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Staphylococcus, Paramyxovirus-like, Rickettsia-like and Other Structures in *Peregrinus maidis* (Homoptera, Delphacidae)

E. D. AMMAR,[1] L. R. NAULT,* W. E. STYER,* AND Y. M. SAIF†

*Department of Entomology, and †Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio 44691

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In an ultrastructural study of the delphacid planthopper, *Peregrinus maidis*, vector of maize mosaic virus (MMV) and maize stripe virus (MStpV), the following structures were found in several organs of MMV-inoculative, MStpV-inoculative, and noninoculative insects: (a) paramyxovirus-like particles (PLP), (b) filamentous rhabdo-like structures (FRS), (c) spiked double-membrane structures (SDS) that were always associated, and sometimes contiguous, with FRS, and (d) rickettsia-like structures (RLS). In salivary gland acini, the PLP, FRS, and SDS were usually found in extracellular spaces between basal infoldings of plasma membranes, whereas the RLS were intracytoplasmic or intranuclear. The PLP and FRS appeared to bud through plasma membranes. The colony of *P. maidis* in which these structures were found suffered from retarded development and premature death. Limited trials to isolate a paramyxovirus from this colony were unsuccessful. However, further trials led to the isolation of *Staphylococcus sciuri*, which proved pathogenic when injected into *P. maidis* and two cicadellid leafhoppers; *Dalbulus maidis* and *Graminella nigrifrons*.

KEY WORDS: Leafhopper, planthopper, maize mosaic virus, maize stripe virus, insect vector, *Staphylococcus sciuri*, paramyxovirus, rickettsia.

INTRODUCTION

During the course of ultrastructural studies to locate maize mosaic (MMV) and maize stripe (MStpV) viruses in their *Peregrinus maidis* (Ashmead) vector (Ammar and Nault, 1985), we found structures unrelated to either virus. Some resembled paramyxoviruses, some resembled rickettsiae, and others were more difficult to identify. The colony of *P. maidis* containing these structures declined rapidly and eventually was lost. This report describes the various structures found in *P. maidis* and trials to isolate pathogens from diseased insects that resulted in the isolation of *Staphylococcus sciuri*, Kloss et al., a bacterium not previously reported to infect invertebrates. Ultrastructural studies on the salivary glands and other organs of *P. maidis* are reported elsewhere (Ammar, 1985, 1986).

[1] Visiting Professor from Department of Economic Entomology, Faculty of Agriculture, Cairo University, Giza, Egypt.

MATERIALS AND METHODS

P. maidis and its preparation for electron microscopy. Two colonies of *P. maidis* were used in the present work: colony F, originally obtained from Florida, and colony H, obtained from Hawaii. The colonies were maintained separately on sweet corn seedlings (cv. Aristogold Evergreen Bantam) at 25°–30°C and 18 hr light/day.

Adults (2–5 weeks old) of *P. maidis* (colony F), prepared for ultrathin sectioning and electron microscopy were either inoculative or noninoculative for MMV and/or MStpV. These adults were dissected live in potassium phosphate buffer (*p*H 7.4). Excised organs were fixed in glutaraldehyde and OsO_4, dehydrated in an ethanol–acetone series, embedded in Spurr's medium, sectioned, stained, and examined as described previously (Ammar

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and Nault, 1985). Negatively stained preparations from the liquid body contents of diseased or dead *P. maidis* were prepared as follows. Small drops from these contents were drawn with a capillary from the abdomen under the integument; these were placed on a coated grid, stained in phosphotungstic acid, and examined by electron microscopy.

