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Microbes Associated with Parthenogenesis in Wasps of the Genus *Trichogramma*

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Cytological evidence is presented for a complete correlation between the presence of microorganisms in eggs and the incidence of revertible parthenogenesis (thelytoky) in wasps of the genus *Trichogramma*. A 2% lacmoid stain was used to visualize microorganisms in wasp eggs. Eggs of all 13 revertible parthenogenetic lines (i.e., lines that can be rendered bisexual by antibiotic treatment) carry microorganisms, while the eggs of all 5 nonrevertible parthenogenetic lines (i.e., lines that cannot be reverted to bisexual reproduction by either temperature or antibiotic treatment) are free of microorganisms. Microorganisms were not detected in eggs of 6 field-collected bisexual (arrhenotokous) lines, nor in those of 3 bisexual lines derived from revertible parthenogenetic lines by antibiotic treatment. The lacmoid stain provides a fast, easy method to detect microorganisms in the *Trichogramma* eggs and may be used in a modified form for the detection of microorganisms in the eggs of other species.

KEY WORDS: Microorganisms; *Trichogramma* spp.; symbionts; parthenogenesis; thelytoky; cytology; lacmoid.

INTRODUCTION

Cytoplasmically inherited microorganisms have been associated with offspring sex ratio distortion and sex conversion in many species of arthropods (Ebbert, 1992; LeGrand *et al.*, 1987). An extreme form of sex ratio distortion is complete parthenogenesis or thelytoky, in which virgin females produce female offspring. Such parthenogenetic lines can exist indefinitely without the involvement of males. Recently, microorganisms have been implicated in complete parthenogenesis in wasps of the genus *Trichogramma* (Stouthamer *et al.*, 1990a,b). Thus far, the evidence for microbial involvement in causing parthenogenesis consisted of showing (i) the extrachromosomal nature of the parthenogenesis trait (Stouthamer *et al.*, 1990a; Stouthamer, 1990) and (ii) the permanent reversion to normal bisexual reproduction when parthenogenetic lines are treated with specific antibiotics (sulfamethoxazole, rifampin, or tetracyclinehydrochloride) or elevated rearing temperatures (>30°C) (Stouthamer *et al.*, 1990a, 1990, 1991). However, no cytological evidence has been presented for the presence of microorganisms in eggs of parthenogenetic females.

Two variants of complete parthenogenesis are now known in *Trichogramma* (Stouthamer *et al.*, 1990a). The first can be reverted to bisexuality by treatment with antibiotics or high temperatures (revertible parthenogenesis), whereas in the second, parthenogenesis appears to be irreversible since neither antibiotics nor high temperatures lead to a reversion to bisexuality (nonrevertible parthenogenesis). Here we use a lacmoid staining technique to determine whether microorganisms are present in the eggs of (i) revertible parthenogenetic females, (ii) nonrevertible parthenogenetic females, (iii) bisexual (arrhenotokous) females from field-collected lines, and (iv) bisexual (arrhenotokous) females derived from revertible parthenogenetic lines by antibiotic treatments.

MATERIAL AND METHODS

Wasp culture origin. Collection details of the North American *Trichogramma* strains are given in Stouthamer *et al.* (1990a,b) and Pinto *et al.* (1991). The European *Trichogramma* are identified by the collection number of the Institute National de Recherches Agronomique (INRA), Antibes, France. Revertible parthenogenetic lines are *T. brevicapillum* (Wapato, WA), *T. cordubensis* (Cordoba, Spain), *T. deion* (Sanderson, Tex.; Belle Fourche, S. Dak.; Mountain Center, CA; Irvine, CA; Hemmet, CA), *T. pretiosum* (Teran, Nuevo Leon, Mexico; Quibor, Lara State, Venezuela; Kauai, HI), *T. oleae* (Strain No. 2 INRA), *T. platneri* (New Casle, CA) and *T. rhenana* (Strain NO. M36 INRA). Nonrevertible parthenogenetic lines are *T. cacoeciae* (Strain No. 91 INRA), *T. embryophagum* (Strain No. 45 INRA), *Trichogramma* sp. (Garberville, CA; Agnew, WA; Washington, DC) The last three strains have not been named because males, needed for species identification, have not been found (Stouthamer *et al.*,

