiukena-gun, 31; Iyo-gun, 5; Onsen-gun, 3; Higashiuwa-gun, 17. (Shi means city and gun means subprefecture.) Most of the silkworm litters were collected from the silkworm-rearing rooms and some were from the mulberry-leaf stock rooms. 

*B. thuringiensis* was isolated according to the method of Aizawa et al. (1961).
One gram of the silkworm litter was suspended in 10 ml of sterilized distilled water and was shaken vigorously for 5 min. The suspended matter was then allowed to stand for 10 min. The upper layer of the suspended matter was transferred to a test tube and heated at 65°C for 30 min. Serial 10-fold dilutions of the heat-treated suspended matter with sterilized distilled water or physiological saline were plated on nutrient agar (pH 7.4). After incubation for 4–7 days at 28°C, the colonies were examined under a phase-contrast microscope or by Möller stain for the isolation of the crystalliferous sporeformer.

**Antiserum.** Seventeen subspecies of *B. thuringiensis*' 12 serotypes (De Barjac and Bonnefond, 1973) were grown in nutrient broth (pH 7.4) at 28°C for 18–24 hr. Vegetative cells were then harvested and suspended in physiological saline containing 1% HCHO at 37°C for 1 hr. Dead bacteria were sedimented and resuspended in saline. Rabbits were immunized with formalin-killed vegetative cells by several intravenous injections at 4-day intervals and bled 7 to 10 days after the last injection. Sera were heated at 56°C for 30 min prior to use. For the preparation of H antisera, OH antiserum was saturated with homologous heat-stable somatic antigen (O antigen). O antigen was prepared by the treatment of vegetative cells at 100°C for 2 hr. Agglutinin titer of each H antiserum to the homologous antigen was 3,200 to 25,600. H factor serum to 3b, 4b, 4c, 5b, and 5c antigen, respectively, was prepared according to Bonnefond and De Barjac (1963), De Barjac and Lemille (1970), and De Barjac and Bonnefond (1972).

**Serology.** H agglutination test was performed with well-motile bacteria selected by Craigie's tube (Craigie, 1931). Slide agglutination test was employed for the identification of H antigen. One drop of the live bacterial suspension was mixed on a glass slide with one drop of the reference *B. thuringiensis* H antiserum which had been diluted 20- to 100-fold with saline depending on the agglutinin titer. Factor sera were used to identify the subfactors of H antigens. Tube agglutination test was employed using formalin-killed bacteria to determine the H agglutinin titer of the antiserum. Agglutinin titer was expressed as the reciprocal of the serum dilution.

**Toxicity of the isolates to the silkworm larvae.** The isolates were grown on nutrient agar slants at 28°C for 7–10 days. Two loopfuls of the culture were suspended in 1 ml of distilled water. Five silkworm larvae, *Bombyx mori*, (3rd or 4th instar) were fed on the bacteria-smeared mulberry leaf and reared for 24 hr at 25°C to examine the qualitative toxicity.

**RESULTS**

**Distribution of B. thuringiensis serotypes**

*B. thuringiensis* was detected in 18 out of 98 sericultural farms examined (Table 1). Among 18 farms, more than 10^8 *B. thuringiensis* spores were contained in 1 g of the silkworm litter in 2 farms and more than 10^4 spores per 1 g of the litter were detected in 8 farms.

Crystalliferous sporeformers were identified by H agglutination test and 146 isolates appeared to belong to the serotypes 3a (subsp. aesti), 4a:4b (subsp. sotto or subsp. dendrolimus), 4a:4c (subsp. kenyae), 5a:5c (subsp. canadensis), 7 (subsp. aizawai), 8 (subsp. morisoni), and 10 (subsp. darnstadiensis). Among them, serotypes 4a:4b, 4a:4c, 7, and 10 were predominantly isolated. In most cases, isolates from a given sericultural
Table 1. H Serotype of Bacillus thuringiensis Isolated from Sericultural Farms of Ehime Prefecture

