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Characteristics of the *Drosophila paulistorum* Male Sterility Agent in a Secondary Host, *Ephestia kuehniella*

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Crosses among the six semispecies of *Drosophila paulistorum* produce sterile male hybrids. This sterility is caused by an agent which has characteristics of a microorganism. It is pathogenic in a secondary host, the larvae of the Mediterranean meal moth, *Ephestia kuehniella,* and can be serially passaged in *Ephestia,* where it is lethal. The agent was passaged back into *D. paulistorum,* where it induced sterility in males of a semispecies different from that of origin of the agent. Infectious particles were obtained from an extract of infected *Ephestia* by ultracentrifugation in a sucrose-Ficoll-metrizamide gradient. Both crude and purified extracts were lyophilized and stored indefinitely without loss of killing power. The agent was destroyed by low pH, lipid solvents, ultraviolet light, and exposure to a temperature of 56°C for 30 min. It appeared to be sensitive to tetracycline and insensitive to penicillin, suggesting that the agent is not a virus, but more likely a cell wall-deficient bacterium or mycoplasma-like organism.

There are six semispecies in the neotropical species complex of the fruit fly, *Drosophila paulistorum.* The semispecies are morphologically indistinguishable, do not normally cross-breed, and remain in reproductive isolation from one another (Fig. 1a). However, when hybrids between the semispecies are produced in the laboratory, male hybrids are sterile (7, 8), and female hybrids produce fertile daughters and sterile sons (Fig. 1b). Nonhybrid females of the paternal semispecies, injected with extracts of testes from sterile male hybrids, produce sterile male progeny (Fig. 1c). Similar results are obtained when extracts of flies of any one semispecies are injected into females of another semispecies. Williamson and co-workers (6, 13, 14) suggested that sterility is transmitted by an infectious agent. All members of the *D. paulistorum* species complex contain intracellular symbionts which morphologically resemble mycoplasmas (mycoplasma-like organisms) and have no discernible effect on their hosts. Large numbers of these mycoplasma-like organisms are seen in the testes of sterile hybrid males (8, 13). In *D. paulistorum* the mycoplasma-like organisms normally pass from mother to offspring via the egg cytoplasm, including the polar cytoplasm, the area of the egg from which the germ cells will arise (5). Thus, hybrids carry only maternal symbionts.

Gottlieb et al. (9) showed that *D. paulistorum* extracts injected into *Ephestia kuehniella* (Mediterranean meal moth) larvae kill the recipients. Extracts of recipient *E. kuehniella* larvae killed not only *E. kuehniella,* but also adult *D. paulistorum.* Some larval *D. paulistorum* recipients of the same *E. kuehniella* extract survived through pupation to adulthood. Fertile adult female recipients produced fertile female and sterile male progeny. The sterility appeared to be due to a specific response in the testes to the presence of material which was genetically incompatible, with respect to the genotype of the recipient (10).

The transmissibility of sterility agent and the presence of the mycoplasma-like organisms led to the suggestion that the agent was the mycoplasma-like organism (6). The production of sterile progeny by surviving *D. paulistorum* larval recipients of extracts of infected *E. kuehniella* indicated that the agent retained specificity through passage. The current report presents results on the physical properties and physiological characteristics of the sterility agent in the secondary host, *E. kuehniella,* and confirms the presence of a microorganism that is involved in the production of hybrid sterility.

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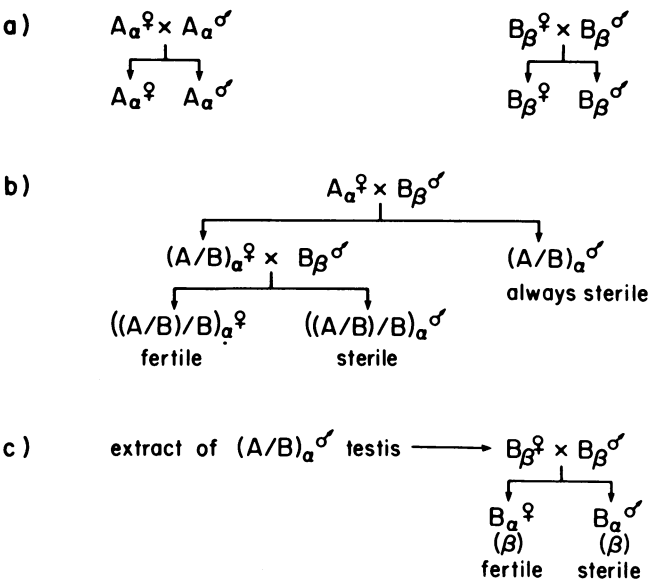