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1990b). Field-collected bisexual lines are *T. deion* (Seven Pines, CA; Marysville, CA), *T. platneri* (New Castle, CA), *T. pretiosum* (Irvine, CA; Kununurra, Australia) and *T. nr. exiguum* (Santa Rita, Mexico). In the antibiotic-treated lines, bisexual lines were derived by treatment with tetracyclinehydrochloride (Stouthamer, 1990a) from the revertible parthenogenetic lines *T. deion* (Sanderson, TX; Irvine, CA) and *T. pretiosum* (Nuevo Leon, Mexico). The wasp cultures were maintained on irradiated *Trichoplusia ni* (cabbage looper) eggs as described in Stouthamer *et al.* (1990b).

Staining procedure of Trichogramma *eggs.* Newly emerged wasps were kept in culture vials, containing several streaks of honey as a food source, for at least 24 hr. Subsequently, 5 to 10 host eggs (*T. ni*) were put in the vial for 30–60 min. Under these conditions the wasps superparasitized the hosts, and up to 30 wasp eggs per host could be found. These superparasitized hosts were dissected in a drop of *Drosophila* ringer solution on a siliconized microscope slide within 2.5 hr after oviposition had taken place. The host eggs debris was removed so that only *Trichogramma* eggs remained in the drop. The chorion of the *Trichogramma* eggs was removed by replacing the drop of *Drosophila* ringer with a drop of bleach ringer (dilution of household bleach in *Drosophila* ringer, 1:100 by volume). The bleach ringer was removed after approximately 3 min and replaced with a drop of freshly made (<24 hr old) Carnoys fixative (3:2.5:1 of 99% ethanol:chloroform:glacial acetic acid). The dechorionated *Trichogramma* eggs were fixed in this solution for about 3 min after which most of the Carnoys was removed without allowing the *Trichogramma* eggs to dry out. Next the eggs were covered with a drop of 70% ethanol which was removed after 2–3 min and replaced with a drop of 2% lacmoid stain [2% lacmoid (Pfaltz & Bauer, Research Chemical Division, Stamford, CT, Cat. No. L06030) in a 1:1:1 solution of water:lactic acid:glacial acetic acid]. A coverslip was placed on the eggs in the lacmoid stain and the excess of stain was removed from under the coverslip which then was sealed with clear acrylic nail polish. Darkly staining microorganisms could be clearly seen under oil at 1000× magnification using this procedure. Sealed slides were kept in the refrigerator and remained usable for several days but over time the background became darker and reduced the contrast between the microorganisms and the cytoplasm. The number of microorganisms were counted in the stained eggs of parthenogenetic strains of *T. deion* (South Dakota and Texas) using an optical micrometer with a grid pattern. The number of microorganisms per grid square (0.01 × 0.01 mm) column was determined by focusing up and down. The total number of microorganisms per egg was determined by summation over all grid squares.

RESULTS

The eggs of all 13 revertible parthenogenetic lines contained microorganisms (see Fig. 1 and examples in Figs. 2A–2C); no microorganisms were visible in the eggs of the 6 field-collected arrhenotokous cultures (example in Fig. 2E), the 4 nonrevertible parthenogenetic lines (example in Fig. 2F), or the 3 arrhenotokous lines derived from parthenogenetic lines by antibiotic treatment (compare Fig. 2A with Fig. 2D). In the eggs of the revertible parthenogenetic lines microorganisms were visible in the tip of the egg away from the micropyle (Fig. 1 and Figs. 2A–2C). The microorganisms surrounded a ball-shaped area (Figs. 1 and 2) known as the germ cell determinant (Tanaka, 1985). Inward from the germ cell determinant, a band of microorganisms was visible. No microorganisms were visible within the germ cell determinant. The germ cell determinant in eggs of all forms also stained to a certain extent, but the dark staining areas (for example, Fig. 2F) form a diffuse web-like structure much less defined than the microorganisms found in the revertible parthenogenetic eggs. Although the microorganisms are concentrated in the tip of the egg, some can be found in the rest of the cytoplasm, where they seem to be present just below the vitelline membrane (Figs. 1 and 2). Their size varied and was generally smallest (about 0.001–0.002 mm long) in the tip of the egg while in the rest of the cytoplasm larger forms could sometimes be found (up to 0.003 mm long). The mean and standard deviation of number of the microorganisms in the eggs of *T. deion* (South Dakota) was 446 ± 132 (n = 14, range 256–668) and in those of *T. deion* (Texas) 319 ± 82 (n = 11, range 289–493).