<table>
<thead>
<tr>
<th>Locality</th>
<th>Farmer's Number</th>
<th>No. of sporeformers/1 g of the litter</th>
<th>No. of B. thuringiensis/No. of sporeformers examined</th>
<th>No. of B. thuringiensis isolates Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ozu-shi</td>
<td>1</td>
<td>$6.4 \times 10^4$</td>
<td>16/24</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>$2.1 \times 10^4$</td>
<td>1/10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>$9.2 \times 10^4$</td>
<td>1/23</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>$1.5 \times 10^4$</td>
<td>1/25</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>$4.7 \times 10^3$</td>
<td>2/19</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>$9.7 \times 10^4$</td>
<td>17/36</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>$6.4 \times 10^4$</td>
<td>10/18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>8</td>
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<td>14/20</td>
<td>14</td>
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<td>9</td>
<td>$2.3 \times 10^4$</td>
<td>4/30</td>
<td>3</td>
</tr>
<tr>
<td>Kamiukena-gun</td>
<td>10</td>
<td>$1.8 \times 10^4$</td>
<td>10/25</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>$3.5 \times 10^4$</td>
<td>17/18</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>$4.1 \times 10^5$</td>
<td>14/26</td>
<td>1a</td>
</tr>
<tr>
<td></td>
<td>13</td>
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<td>1/22</td>
<td>1a</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>$5.6 \times 10^5$</td>
<td>23/25</td>
<td>23</td>
</tr>
<tr>
<td>Iyo-gun</td>
<td>15</td>
<td>$1.8 \times 10^4$</td>
<td>4/19</td>
<td>4</td>
</tr>
<tr>
<td>Ochi-gun</td>
<td>16</td>
<td>$2.9 \times 10^3$</td>
<td>1/21</td>
<td>1a</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>$3.1 \times 10^4$</td>
<td>8/20</td>
<td>8</td>
</tr>
<tr>
<td>Kita-gun</td>
<td>18</td>
<td>$1.0 \times 10^5$</td>
<td>2/24</td>
<td>2a</td>
</tr>
</tbody>
</table>

* Nontoxic to the silkworm larvae, *Bombyx mori*.

Table 2. H Agglutination of Bacillus thuringiensis Isolates

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>H serotype</th>
<th>H agglutinin titer of the reference B. thuringiensis antiserum by the isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>74- E-3-2</td>
<td>8</td>
<td>6,400</td>
</tr>
<tr>
<td>74- E-21-1</td>
<td>7</td>
<td>12,800</td>
</tr>
<tr>
<td>74- E-29-3</td>
<td>8</td>
<td>6,400</td>
</tr>
<tr>
<td>74- E-65-4</td>
<td>4a:4b</td>
<td>6,400</td>
</tr>
<tr>
<td>74- E-71-3</td>
<td>7</td>
<td>12,800</td>
</tr>
<tr>
<td>74- E-74-7</td>
<td>5a:5c</td>
<td>6,400</td>
</tr>
<tr>
<td>74- E-78-1</td>
<td>3a</td>
<td>51,200</td>
</tr>
<tr>
<td>74- E-80-2</td>
<td>10</td>
<td>6,400</td>
</tr>
<tr>
<td>74- E-81-2</td>
<td>10</td>
<td>6,400</td>
</tr>
<tr>
<td>74- E-82-1</td>
<td>10</td>
<td>6,400</td>
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<td>74- E-83-8</td>
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<td>25,600</td>
</tr>
<tr>
<td>74- E-86-1</td>
<td>4a:4c</td>
<td>25,600</td>
</tr>
<tr>
<td>74- E-86-23</td>
<td>8</td>
<td>6,400</td>
</tr>
<tr>
<td>74- E-93-2</td>
<td>4a:4b</td>
<td>12,800</td>
</tr>
<tr>
<td>74- E-93-24</td>
<td>8</td>
<td>6,400</td>
</tr>
</tbody>
</table>

farm all belonged to the same serotype. However, simultaneous distribution of 2 serotypes was demonstrated in 2 sericultural farms.

As shown in Fig. 1, it was found that *B. thuringiensis* is distributed widely in Ehime
Fig. 1. Distribution of Bacillus thuringiensis serotypes in Ehime Prefecture. The empty circle indicates the sericultural farm in which B. thuringiensis was not detected. The number in the circle shows the serotype of B. thuringiensis which was isolated in the indicated sericultural farm.