Infectivity tests. For tests 1–3, extracts from diseased or dead insects of *P. maidis* (colony F), prepared by macerating insects in PBS (phosphate-buffered saline) or PBST (PBS + Tween) buffers (*p*H 7), were tested for infectivity by injection into healthy *P. maidis* nymphs (colony H). Azide was not added to the PBS or PBST, since earlier tests proved that azide is harmful to test insects. In tests 4 and 5, suspensions of various bacterial isolates grown in broth for 24 hr, were injected into nymphs of *P. maidis* (colony H) and of two leafhopper species, *Dalbulus maidis* (DeLong & Wolcott) and *Graminella nigrifrons* (Forbes), both Cicadellidae. Nymphs (fourth to fifth instars) were lightly anesthetized with CO_2 and then injected abdominally with ca. 0.025 μl of inoculum/insect, using microcapillary glass needles. In test 5, bacterial suspensions in broth were also tested by feeding them, with 10% sugar added as a feeding stimulant, to nymphs of *P. maidis* (colony H) for 24 hr through a parafilm membrane. In tests 1–3, control insects were injected with similar extracts from healthy insects (colony H), and in tests 4 and 5 control insects were injected or fed with sterile broth. In each test, 28–40 insects/treatment were used. After treatment, test insects were maintained on sweet corn seedlings at 25.5°C. Dead or diseased insects were collected once or twice a day, examined immediately for symptoms (see Results) using a stereomicroscope, and then placed in a freezer until used to obtain disease extracts. χ^2 analysis was used to compare mortality rates in various treatments.

Testing for paramyxovirus. Crude PBST extracts of diseased or dead *P. maidis* that had been injected with extracts from diseased nymphs of colony F in infectivity test 1 were centrifuged at 5000 rpm. The supernatant was filtered through a 0.45 μm filter and inoculated into the amniotic cavity of 10-day-old embryonating specific-pathogen-free chicken eggs (SPAFAS Inc., Norwich, CN). Incubation was continued for 96 hr and the eggs were candled daily for embryo mortality. The amniotic/allantoic fluid was harvested at the end of the incubation period and tested for hemagglutinating activity (HA) using chicken red blood cells.

Culturing of bacteria. Crude PBS extracts of diseased or dead *P. maidis* that had been injected with extracts from diseased insects in infectivity test 2 were cultured on bovine blood (5%) agar and transferred to brain heart infusion broth.

RESULTS

Structures found in P. maidis

The following structures were frequently observed in organs of adult *P. maidis* (colony F); structures were observed in MMV-inoculative, MStpV-inoculative, and noninoculative adults of *P. maidis* (colony F).

Paramyxovirus-like particles (PLP). These were quasi-spherical bodies, bound by a single (trilamellar) membrane, and covered with spikes (surface projections) (Fig. 1). The diameter of these particles, including spikes, usually ranged from 112 to 160 nm (mean ± SE = 134.4 ± 2 nm). However, a few particles were much larger, up to 363 nm in diameter. Contents of the PLP were electron semiopaque with some opaque areas. These particles were found in the salivary glands, nerve ganglia, ovarioles, and leg muscles. In the salivary glands, they were usually found in extracellular spaces between basal infoldings of the plasma membrane near the periphery of acini (Fig. 2). In MMV-inoculative insects, the PLP were found in these spaces along

with MMV particles (Fig. 3). Occasionally in some tissues, e.g., ovarioles and connective tissues, structures resembling the PLP appeared to bud through plasma membranes (Fig. 4).

Filamentous rhabdo-like structures (FRS). These were filamentous, flexible, sometimes branched structures, that reached 800 nm in length (Fig. 5). They were apparently bound by a single membrane covered with spikes (Figs. 6, 7). In cross sections, their diameter, including the spikes, averaged 77.6 ± 3.7 nm (range = 64–85 nm). However, some of the FRS had a bulbous end that averaged 103.7 ± 7.7 nm in diameter (range = 92–117 nm). The FRS were found in the same organs of *P. maidis* as the PLP. Both structures had similar accumulation sites, and were sometimes found together in the salivary glands (Fig. 6). Also, some of the FRS appeared to develop through plasma membranes (Figs. 7, 8). Usually, the FRS were much more abundant than the PLP, particularly in older adults.

