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Symbiotes of Planthoppers : I. The Isolation of Intracellular Symbiotes from the Smaller Brown Planthopper, *Laodelphax striatellus* FALLÉN (Hemiptera : Delphacidae)

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Two yeast-like microorganisms were isolated from the eggs of the smaller brown planthopper, *Laodelphax striatellus* FALLÉN. They grew both on solid and liquid media. By using immunological techniques with the antisera against those strains, they could be identified with the intracellular symbiotes present in the eggs, the ovaries of adult female, and the fat bodies of abdomen of the insects.

INTRODUCTION

Intra- and extra-cellular symbiotic microorganisms have been observed in various insects (BUCHNER, 1965). As these symbiotes are thought to play important physiological roles in their hosts (BROOKS, 1962: BUCHNER, 1965: KOCH, 1967), many investigators have tried to isolate them. However, most of the work relating to the isolation of intracellular symbiotes from insects is not reproducible nor reliable.

In the smaller brown planthopper, *L. striatellus*, yeast-like symbiotes were observed in the mycetocytes of eggs, fat bodies of abdomen and ovaries of adult female (NASU, 1963). These symbiotes are transmitted to their progeny by incorporating the yeast-like symbiotes into the eggs in the ovary (NASU, 1963). Although MITSUHASHI (1975) succeeded in isloating a yeast-like organism from the medium of the cultured cells of this insect, he failed to keep it growing.

To elucidate the physiological repercussions of intracelluar symbiotes on the host, it was deemed necessary to develop a method for the isolation of the intracellular symbiotic microorganisms from various insects. The isolation of intracellular yeast-like symbiotes from the eggs of *L. striatellus* and their identification with the symbiotes living in the eggs by using immunological techniques are described in this report.

MATERIALS AND METHODS

Biological Materials. The smaller brown planthopper, *L. striatellus*, was reared on

Table 1. MICROORGANISMS USED FOR THE TEST OF THE SPECIFICITY OF ANTISERUM AGAINST ISOLATED SYMBIOTES

Hansenula anomala	*Candida parapsilosis var. intermedia*
Saccharomyces rouxii Boutroux	*Schizosaccharomyces pombe Lindner*
Rhodotorula glutinis	*Saccharomyces cerevisiae Hansen*
Pichia membranaefaciens Hansen	*Saccharomyces bisporus var. mellis Van der Walt*
Candida albicans	

rice seedlings at 25°C. Original insects used were made available by Prof. Shojiro ISHII, Kyoto University. Prior to the isolation of symbiotic microorganisms, the eggs of the insects were sterilized by immersion for 3 minutes in 0.2% Hyamine T solution (Sankyo Co., Tokyo), followed by washing with sterile distilled water and by dipping into 75% ethanol for 3 minutes. Microorganisms used for the tests of specificity of antiserum against isolated symbiotes are listed in Table 1. These microorganisms are known to carry surface antigens differing serologically.

Media. For the isolation of symbiotic microorganisms, Grace TC medium (GRACE, 1962) containing 50 μg per m*l* of both penicillin and streptomycin, but not supplemented with immobilized hemolymph. Bacto YM agar and Bacto YM broth (Difco Laboratories, Detroit, Michigan) were used as stock and for the culture of the isolated microorganisms, respectively.

Antisera. Antiserum against the microorganisms isolated was prepared by repeated injection of 0.1 m*l* aliquots of cultured cells suspended in saline (ca. 10^{10} cells/m*l*) every 3 days into the footpads of guinea pigs. The blood was collected one week after the eighth injection. Immunoglobulin G was precipitated by the addition of solid ammonium sulfate to the sera up to 50% saturation and the precipitate was dissolved in PBS[1] to give one tenth of the original volume. FITC-conjugated rabbit antiserum against guinea pig IgG (Miles Laboratories Inc., Kankakee, Illinois) was used for the indirect fluorescent antibody staining of the symbiotic microorganisms.

Indirect Fluorescent Antibody Staining. Indirect fluorescent antibody staining was performed by the modified method of WELLER et al. (1954). Mycetocytes isolated from either the embryos or the fat bodies of the abdomen of the insects were placed on a glass slide and smears were prepared. After treatment in 90% ethanol for 10 minutes, the smears were incubated with 0.1 m*l* of the partially purified antibodies against the isolated microorganisms for 30 minutes at 37°C, followed by washing with PBS, then treated with 0.05 m*l* of diluted FITC-conjugated rabbit anti-guinea pig IgG antibodies (1:50) for 30 min at 37°C, and washed with PBS. Prior to the examination under fluorescent microscopy, an adequate supply of a mixture of 0.5 *M* carbonate-bicarbonate buffer (pH 9.0): glycerol (1:9) was placed on the slide.