Fig. 2. Phase-contrast photograph of the spores and the parasporal inclusions of Bacillus thuringiensis serotype 5a:5c isolate, 74-E-74-7. Seven-day culture at 28°C. Note the spherical parasporal inclusions (P). S, spore. Scale=5 μm.
Prefecture. No concentrated distribution in any particular region was observed. High titers of \( H \) agglutinin of the reference \( B. \) thuringiensis \( H \) antisera were shown by these isolates (Table 2).

Toxicity of \( B. \) thuringiensis isolates to the silkworm larvae

Toxicity of the isolates was qualitatively examined. Most of the isolates produced typical rhomboidal parasporal inclusions and showed toxicity to the silkworm larvae, \( B. \) mori. However, an isolate belonging to serotype 5a:5c and 3 isolates belonging to serotype 10 showed no toxicity to the silkworm larvae. These nontoxic isolates produced unusual parasporal inclusions and spherical inclusions were formed in an isolate from Ochi-gun belonging to serotype 5a:5c (isolate No.: 74-E-74-7) (Fig. 2).

DISCUSSION

Since Ishiwata (1901) isolated “Sotto-Bacillen” from diseased silkworm larvae at Nishigahara, Tokyo, several investigators have surveyed the distribution of \( B. \) thuringiensis in sericultural farms. Chigasaki (1919) demonstrated a wide distribution of this kind of bacterium in the prefectures of Tokyo, Nagano, Tottori, and Kyoto. An extensive study was performed by Aizawa et al. (1961). They showed a frequent isolation of \( B. \) thuringiensis from sericultural farms of several prefectures: Shizuoka, Gunma, Saitama, Ibaraki, and Yamagata. In some farms, they found that more than \( 10^6 \) \( B. \) thuringiensis spores were contained in 1 g of the dust of the silkworm-rearing room. Subsequently, the distribution of \( B. \) thuringiensis was investigated in sericultural farms of Aichi Prefecture (Ishikawa et al., 1964) and Saitama Prefecture (Ono, 1968).

In parallel with the surveys mentioned above, serological studies were done by Aoki and Chigasaki (1916a, b, 1919). They described a nontoxic “Sotto-Bacillen” strain which was serologically identical to the toxic strain (Aoki and Chigasaki, 1916b) and a toxic sporeformer which was closely related to but was serologically different from “Sotto-Bacillen” (Aoki and Chigasaki, 1919). Although their findings suggested a possible serological classification of this bacterial group, no further investigation was attempted.

Ohba and Aizawa (1978) identified 241 \( B. \) thuringiensis isolates from sericultural farms of Japan and demonstrated the distribution of \( B. \) thuringiensis serotypes 1, 3a, 4a:4b, 4a:4c, 7, 8, and 11. In the present study, 146 crystalliferous sporeformers from 18 sericultural farms of Ehime Prefecture were analyzed by \( H \) agglutination test. The result shows that serotypes 3a, 4a:4b, 7, and 8 are distributed in this prefecture as well as in other sericultural prefectures. Since serotype 4a:4c has been rarely found in Japan (Ohba and Aizawa, 1978) and serotype 10 has not been isolated from other regions of Japan, frequent isolation of these serotypes in Ehime Prefecture is ecologically noteworthy. Norris (1969) suggested that the distribution of serotype 4a:4c related to the stored products environment, however, our result shows the distribution of serotype 4a:4c in an environment other than that of stored products.

Isolation of inclusion-forming serotype 5a:5c in Japan has not been reported previously, whereas acrystalliferous sporeformers possessing 5a:5c \( H \) antigen have been isolated from the soils of Japan (Ohba and Aizawa, 1978).

These results mentioned above suggest that the flora of \( B. \) thuringiensis serotypes varies considerably depending on the locality. Further investigation on the distribu-
tion of *B. thuringiensis* serotypes in Japan and Asian countries will demonstrate geographical characteristics in the ecology of *B. thuringiensis* and related bacteria.

It is interesting that no toxicity to the silkworm larvae was detected in 4 isolates belonging to serotypes 5a:5c and 10. Comparative study between these isolates and the reference strains is in progress.

REFERENCES


De Barjac, H. and A. Bonnefoi (1972) Presence of H antigenic subfactors in serotype V of *Bacillus thuringiensis* with the description of a new type: *Bacillus thuringiensis* var. canadensis. *J. Invertebr. Pathol.* **20**: 212–213.


