Serological Comparison of Three Milky Disease Isolates

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Two European milky disease isolates, Bacillus frisbourgensis and Bacillus popilliae melolontha, were compared serologically with Bacillus popilliae. A close relationship exists between all three pathogens. Double diffusion experiments and a complement fixation test suggest that the European milky disease pathogens are identical whereas B. popilliae shows distinct differences. Two antigens of the European isolates are not shared by B. popilliae, which has one specific antigen that is not present in B. frisbourgensis or B. popilliae melolontha.

INTRODUCTION

The first isolation of a milky disease pathogen was reported from North America by Dutky (1940). Since then insects naturally infected with milky disease were found in Europe (Hurpin, 1955; Wille, 1955), in Australia (Beard, 1956) and in New Zealand (Dumbleton, 1945). The milky disease pathogens exhibit a number of common properties which are reviewed by Dutky (1963).

Serology plays an important role in the identification of insect pathogens within the species of Bacillus thuringiensis. De Barjac and Bonnefoi (1963) introduced a very useful system based on flagellar antigens. Krywienczyk and Angus (1960) investigated the antigenic properties of the endotoxin of B. thuringiensis. The only serological study conducted on milky disease organisms is that of Hrubant and Rhodes (1968). They compared strains of B. popilliae and B. lentimorbus with other spore-forming insect pathogens.

In recent studies on milky disease pathogens, a distinct difference between the European isolates and B. popilliae was found (Lüthy, 1968; Lüthy et al., 1971). In the work reported here a serological method was used to obtain additional information on the relationship among these pathogens.

1 Contribution No. 164 from this Institute.

MATERIALS AND METHODS

The following milky disease pathogens were used:

Bacillus frisbourgensis (Wille, 1956), strain LBG B 4263, Institute of Microbiology, Swiss Federal Institute of Technology, Zurich, Switzerland.

Bacillus popilliae melolontha (Hurpin, 1955), Laboratoire de Biocénétique et de Lutte Biologique, La Minierre par Versailles, France.

Bacillus popilliae (Dutky, 1940), strain B 2309 S, Northern Utilization Research and Development Division, U. S. Department of Agriculture, Peoria, Illinois.

All three strains were grown in test tubes containing a biphasic egg yolk medium (Lüthy, 1968). The liquid phase of the medium had the following composition: 0.05% glucose (added after sterilization of the medium), 0.2% yeast extract (Difco), 0.2% casamino acids (Difco), 0.2% K2HPO4·3H2O, 0.2% KCl, 0.05% MgSO4·7H2O, 0.005% CaCl·2H2O. The pH was adjusted to 8.0 with KOH before autoclaving in order to reach a pH value of 7.3 after sterilization. In vivo cultured spores were used as inoculum. They were heat-shocked in the medium for 20 min at 80°C prior to incubation. The incubation temperature was 28°C.
Cells were harvested by centrifugation in the middle of the logarithmic growth phase. At this stage most of the cells were still motile. The sediments were washed three times in 0.1 M potassium phosphate buffer, pH 7.2. The washed pellets were resuspended in buffer and adjusted to a cell concentration of $2 \times 10^9$ cells/ml. Subsequently the cells were ruptured by sonication (MSE ultrasonic disintegrator, 60 W, 20 kcycles/sec, Measuring and Scientific Equipment Ltd., London, England). The total time of the ultrasonic treatment, carried out under ice-water cooling, was 2 min, divided into four 30-sec periods at intervals of 1 min to avoid overheating of the suspension. Microscopic examination showed that the disintegration was complete. The suspensions were stored at $-18^\circ$C until they were used as antigens.

Rabbits were injected subcutaneously four times with progressively higher volumes of antigenic suspensions (0.1, 0.2, 0.4, and 0.8 ml). The injections were given in complete Freund’s adjuvants at intervals of 1 wk. Beginning 20 days after the last subcutaneous injection, intravenous injections were given every third or fourth day during 3 wk. Finally the animals were bled and the sera were separated and stored at $-18^\circ$C without additives.

Double diffusion tests were performed using agarose and cellulose acetate membranes (Millipore Filter Corp., Bedford, Massachusetts). For detailed description of the methods see Krywienczyk and Bergold (1961) and Krywienczyk et al. (1969).

The method used for the complement fixation was previously described by Krywienczyk and Bergold (1960). The tests were performed in small disposable trays.

### TABLE 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>$B. fribourgensis$</th>
<th>$B. popilliae melolontha$</th>
<th>$B. popilliae$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$B. fribourgensis$</td>
<td>28,000</td>
<td>20,000</td>
<td>7,880</td>
</tr>
<tr>
<td>$B. popilliae melolontha$</td>
<td>5,120</td>
<td>4,500</td>
<td>2,560</td>
</tr>
<tr>
<td>$B. popilliae$</td>
<td>2,550</td>
<td>3,840</td>
<td>10,240</td>
</tr>
</tbody>
</table>

* Reciprocal values of the highest dilution of antiserum giving fixation of 25–50% with the optimal dilution of the antigen.
(Linbro Chemical Co. Inc., New Haven, Connecticut) with cup capacities of 0.3 ml. The total volume of the reactants including sheep blood cells amounted to 0.09 ml per well. Two units of complement and a 0.5% suspension of sheep erythrocytes were used. The reciprocal dilutions of the antisera were compared on a level that gave 25–50% fixation. The optimal dilution of the antigens ranged between 1:200 and 1:300.

RESULTS AND DISCUSSION

The serological experiments show that the European milky disease isolates are related to B. popilliae. However, B. fribourgensis and B. popilliae melolontha have two specific antigens, a and b, that are not shared by B. popilliae (Fig. 1). One antigen, c, of B. popilliae is not present in the other two pathogens (Figs. 1, 2). The antigenic pattern of B. fribourgensis and B. popilliae melolontha suggests a high degree of similarity or even identity. Compared with B. fribourgensis, the antiserum against B. popilliae melolontha was not strong enough to yield precipitation lines with antigens a and b in the double diffusion experiment.

The results of the complement fixation which are presented in Table 1 confirm the double diffusion experiments. The antisera against B. fribourgensis and B. popilliae melolontha fix the complement with B. popilliae at an appreciably lower level than in their homologous and heterologous reaction.

The results of the serological comparison which are presented in Table 1 confirm the double diffusion experiments. The antisera against B. fribourgensis and B. popilliae melolontha fix the complement with B. popilliae at an appreciably lower level than in their homologous and heterologous reaction.

The results of the serological comparison are in accordance with investigations conducted on the morphology of the parasporal bodies, on the behavior of in vitro cultures, and on host specificity (Lüthy et al., 1971). B. popilliae melolontha and B. fribourgensis always demonstrated a high degree of correspondence whereas B. popilliae distinctly differed from the two European isolates.

REFERENCES