Spiked double-membrane structures (SDS). These structures resembled the PLP in being quasi-spherical bodies covered with spikes, but differed in being much larger and normally bound by two membranes (Figs. 5–8). The outer membrane of the SDS was more prominent than the inner membrane, which, in some smaller SDS, was difficult to visualize (Fig. 7). The SDS averaged 394 ± 16 nm in diameter (range = 213–480 nm). They varied in electron opacity, and appeared to contain granular and, in some cases, fibrous material (Fig. 6). In a few instances, the SDS appeared to invaginate slightly on two opposite sides, resembling early stages of division (Fig. 5, arrow). The SDS were always associated with the FRS. Frequently, the latter structures appeared contiguous with, and extending from, envelops of the SDS (Figs. 5, 7).

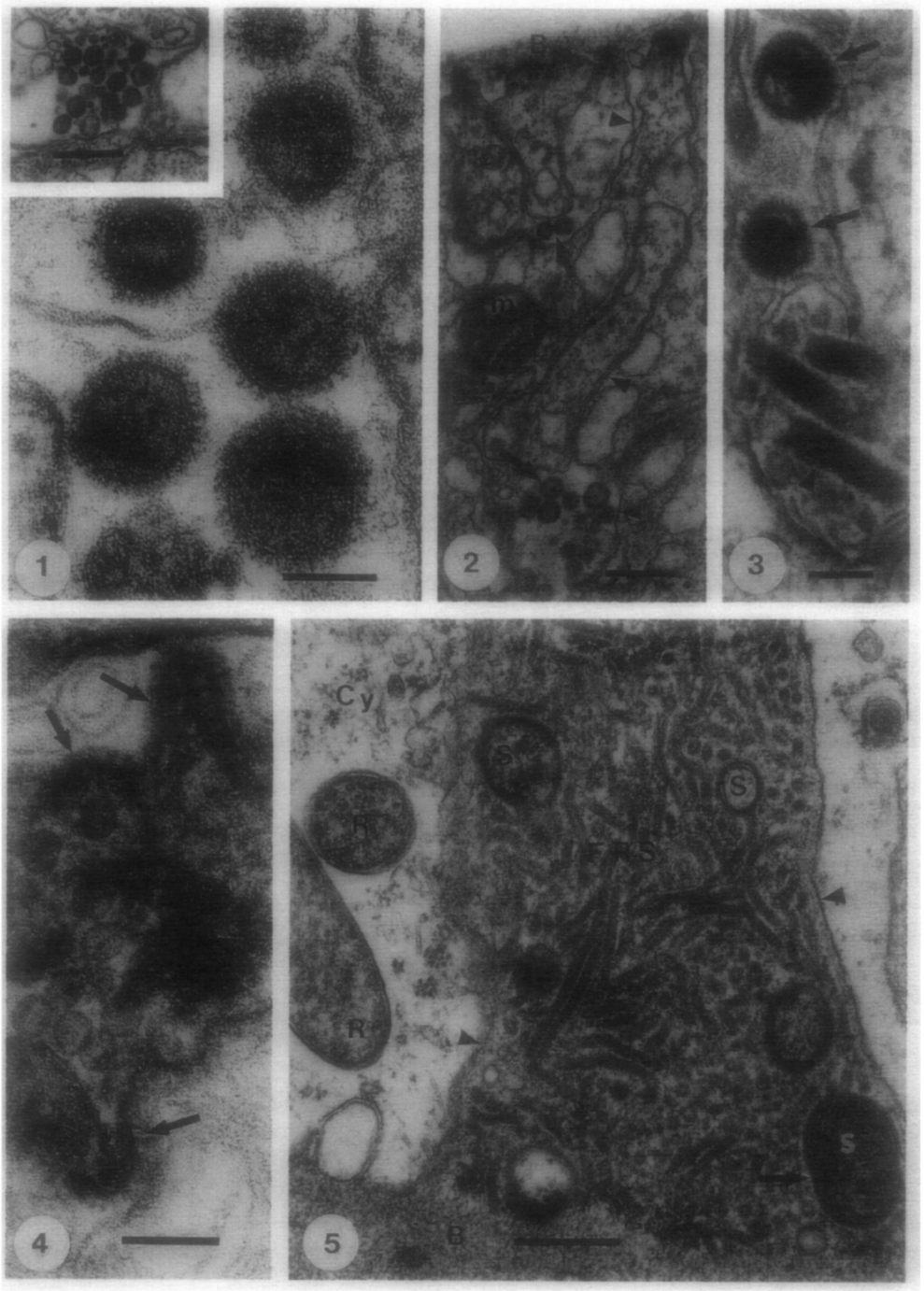
Rickettsia-like structures (RLS). These were elongated bodies bound by two distinct membranes with no spikes (Figs. 5, 9). The RLS reached 1.7 μm in length and their diameter averaged 457.5 ± 15.6 nm (range = 352–686 nm). They were usually surrounded by an electron-lucent area, probably occupied in vivo by the slime layer known for several rickettsiae (Weiss, 1982). The RLS were found in several tissues of *P. maidis* including the salivary glands, midgut epithelium, ovarioles, and fat tissue. They were located in the cytoplasm, either free (Fig. 5) or enclosed as a group within a single-membrane bound vesicle. Occasionally, they were also found clustered in the nucleus (Fig. 9).