RESULTS

Isolation and Properties of Symbiotes from Eggs of the Smaller Brown Planthopper

The surface sterilized eggs of the insects, *L. striatellus*, were suspended in 0.5 m*l* of sterile water and homogenized in a Potter-Elvehjem type teflon homogenizer. 0.1

[1] Abbreviation used: PBS, phosphate buffered saline solution; FITC, fluorescein isothiocyanate; IgG, immunoglobulin G.

m*l* aliquots of the homogenate were placed on the agar plate containing the modified Grace TC medium and were incubated at 25°C. Two different yeast-like symbiotic microorganisms were isolated from the colonies growing on the plates.

One of them (Ls–1) multiplied by budding and formed white colonies. The cells of these colonies were ellipsoidal shaped (4.7×2.2 μm) (Fig. 1a). The other strain (Ls–2) also grew by budding and formed yellowish white colonies. The shape of the cell consisted of elongated rods (1.5×7.6 μm) (Fig. 1b). The cells of Ls–1 and Ls–2 were prone to undergo lytic processes during the incubation. Both strains changed their cell shapes from yeast-like ellipsoidal and rod forms into filamentous ones as their colonies became older (Figs. 2a and 2b). Characteristics of both Ls–1 and Ls–2 were kept unchanged over 5 years on YM agar slants.

Immunological Identification of the Isolated Microorganisms with Those Living inside the Eggs

For the identification of the isolated Ls–1 and Ls–2 with the yeast-like symbiotes of the smaller brown planthopper, the specificity of the ammonium sulfate fractionated guinea pig IgG against these microorganisms was examined by using FITC-conjugated rabbit anti guinea pig IgG antibodies. As is obvious from the results shown in Table 2, the antibodies against either Ls–1 or Ls–2 were absorbed specifically onto the surface of the cells used for immunization, though there were some indication that they have certain antigens in common. In addition, these antibodies were not adsorbed by any other microorganisms listed, see Table 1.

As the strict specificity of the antibodies against the isolated yeast-like microorganisms had been confirmed, the immunological identity with the symbiotes living inside the eggs was then examined with these antibodies. The yeast-like symbiotes

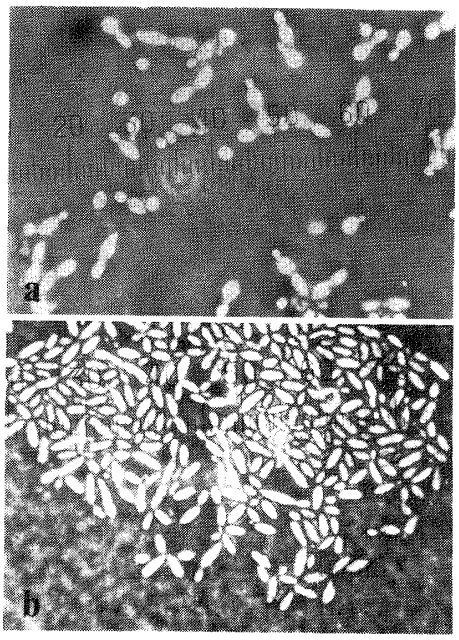

Fig. 1. Yeast-like symbiotes isolated from *L. striatellus*. (a) Eillipsoidal shaped cells (Ls-1), light-microscopy, ×400. (b) Elongated rods cells (Ls-2). ×400.

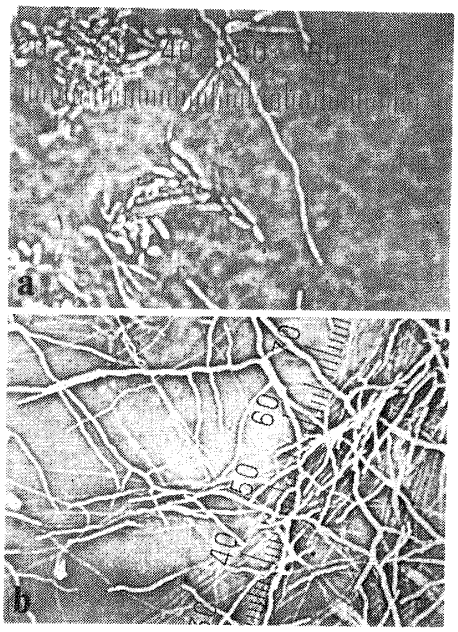

Fig. 2. Filamentous forms of yeast-like ellipsoidal (Ls-1) (a) and rod (Ls-2) (b) cells, light-microscopy, ×200.

Table 2. SPECIFICITY OF ANTIBODIES AGAINST Ls-1 AND Ls-2 AS SHOWN BY THE INDIRECT IMMUNOFLUORESCENT METHOD

Antibody	Microorganisms tested	Staining reaction[a]
anti Ls-1	Ls-1	+
anti Ls-1	Ls-2	±
anti Ls-1	symbiotes	+
anti Ls-1 adsorbed with Ls-1	symbiotes	−
anti Ls-1 adsorbed with Ls-2	symbiotes	±
anti Ls-2	Ls-1	±
anti Ls-2	Ls-2	+
anti Ls-2	symbiotes	+
anti Ls-2 adsorbed with Ls-1	symbiotes	+
anti Ls-2 adsorbed with Ls-2	symbiotes	−

[a] +, Stained well; ±, Stained faintly; −, Not stained.