DISCUSSION

The evidence presented here showed a complete correlation between the presence of microorganisms and

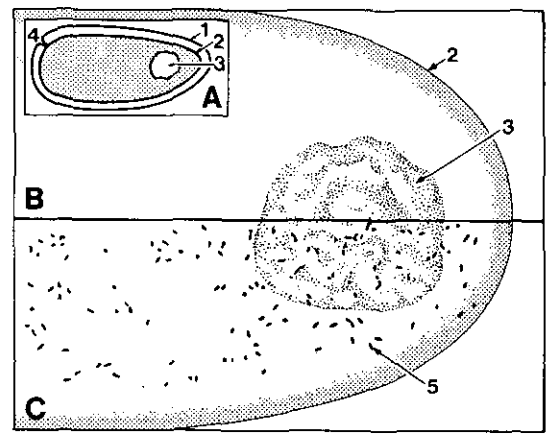

FIG. 1. Diagram of *Trichogramma* egg showing (A) Complete egg with chorion, (B) half of posterior tip of a dechorionated egg free of bacteria, and (C) half of posterior tip of dechorionated egg with bacteria. (1) Chorion, (2) vitelline membrane, (3) germ cell determinant, (4) micropyle, (5) bacteria.

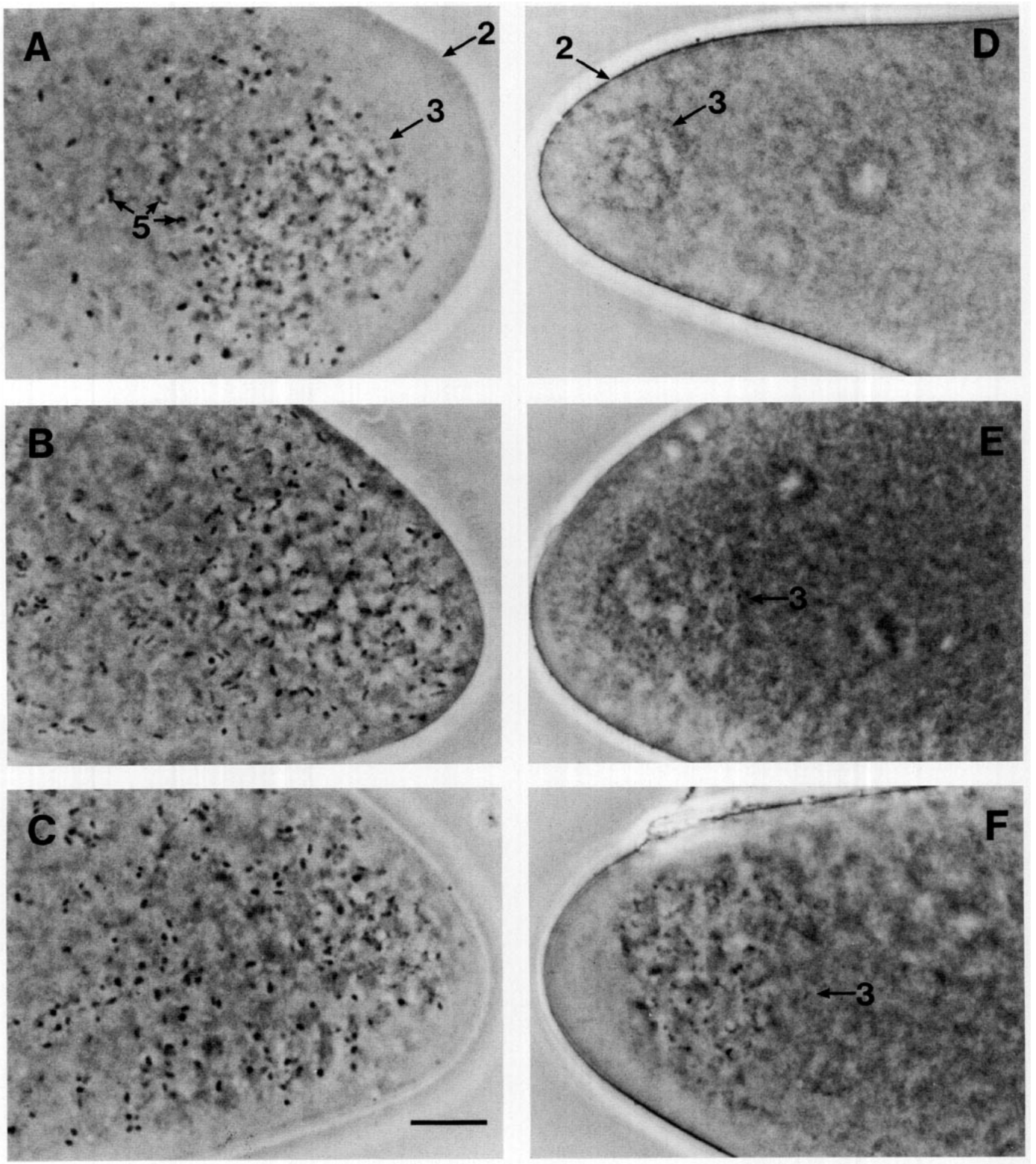

FIG. 2. Light micrographs showing the posterior tip of *Trichogramma* eggs. Eggs of microbe-associated parthenogenetic lines (A) *T. deion* (Sanderson, TX), (B) *T. cordubensis* (Cordoba, Spain), (C) *T. oleae* (INRA No. 2); eggs free of microbes are shown in (D) antibiotic-treated form of microbe-associated parthenogenetic *T. deion* (Sanderson, TX), (E) field-collected bisexual *T. deion* (Seven Pines, CA) and (F) nonrevertible parthenogenetic *T. cacoeciae* (INRA No. 91). For explanation of numbers see legend to Fig. 1 (bar = 10 μm).

the incidence of revertible parthenogenesis. In no case were microorganisms detected in eggs of field-collected arrhenotokous females nor in those of nonrevertible parthenogenetic females.

The location of the microorganisms around the germ cell determinant corresponded to that reported for the cytoplasmic incompatibility microorganisms found in several species of the parasitic wasp genus *Nasonia* (Breeuwer and Werren, 1990). We detected no evidence for movement of the microorganisms out of the germ determinant area during the early part of the embryonic development (i.e., up to the 128 nuclei stage). Nor did we find an association of the microorganisms with nuclei as reported for the cytoplasmic incompatibility microorganisms of *Drosophila melanogaster* (O'Neill and Karr, 1990).

The ultimate proof that the microorganisms seen in the eggs are indeed the cause of parthenogenesis awaits our ability to culture them in some medium. However, the circumstantial evidence presented so far shows a clear correlation between the presence of the microorganisms and the occurrence of revertible parthenogenesis. How widespread the correlation is between microbial infection and parthenogenesis remains to be determined, but more cases are also reported for other wasp families (Stouthamer, 1991; Zchori-Fein *et al.*, 1992). The staining procedures described here can be used as a fast method to establish whether microorganisms are present in the eggs of other strains of parthenogenetic *Trichogramma*. In addition, these methods may be modified to detect microorganisms in the eggs of other insects.

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