Infectivity Tests and Trials to Isolate a Pathogenic Agent

The colony F of *P. maidis,* in which the above described structures were found, was apparently suffering from a disease manifested in unusually high mortality and retarded nymphal growth. Consequently, in a few months, despite vigorous efforts to maintain this colony, numbers declined from a few thousands to only two late-instar nymphs. These last survivors were used to propagate the suspected disease in healthy *P. maidis* of colony H and to perform the following infectivity tests.

Test 1. Crude extracts, prepared by macerating the 2 surviving nymphs from colony F in 0.5 ml PBS, were injected into 30 nymphs of healthy *P. maidis* (colony H). Three days later, mortality of treated nymphs was 93%, compared to 3% in control nymphs ($p < 0.001$). Treated nymphs were much darker and their integument was abnormally distended and fragile. In negatively stained preparations from these nymphs, no paramyxovirus or other virus-like particles were detected but massive numbers of quasi-spherical, cell-walled structures, that averaged 684 ± 30 nm in diameter were found.

Test 2. In this test, the disease inoculum (DI) was prepared by macerating 9 diseased or dead insects (obtained in test 1) in 3 ml PBST, followed by centrifugation at 5000 rpm to remove particulates. A similar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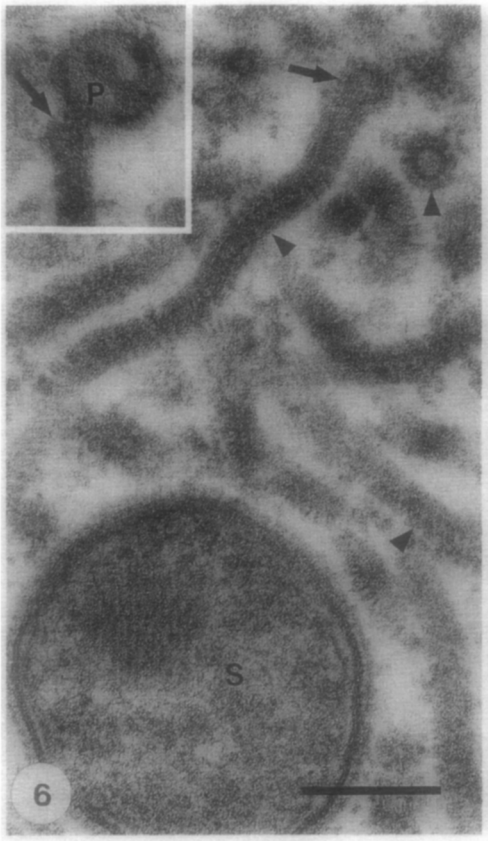
preparation from healthy insects was used as the control inoculum (CI). Four groups of healthy *P. maidis* nymphs (colony H) were each injected with one of the following (28 insects/treatment): (A) the supernatant of DI; (B) pellet of DI, resuspended in PBST; (C) the supernatant of CI, and (D) the resuspended pellet of CI. Mortalities at 4 and 8 days, respectively, for these groups were: (A) 35.7 and 42.9%, (B) 57.1 and 71.4%, (C) 10.7 and 17.9%, and (D) 10.7 and 17.9%. The differences between treatments (A) and (C) were significant ($p < 0.05$) only on Day 4, whereas those between treatments (B) and (D) were highly significant on both Days 4 and 8 ($p < 0.01$ and 0.001, respectively). Negatively stained preparations from insects that died in treatment (B) only, contained great numbers of the spherical cell-walled structures described in test 1. When the supernatant of DI was injected into embryonated chicken eggs, no mortality occurred in the embryos and no HA activity was detected in the allantoic fluid. Three blind passages in embryonating eggs yielded negative results.