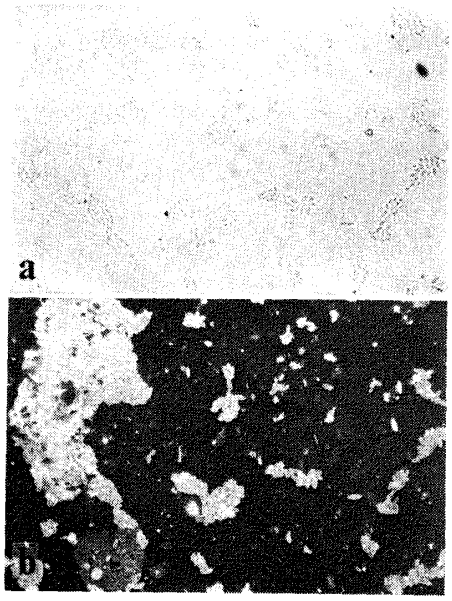

Fig. 3. Stained symbiotes in the mycetocytes of *L. striatellus* by anti Ls-1 IgG. After staining by the indirect immunofluorescent method using specific anti Ls-1 IgG, microphotographs were taken under ordinary (a) and UV-light (b), ×200.

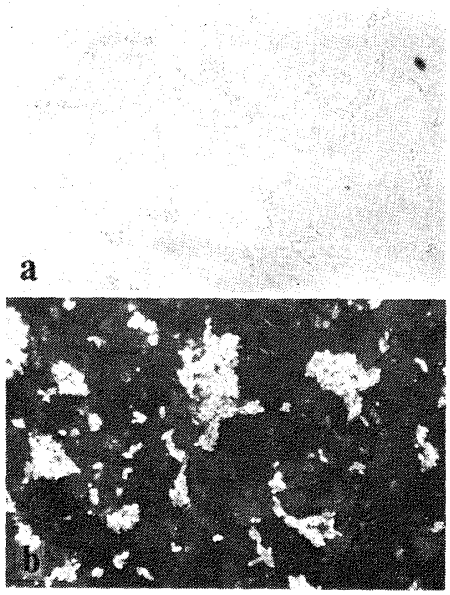

Fig. 4. Stained symbiotes in the mycetocytes *L. striatellus* by anti Ls-2 IgG. Microphotographs were taken under ordinary (a) and UV-light (b), ×200.

found in the mycetocytes of insects were treated first with either one of the guinea pig IgG prepared, followed by the treatment with FITC-conjugated rabbit anti guinea pig IgG antiserum. Photographs shown in Figs. 3a and 3b were taken under ordinary and UV-light after treatment with anti Ls-1 antibodies. These photographs demonstrate clearly that some of the symbiotic microorganisms carry surface antigens in common with Ls-1. When treated with anti Ls-2 antibodies in the same way, most of the yeast-like symbiotes adsorb the antibodies against Ls-2 (Figs. 4a and 4b). In addition, as the antibodies against Ls-1 and Ls-2 are applied simultaneously to the

smear of the mycetocytes of the insect, the symbiotic microorganisms are stained without exception. These observations strongly suggest that the two yeast-like microorganisms isolated from eggs of the smaller brown planthopper are the microorganisms harboured in the mycetocytes of the host insects.

DISCUSSION

The characteristics of intracellular symbiotic microorganisms attracted the attention of entomologists long ago. In spite of the abundant literature relating to the existence of such symbiotes, there are very few reports dealing with the isolation of these microorganisms except those published in the early 1900s. Yeast-like symbiotes have been found by NASU (1963) in the smaller brown planthopper and the isolation of a symbiotic microorganism has been reported by MITSUHASHI (1975) during the cultivation of cells derived from the insects. The present paper appears to be the first to report the successful isolation of the intracellular symbiotes from the same insect. The two strains of symbiotes, Ls–1 and Ls–2, grow by budding and undergo lytic processes when their growth is hindered by unfavorable conditions which may account for the difficulty in isolating the intracellular symbiotic microorganisms.

The isolated microorganisms were identified with those living inside insect cells by using the indirect fluorescent antibody staining method. Antibodies against these two microorganisms are adsorbed specifically by the cells injected for immunization, which indicates that the antigens located on the surface of these strains are not shared by any other microorganisms tested. Furthermore, as the antibodies against Ls–1 and Ls–2 are applied simultaneously on the smear of the mycetocytes of the insects, the symbiotic microorganisms are always stained. These results indicated that the two yeast-like microorganisms are the symbiotes of the smaller brown planthopper, *L. striatellus*, and at least two symbiotic microorganisms are harboured inside the mycetocytes of the smaller brown planthopper.

A paper describing the effect exerted by these microorganisms on the host insect is in preparation.

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