New Japanese Isolates of *Bacillus popilliae* Isolated from Milky Diseased Larvae of *Popillia japonica*, *Anomala rufocuprea* and *Anomala daimiana* (Coleoptera: Scarabaeidae)

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Three new pathogenic bacteria were isolated from milky diseased larvae of *Popillia japonica* Newmann, *Anomala rufocuprea* Motschulsky and *Anomala daimiana* Harold (Coleoptera: Scarabaeidae) reared in the laboratory after field collection from golf courses in Sapporo in 1993. The morphological features regarding bacterial spores, infectivity host range and plasmid DNA profiles in these isolates showed different types of strains of *Bacillus popilliae*. Milky diseased larvae of *P. japonica* and other beetles have not yet been reported in Japan. This report is therefore the first of its kind in Japan.

*Key words:* milky disease, *Bacillus popilliae*, Japanese beetle, *Popillia japonica*, new isolates

INTRODUCTION

After Dutky's (1940) first description of milky disease in *Popillia japonica* (Coleoptera: Scarabaeidae), the disease was found in other insect species in New Zealand (Dumbleton, 1945; Fowler, 1972), Australia (Beard, 1956; Milner, 1974), India (David and Alexander, 1975) and Europe (Wille, 1956). However, there has, to date, been no report of milky disease in Japan. Many golf courses have been established over the last decade in Japan, and grass damage by Japanese beetles has also increased recently. Therefore, surveys for pathogenic bacteria were conducted in the more damaged golf courses, in an effort to determine valuable pest control agents.

In 1993, thousands of larvae were collected from golf courses in Sapporo and reared in the laboratory. They were identified as the Japanese beetle (*Popillia japonica*), soybean beetle (*Anomala rufocuprea*) and cherry chafer (*Anomala daimiana*). During the rearing period, milky diseased grubs were found among these larvae. The pathogenic bacteria were isolated from the 3 insect species.

In this report, the morphological characteristics, infectivity host range and plasmid DNA profiles of the newly isolated bacterial pathogens are described.

MATERIALS AND METHODS

*Bacterial strains.* After field collection, third-stadium larvae of *Popillia japonica*, *Anomala rufocuprea* and *Anomala daimiana* were reared in the laboratory. A few larvae from all 3 species were infected with the milky disease. Bacteria were isolated from infected larvae and named *Bacillus popilliae* var. *popilliae* Mame, var. *popilliae* Hime and var. *popilliae* Sakura, after the

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host beetles from which the bacteria were isolated. The reference strain was var. *popilliae*, which was provided by Fairfax Biological Laboratory in 1980 and refrigerated at 4°C until this experiment.

![Image of scanning electron micrographs](image)

**Fig. 1.** Scanning electron micrographs of *B. popilliae* and the newly isolated strains. a: reference strain, *B. popilliae* var. *popilliae*; b: Sakura strain, var. *popilliae* Sakura; c: Hime strain, var. *popilliae* Hime; d: Mame strain, var. *popilliae* Mame.
SEM observation. Spores were isolated from cadavers of milky diseased insects by centrifugation. The procedure of centrifugation was that followed by Baba et al. (1990). Isolated bacterial spores were gold-coated and observed for morphological characteristics with a Hitachi S-800 Scanning Electron Microscope (SEM).

Bioassays. Bioassay procedures were basically those of St. Julian et al. (1963, 1968). Larvae of the Japanese beetle (Popillia japonica), soybean beetle (Anomala rufocuprea), cherry chafer (Anomala diamiana) and striped chafer (Anomala testaceipes) were collected from the Makomanai Country Club in Sapporo. Larvae of 2 other beetles, spotted chafer (Blitopertha orientalis) and yellowish elongate chafer (Heptophylla picea), were collected from the courtyard of the Faculty of Agriculture at Hokkaido University and used for bioassay. In order to determine the host ranges of these spores, 6 larvae of each host species were used and 1 million spores were injected per larva per os or injection by microsyringe.

Disease symptoms. The extent of infection in an intact larva could be determined visually by gently pressing the larva’s posterior toward its anterior and observing the opacity and color of its hemolymph at the posterior.

Rearing of larvae. Larvae were individually reared on turf grass grown on autoclaved soil in plastic cups at room temperature (25°C).

Isolation of plasmid DNA. Plasmid DNA was isolated from var. popilliae and 3 other strains by a modified procedure of Valyasevi et al. (1990). Spores of these strains were cultured on a high-phosphate plate, and cells were then pelleted by centrifugation (10,000 rpm, 5 min) and suspended in 6.7% sucrose-Tris EDTA-salt buffer. The suspension was stored at −80°C in a deep freezer for 10 min, and then incubated for 30 min at 37°C. Cells were mixed with lysozyme (20 mg/ml), incubated at 37°C for 60 min, and lysed by addition of alkaline SDS. An equal volume of 5 M NaCl in TES buffer was added to the cleared lysate, and the mixture was repeatedly deproteinized by phenol until the aqueous phases became translucent. After treatment by chloroform:isoamyl alcohol (24:1), DNA was precipitated by addition of 2 volumes of 95% cold ethanol (−20°C) and centrifuged down (15,000 rpm, 30 min).