FIG. 1. *Flow diagram of the passage of infectious agent in D. paulistorum. (a) Results of crosses within each of two semispecies; (b) results of a cross between semispecies and of tests of the resulting hybrids; (c) passage of infectious agent into nonhybrid females of the paternal semispecies (B), as a result of the injection of an α containing extract of testes of sterile hybrid males. Key: "A" and "B" represent two different semispecies of D. paulistorum; "A/B" represents a hybrid from a cross between an "A" female and a "B" male; "α" and "β" represent the infectious agent resident in semispecies "A" and semispecies "B," respectively.*

MATERIALS AND METHODS

Organisms. The primary sources of the sterility agent were *D. paulistorum* of the Mesitas or the Santa Marta strains (members of two different semispecies) or hybrids between them. Flies were cultured on either instant media (Carolina Biological or Fisher Scientific Co.) or on freshly made cornmeal-brewer's yeast-agar medium (4).

Larvae of four inbred laboratory strains of *E. kuehniella*, two wild-type strains, and two eye pigmentation mutant strains were used. *E. kuehniella* were cultured at 25°C in plastic containers on commercial cornmeal. Last instar larvae were collected from mass matings and stored for up to a year (2) in the refrigerator at 4°C in petri plates.

Injection procedure. Healthy last instar *E. kuehniella* larvae are approximately 10 to 12 mm long and 2 mm in diameter. Animals used for injection were returned to room temperature (25°C) and etherized for 6 to 7 min. Injection needles were produced by pulling 1-mm-outside-diameter, thin-wall glass microcapillary tubing (Drummond). The microneedle was inserted along the central axis of one of the cylindrical abdominal prolegs of the larva. When the needle was withdrawn, the proleg acted as a self-sealing valve and prevented loss of the injected material. A Hamilton microliter syringe, a repeating dispenser fitted with a three-way valve, and Tygon tubing were used to deliver precise doses of 2 μl. After injection, larvae were placed in petri dishes containing pleated filter paper and a small amount of cornmeal.

Extract preparation. *E. kuehniella* or *D. paulistorum*, or parts selected from them, were ground with a Teflon pestle in a glass homogenizer containing either sterile Beadle-Ephrussi-Ringer (BER) solution (1) (for *Drosophila* and *Ephestia*) or sterile glass-distilled water (for *Ephestia*). Osmotic protection for the agent was provided by the high concentration of amino acids and lipids in the insect homogenates. Suspensions were subjected to centrifugation for 15 min at 1,050 × *g* in a Sorvall centrifuge to remove cell debris and other heavy particulate material. The supernatant from this centrifugation, referred to as a crude extract, served as inoculum in many experiments. A clarified extract was obtained by subjecting the supernatant to refrigerated centrifugation at 27,300 × *g* for 45 to 90 min and resuspending the pellet. The formation of phenols by the action of enzymes in the hemolymph caused *E. kuehniella* extracts to turn black upon prolonged exposure to the air. To inhibit the formation of toxic phenols, we added 2 to 3 crystals of recrystallized phenylthiourea per ml of extract.

Fresh extracts stored at 4°C for 1 or 2 days showed gradual loss of killing power. For preservation over longer periods extracts were divided into aliquots and frozen at either −17°C or −90°C. At −17°C killing power decreased over a period of months; at −90°C there was an initial drop but no further decline over a 6-month period (9). To assess the suitability of lyophilization for prolonged storage without loss of lethality, extracts were shell frozen in a dry ice-isopropanol slurry, lyophilized, and stored under vacuum at 4°C.