Test 3. The disease inoculum was prepared by macerating 18 diseased or dead insects (obtained from treatments (A) and (B) of test 2) in 3 ml PBS. This crude extract was filtered through a 0.45 μm filter. Three groups of healthy nymphs of *P. maidis* were each injected with one of the following: (40 insects/treatment), (A) filtered DI, (B) unfiltered DI, (C) unfiltered crude extract from healthy nymphs. Mortality 4 and 8 days following these treatments, respectively, were: (A) 5 and 7.5%, (B) 87.5 and 90%, and (C) 17.5 and 22.5%. The differences in mortality rates, on both dates, between treatments (A) and (C) were nonsignificant, but those between (B) and (C) were highly significant ($p < 0.001$).

Test 4. The results of tests 1–3 indicated that a quasi-spherical, cell-walled, rather large and filterable agent was most probably involved in the disease of *P. maidis*. Thus, crude PBS extracts of diseased or dead insects, obtained from treatments (A) and (B) of test 2, were cultured on blood agar. Four distinct types of bacterial colonies were isolated. Arranged descendingly according to their abundance in these cultures, these were: (1) gram-positive cocci, (2) mucoid colonies of gram-negative cocci, (3) small gram-negative bacilli, and (4) large gram-variable (mostly negative) bacilli. Broth cultures of these isolates were injected into healthy nymphs of *P. maidis*, and control nymphs were injected with sterile broth. Mortalities on Days 4 and 8, respectively, following injection with these isolates (40 insects/treatment), were: (1) 92.5 and 92.5%, (2) 42.5 and 47.5%, (3) 17.5 and 32.5%, (4) 35 and 50%; and control, 17.5 and 30%. Differences in mortality rates between treated and control nymphs were significant only with isolate 1 ($p < 0.001$). When extracts from diseased or dead insects obtained in this treatment were cultured on blood agar, only isolate 1 resulted. This isolate, which proved to be coagulase negative, mannitol and DNase

FIGS. 1–4. Paramyxovirus-like particles (PLP) in *Peregrinus maidis:* (1) PLP at high and low (inset) magnifications; bars = 100 and (inset) 500 nm. (2) PLP (arrows) in extracellular spaces between basal infoldings of plasma membrane (arrowheads) of a salivary gland secretory cell; B, basal lamina; m, mitochondrion; bar = 500 nm. (3) PLP (arrows) with particles of maize mosaic virus (arrowheads); bar = 100 nm. (4) PLP apparently budding (arrows) from a plasma membrane of an ovarian cell; bar = 100 nm.