Table 1. Host range of three Bacillus popilliae isolates bioassayed against six beetles

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Type of inoculation</th>
<th>Percentage of infection (infected larvae/tested larvae)</th>
<th>var. popilliae Mame</th>
<th>var. popilliae Hime</th>
<th>var. popilliae Sakura</th>
</tr>
</thead>
<tbody>
<tr>
<td>Popillia japonica</td>
<td>injection</td>
<td>50.5% (48/95)</td>
<td>35% (7/20)</td>
<td>30% (6/20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>per os</td>
<td>5 (1/20)</td>
<td>15 (3/20)</td>
<td>5 (1/20)</td>
<td></td>
</tr>
<tr>
<td>Anomala daimiana</td>
<td>injection</td>
<td>5 (1/20)</td>
<td>10 (1/10)</td>
<td>35 (7/20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>per os</td>
<td>0 (0/20)</td>
<td>10 (1/10)</td>
<td>0 (0/20)</td>
<td></td>
</tr>
<tr>
<td>Anomala rufocuprea</td>
<td>injection</td>
<td>0 (0/5)</td>
<td>40 (2/5)</td>
<td>20 (1/5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>per os</td>
<td>0 (0/5)</td>
<td>20 (1/5)</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Mimela testaceipes</td>
<td>injection</td>
<td>6.7 (1/15)</td>
<td>20 (2/10)</td>
<td>6.6 (1/15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>per os</td>
<td>0 (0/15)</td>
<td>0 (0/10)</td>
<td>0 (0/15)</td>
<td></td>
</tr>
<tr>
<td>Blitopertha orientalis</td>
<td>injection</td>
<td>40 (24/60)</td>
<td>58.3 (35/60)</td>
<td>73.3 (44/60)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>per os</td>
<td>31.7 (19/60)</td>
<td>33.3 (20/60)</td>
<td>43.3 (26/60)</td>
<td></td>
</tr>
<tr>
<td>Heptophylla picea</td>
<td>injection</td>
<td>0 (0/10)</td>
<td>0 (0/10)</td>
<td>0 (0/10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>per os</td>
<td>0 (0/10)</td>
<td>0 (0/10)</td>
<td>0 (0/10)</td>
<td></td>
</tr>
</tbody>
</table>

* One million spores were inoculated per larva.

b Number of larvae.

c Not tested.
RESULTS

Isolation of 3 B. popilliae var. popilliae strains

Field-collected larvae developed obvious symptoms typical of milky disease in the laboratory. In addition, the microscopic observations revealed opacity and whitened hemolymph at the posterior of the larvae. Isolated spores from each milky diseased larva are shown in Fig. 1. Morphological differences between isolates were observed in spores and parasporal bodies. The size of the parasporal bodies in var. popilliae was smaller than those of the 3 other var. popilliae strains. Though the morphological shapes with spores and parasporal bodies in var. popilliae and var. popilliae Sakura were similar, the parasporal bodies were directly attached on the top of the spores, and those of var. popilliae Hime and var. popilliae Mame strains were irregularly attached.

Host range of var. popilliae strains

Three bacterial strains isolated from P. japonica, A. rufocuprea and A. daimiana were tested using 6 beetle species (Table 1). In the case of infectivity of var. popilliae Mame, larvae of P. japonica developed the highest infection rate (50.5%), followed by B. orientalis (40%). The infection rates for the 3 other beetles were too low to calculate.

The numbers of larvae which tested positive against A. rufocuprea were fewer than those against the other species, especially for var. popilliae Hime, because of the difficulty in collecting larvae of A. rufocuprea in the field. However, infectivity for larvae of A. rufocuprea, B. orientalis and P. japonica were revealed, indicating a wider host range than for var. popilliae Mame. Only larvae of Heptophylla picera showed no infection.

The results for var. popilliae Sakura indicated that larvae of B. orientalis developed high

![Fig. 2. Plasmid DNA profiles of three isolated strains of Bacillus popilliae. Lane 1: λ-HindIII digested marker; lane 2: B. popilliae var. popilliae Mame; lane 3: var. popilliae Hime; lane 4: var. popilliae Sakura.](image)
infectivity (73%), followed by larvae of *A. daimiana* (35%) and *P. japonica* (30%).

**Plasmid DNA profiles of three strains of var. *popilliae***

The plasmid DNA profiles of these 3 bacterial strains and var. *popilliae* were examined. The results of the agarose gel electrophoresis analysis of DNAs from the 3 isolates are shown in Fig. 2. The DNA profile of var. *popilliae* was the same as that for var. *popilliae* Mame. The Hime isolate demonstrated no detectable plasmid DNA. Therefore, the parasporal body gene should be located on chromosomal DNA in the Hime isolate. The plasmid DNA profiles of the Mame and Sakura isolates were indistinguishable, having 9.8 and 5.5 kb plasmids, respectively.

**DISCUSSION**

Milky diseased larvae of *P. japonica* and other beetles have not yet been reported in Japan. In this study, milky diseased larvae from 3 beetles, *P. japonica*, *A. rufocutrea* and *A. daimiana*, were found. Bacterial spores of these isolates showed different morphological features from the isolate of *P. japonica*. Infectivity and host range of these isolates against beetles were also different. These isolates were named var. *popilliae* Mame, var. *popilliae* Hime and var. *popilliae* Sakura, according to spore morphology and host ranges from which bacteria were isolated.

Milner (1974) reported the shape of parasporal bodies of *Bacillus popilliae* var. *melolontha* and var. *rhopaea*, as cuboidal. In our isolates, the var. *popilliae* Mame and Sakura parasporal bodies form had rhomboidal forms and were attached to the spores. The plasmid patterns of these 3 isolates also differed among var. *popilliae* Mame and Sakura and var. *popilliae* Hime.

Dingman (1994) demonstrated plasmid patterns of several *B. popilliae* strains. The plasmid profiles of var. *popilliae* Mame and Sakura were similar to the KLN3 and KLN4 reported by Dingman (1994). The var. *popilliae* Hime strain has no plasmid. Since this strain produces parasporal bodies, parasporal body genes should be located on chromosomal DNA. In this study, 3 new *B. popilliae* strains were discovered in Sapporo. In addition, their infectivities against the beetle larvae were different, and the discovery of these 3 strains presented the possibility of their use in pest control against beetles sensitive to these isolates.

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