Size of agent. Extracts containing the infectious agent were passed through sterile (Millipore or Metricel) cellulose filters, ranging in pore size between 0.2 and 1.2 μm, or (Nuclepore or Uni-pore) polycarbonate membrane filters, having 0.5-, 0.4-, and 0.2-μm porosities. The killing power of each filtrate was assessed by injection into *E. kuehniella* larvae.

To demonstrate the presence of intact agent on the 0.20-μm Nuclepore membrane filters after they had been used to filter an homogenate, sample filters were placed in sterile 9% sucrose solution and shaken with a Vortex mixer. The solution was injected into *E. kuehniella* larvae.

Reduction of host material. Initially a crude extract was subjected to centrifugation in multiple-step discontinuous gradients. The crude material was layered over either a single 1 M sucrose layer, over a two-layer gradient (1 and 2 M sucrose) or over a three-layer gradient (1, 2, and 3 M sucrose) and centrifuged under refrigeration for 1 to 2 h at 27,300 × *g*, in an H-7 swinging bucket head on a Sorvall RC2-B. Subsequently, we employed a different system in a three-step gradient. A crude extract was layered over the following aqueous solutions of increasing density: (i) 30% sucrose; (ii) 30% sucrose plus 20% Ficoll (Pharmacia Fine Chemicals); (iii) 30% sucrose plus 20% Ficoll plus 30% metrizamide (Sigma Chemical Co.). Twenty percent Ficoll in 1 M sucrose had a density approaching that of 2 M sucrose but with lower osmolarity. Thirty percent metrizamide in sucrose-Ficoll, with a density roughly equivalent to that of 3 M sucrose, was used as the bottom layer to prevent pelleting. Centrifugation was carried out under refrigeration for 15 h at 52,112 × *g*, on a Beckman L2-65B with a Ti 27 swinging bucket rotor.

Preparation of agent for scanning electron microscopy. Material to be examined was collected on the surface of a Nuclepore filter and then fixed with 3% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2. The material on the filter was dehydrated in an ethanol series. The ethanol was replaced with liquid CO_2, and the filter was dried by the critical-point method (3). The dry filter was mounted on an aluminum stub, sputter-coated with gold, and examined in an AMR 1000 scanning electron microscope.

pH sensitivity. Sterility agent-containing extracts were diluted with 0.1 M citrate-phosphate or Veronal buffer solutions of the desired pH, incubated for 3 h at 25°C, and injected into *E. kuehniella* larvae. For comparison, an extract was diluted with BER solution. Buffers without added sterility agent were included in the tests.

Lipid-solvent sensitivity tests. A 1-ml amount of extract was tested in the presence or absence of 0.1 ml of analytical grade chloroform (11). The mixtures were shaken for 10 min and then centrifuged at 1,000 × *g* for 5 min. Samples of the aqueous phase were injected into *E. kuehniella* larvae.

To determine sensitivity to diethyl ether, 1 ml of extract and 0.25 ml of distilled, anhydrous diethyl ether were mixed in a sealed tube and kept for 24 h at 4°C. For comparison, an extract without ether was treated in the same manner. The mixtures were poured into petri plates and swirled for 10 min to remove the ether. The remaining fluids were injected into *E. kuehniella* larvae.

Antibiotic sensitivity. Sterility agent was reconstituted from lyophilized material and mixed with either tetracycline hydrochloride (Pfizer Inc.) or penicillin G (E. R. Squibb & Sons). Mixtures were kept on ice for 30 min before injection of 2-μl samples into recipient *E. kuehniella* larvae. Larvae were injected with either mixture, antibiotic alone or untreated extract to establish killing power or toxicity.

Ultraviolet light sensitivity. Sterility agent-containing extracts were placed in open, sterile petri plates and exposed to 280 or 157.5 μW of ultraviolet light per cm^2 at 254 nm produced by a UVS-12 mineral lamp (UltraViolet Products). Larvae were injected with extracts withdrawn at intervals.