FIG. 5. A large aggregate of filamentous rhabdo-like structures (FRS) between basal infoldings of plasma membrane (arrowheads) of a salivary gland duct cell; B, basal lamina; Cy, cytoplasm; R, rickettsia-like structures; S, spiked double-membrane structures; one (arrow) is invaginated on opposite sides; bar = 500 nm.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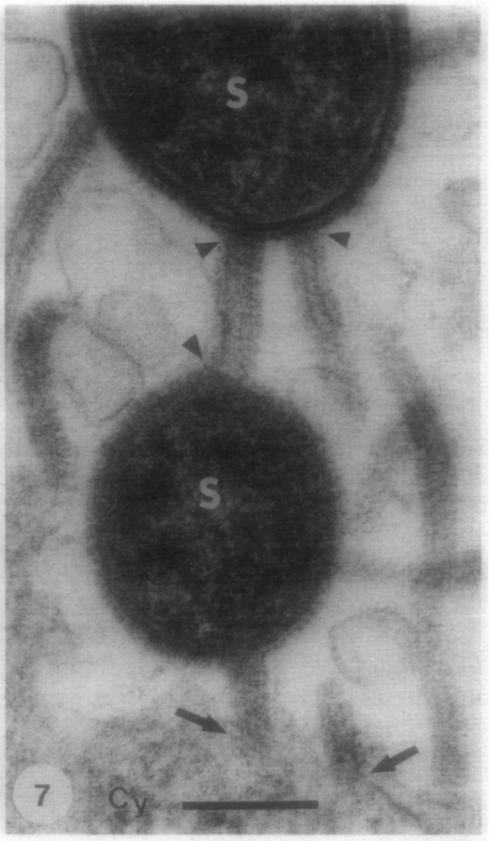
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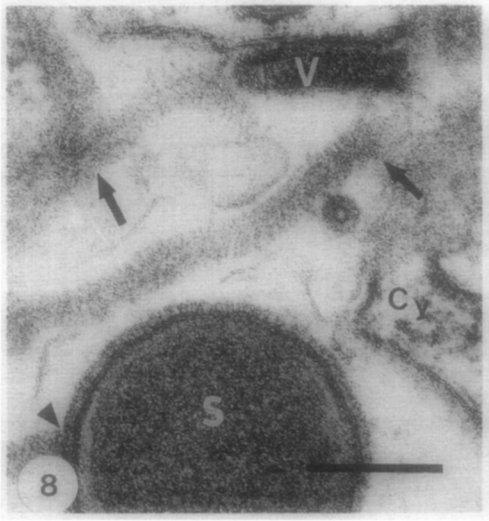
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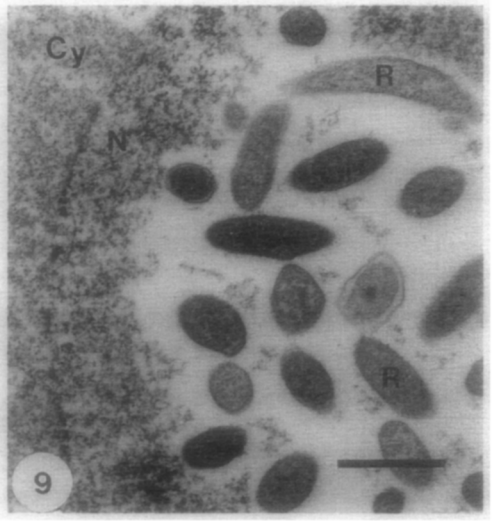
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positive, was identified by the National Veterinary Services Laboratory (Ames, IA), as *Staphylococcus sciuri* Kloss et al.

Test 5. Broth cultures of *S. sciuri* were injected into nymphs of *P. maidis*, *D. maidis*, and *G. nigrifrons* (40 nymphs/species). Four days later, mortality of treated insects was 100% in all three species, whereas mortality in the controls was 5% in *P. maidis* and 25% in the other two species. However, when broth cultures of *S. sciuri* with added sugar were fed to 30 nymphs of *P. maidis*, mortality in treated insects 8 days later was 16.7%, not significantly higher than that in the control (6.7%).