RESULTS

Serial passage of the infectious agent. Homogenates of *D. paulistorum* were used to prepare inocula for injection into *E. kuehniella* larvae. The larvae were injected with either crude or clarified extract. Recipients died within 2 days (Table 1). Moribund larvae from the injected group were homogenized to produce extracts for a second serial passage in *E. kuehniella*; recipients died within 12 h. Subsequent passages gave similar results. Full-strength homogenates of infected *E. kuehniella* kill adult *D. paulistorum* recipients of any semispecies. This result contrasts with the observation that the *D. paulistorum* homogenates never kill *D. paulistorum* recipients.

Potency of lyophilized preparations. Homogenates of *E. kuehniella* infected with Mesitas sterility agent from *D. paulistorum* were lyophilized. Lyophilized material was reconstituted with water to contain the equivalent of three larvae per ml. Potency, as measured by killing power, was determined by injection into *Ephestia* larvae. Resuspended material killed all recipients within 12 h. The 50% lethal dose for reconstituted material, as determined by the Reed and Muench procedure (12), ranged from

TABLE 1. *Effect of serial passage on mortality of inoculated E. kuehniella larvae*

Passage no.	Extract		% Dead[c] at day postinjection:			
	Source and recipient[a]	Type[b]	½	1	1½	2
1	Dp ⇒ Ek	CL	0	65	90	100
		CR	0	60	90	100
2	Ek ⇒ Ek	CL	100			
3	Ek ⇒ Ek	CL	100			

[a] Dp ⇒ Ek, *D. paulistorum* (Mesitas) to *E. kuehniella*, 8 *D. paulistorum* heads per ml. Ek ⇒ Ek, *E. kuehniella* to *E. kuehniella*, 3.0 *E. kuehniella* larvae per ml.

[b] CL, Clarified extract; CR, crude extract.

[c] Twenty larvae per set.

10^5 through 10^7. There was a "tailing effect," i.e., a disproportionately high number of deaths at high dilutions when larvae had survived more concentrated doses. Presumably the aggregation of organisms was responsible for these results. Lyophilization appeared to be an excellent method for maintaining the potency of the sterility agent.

Size of the agent. Homogenates from infected *E. kuehniella* were subjected to passage through either cellulose or polycarbonate filters. Filtrates of homogenates retained killing power after passage through cellulose membranes of 1.2- or 0.65-μm porosity or through polycarbonate membranes of 0.5- or 0.4-μm porosity. Two sterilizing filters, the 0.45-μm cellulose filter and the 0.2-μm polycarbonate filter, prevented passage. Killing power was recovered from these filters when they were blended in a Vortex mixer in sucrose solution. Results were similar when primary *D. paulistorum* extracts were filtered. The sterility agent appeared to be of a size between 0.4 and 0.2 μm.

Reduction of host material. Sterility agent from *D. paulistorum* or infected *E. kuehniella* passed through 2 M sucrose, but not through 3 M sucrose. To avoid the high viscosity and high osmolarity of 2 and 3 M sucrose, we shifted to a sucrose-Ficoll-metrizamide gradient system. As indicated by potency, the agent remained intact in this system.

Extracts of infected *E. kuehniella* subjected to these three-step gradients yielded two regions containing potent material. Material from the interface of the heavy layers contained most of the infectious agent; material recovered from the interface of the less dense layers had some killing power.

Bottom-layer material from infected and from uninfected animals was collected on 0.2-μm porosity Nuclepore filters and prepared for examination by scanning-electron microscopy. Numerous smooth, featureless, nearly spherical particles, of sizes between 0.23 and 0.38 μm in diameter, were seen on the filters containing material from clarified extracts of infected *E. kuehniella*. Similar particles were not found on filters containing clarified extracts of uninfected animals. Thus, some separation from host material could be accomplished by centrifugation.

Physiological properties of the agent. The agent from *Drosophila* or *Ephestia* extracts survived −70°C for prolonged periods, since 4-year-old frozen samples still maintain killing power. The agent tolerated temperatures up to 40°C for 2 h, but not 56°C for 30 min. Repeated (five or more) freeze-thaw cycles or exposure to chloroform or diethyl ether completely destroyed killing power. Killing power was retained in the pH range of 4 to 9 (Table 2). Ultraviolet light destroyed the killing power of the agent; as little as 157.5 μW of 254-nm ultraviolet light per cm^2 for 30 s was effective.