DISCUSSION

Previous ultrastructural studies on the planthopper *P. maidis* revealed the presence of rhabdovirus particles of MMV (Herold and Munz, 1965; Ammar and Nault, 1985), a spherical "latent" *Peregrinus* virus (Herold and Munz, 1967), and apparently symbiotic yeast-like organisms (Ammar, 1985). None of these agents is similar to the PLP, FRS, or SDS reported here.

The PLP resemble animal paramyxoviruses in shape, size, general ultrastructure, and apparent budding from plasma membranes (Compans and Choppin, 1973; Palmer and Martin, 1982). Paramyxoviruses have not been reported previously from insects, although "myxovirus-like" particles were found in extracts from diseased honey bees in Ohio (Kulincevic et al., 1969), and an orthomyxovirus, swine influenza virus, apparently can multiply in an invertebrate, the swine lung worm (Bellett et al., 1973).

The FRS resemble the filamentous forms of some paramyxoviruses, e.g., respiratory syncytial virus (Plate 5, p. 57 in Palmer and Martin, 1982). Additionally, the similarity of accumulation and budding sites and occasional association between the FRS and the PLP suggest that the FRS are probably the filamentous forms of the PLP. FRS probably is not a developmental stage of MMV or other rhabdovirus because (a) FRS occurred in insects never exposed to MMV, and (b) no rhabdovirus particles were found in these insects.

The SDS are difficult to associate with other known organisms. Their larger size, double membrane, and possible division suggest a nonviral agent. But their spiked envelope, constant association, and apparent contiguity with envelopes of the FRS are perplexing. Ultrastructure of the inner contents of some SDS seemed similar to the RLS, but the latter structures, compared to the former, were larger, more elongated, not covered with spikes, and had different accumulation sites. The RLS resemble several rickettsiae in size and ultrastructure, and resemble *Rickettsia rickettsii* in being intranuclear as well as intracytoplasmic (Weiss, 1982). Rickettsiae have been reported in other invertebrates as pathogens and symbiotes (Weiss and Dasch, 1981).

Staphylococcus sciuri, but not three other bacteria, isolated from *P. maidis* was pathogenic when injected into *P. maidis*, *D. maidis*, and *G. nigrifrons*. *S. sciuri*, recently elevated to species status from subspecies *S. scuiri scuiri* (Schileifer et al.,

FIGS. 6–8. Filamentous rhabdo-like structures (FRS) and spiked double membrane structures (SDS) (bars = 200 nm): (6) FRS (arrowheads), some with a bulbous end (arrows), associated with the SDS (S) and with paramyxovirus-like particle (P in inset). (7, 8) FRS, apparently contiguous with envelopes of the SDS (arrowheads), or extending from obliquely sectioned plasma membranes (arrows) in salivary gland. Cy, cytoplasm; S, SDS; V, maize mosaic virus.

FIG. 9. Rickettsia-like structures (R) in the nucleus (N) of a midgut cell. Cy, cytoplasm; bar = 1 μm.

1983), was previously isolated from human and animal skin (Kloos et al., 1976; Kloos and Schileifer, 1981) and from bovine intramammary infections (Brown, 1983). Thus it appears that *S. scuiri* invades mainly epidermal and subepidermal tissues, which may explain why it was not encountered in thin sections of "excised" internal organs of *P. maidis* in the present work. Another possible explanation is that sections of *S. scuiri* were confused with those of the RLS. However, Briggs (1958) killed *Bombyx mori* larvae with injected *Staphylococcus aureus*. A *Staphylococcus* sp. was reported to be pathogenic to *Trichogramma* spp. in China (Zhelong and Yi, 1984). The disease symptoms in *Trichogramma* are similar to those found in *P. maidis*. In the latter case, although pure cultures of *S. sciuri* induced disease in tests 4 and 5, it is uncertain that this bacterium was the agent or the sole agent responsible for the disease condition originally found in colony F of *P. maidis*. It is possible that other agents, e.g., a paramyxovirus or a rickettsia as suggested by ultrastructural observations, might have been involved. Failure to isolate them may have been due to their lower concentration in initial extracts or to the unsuitability of methods used to isolate and propagate them.

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