Antibiotic sensitivities. All larvae receiving agent without antibiotic were dead within 1 day. *E. kuehniella* larvae were sensitive to penicillin and tetracycline and showed 80% mortality 6 days after injection with either antibiotic (Table 3). The agent exhibited sensitivity to tetracycline since there was survival of larvae past 1 day when the injection mixture contained tetracycline. After 6 days survival of larvae injected

TABLE 2. *Effects of pH on infectious agent*

Material injected[a]	Days post-injection[b]	No. of *Ephestia* larvae surviving injection at pH:								
		3	3.5	4	4.5	5	6	7	8	9
Veronal buffer	0	21		19		21	18	20	20	20
	½	21		19		21	18	18	19	20
	1	21		17		21	18	16	19	20
	2	21		17		21	18	16	19	19
	3	21		17		21	18	15	19	17
Veronal buffer + extract	0	25		25		25	25	25	25	25
	1	25		25		20	25	24	25	23
	2	24		5		0	0	0	0	0
	3	24		4						
Citrate buffer + extract	0	20	20	20	20	19				20[c]
	½	19	18	20	16	15				16
	1	19	18	0	0	0				4
	2	12	15							0
	3	6	13							

[a] Extracts diluted with 0.1 M citrate or Veronal buffer, incubated 3 h at 25°C.
[b] Zero day indicates number of larvae injected.
[c] Extract plus BER, no buffer added.

TABLE 3. *Effect of tetracycline and penicillin on infectious agent*

Extract concn	Antibiotic[a]	Amt (μg/μl) of antibiotic-extract mixture injected	% Dead[b] at day postinjection			
			½	1	2	6
None	Tetracycline	5	33	33	33	78
10^{-1}	Tetracycline	5	0	53	73	87
10^{-2}	Tetracycline	5	0	0	29	71
10^{-4}	Tetracycline	5	0	53	60	73
None	Penicillin	0.5	0	0	50	80
10^{-1}	Penicillin	0.5	69	100		

[a] Equal quantity of extract was mixed with antibiotic solution to produce the antibiotic-extract mixture. After 30 min of incubation, 2 μl was injected into each *Ephestia* larva. When no antibiotic was added, 10^{-1} and 10^{-2} dilutions killed all larvae within ½ day; 10^{-4} killed all larvae within 1 day.

[b] Ten to fifteen larvae per set.

with the mixture of tetracycline plus extract was the same as that of animals receiving tetracycline alone. This suggests that tetracycline prevented the expression of the agent. Penicillin had little, if any, protective effect.

DISCUSSION

Our evidence strengthens the suggestion that the sterility agent from *D. paulistorum* is a microorganism. The sterility agent present in the extracts of sterile hybrid *D. paulistorum* males can be transferred by injection to *E. kuehniella* larvae, where it causes death (9). The killing power is increased in serial passages through *Ephestia*. The agent induces male sterility in nonhybrid *D. paulistorum* of a different semispecies (9). The alternate host allowed us more easily to determine the properties of the sterility agent, separated from the *D. paulistorum*, with lethality as the criterion of its presence.

The filtration experiments suggest that the agent is larger than 0.2 μm in diameter and less than 0.4 μm. It is inactivated at 56°C for 1/2 h, is sensitive to ultraviolet light and organic solvents, and is stable in the pH range 4 to 9. Our work provides substantial evidence that the sterility agent is a microorganism. The size, the solvent sensitivities, and the fact that tetracycline restricts the killing power whereas penicillin has little effect point to a cell wall-deficient organism, rather than a toxin or viral particle, as the sterility agent.

An improved centrifugation procedure has enabled us to remove much of the host components of extracts. The resulting material containing the agent can be lyophilized and stored indefinitely without loss in potency.

The work with *E. kuehniella* supports the idea that the sterility agent from *D. paulistorum* is a cell wall-deficient organism and that the mycoplasma-like organisms seen in electron micrographic studies of *D. paulistorum* sterile hybrid males (7, 8) could be one and the same.

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