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Streptococcal L-forms isolated from *Drosophila paulistorum* semispecies cause sterility in male progeny

(endosymbiont/hybrid sterility/mycoplasma-like/streptococci)

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ABSTRACT The *Drosophila paulistorum* complex contains six semispecies that do not normally interbreed. In the laboratory, crosses between semispecies produce fertile daughters and sterile sons. Microbial endosymbionts have been observed in all *D. paulistorum* flies that display this male sterility. Streptococcal L-forms have been isolated from the Andean-Brazilian (Mesitas) and Transitional (Santa Marta) semispecies and cultured in artificial medium. Transfer of these L-forms from their native hosts into reciprocal semispecies resulted in sterile male progeny. When L-forms were inoculated into the semispecies from which they had been isolated, most of the male progeny were fertile. Control streptococcal L-forms did not show this sterility pattern.

The term "semispecies" refers to subgroups derived from a single species and implies that the process of speciation is in progress. The six semispecies constituting the neotropical *Drosophila paulistorum* complex have been separated and designated by their geographical distribution. In nature these semispecies do not normally interbreed (Fig. 1*a*), but among hybrids produced in the laboratory, the males are sterile and the females are fertile (Fig. 1*b*). When these hybrid females are backcrossed to males of either parental semispecies, their male progeny are still sterile and their female progeny are fertile (2).

Factors responsible for this sterility can be artificially transmitted (3). When extracts of male flies from one semispecies were injected into females of another, the male progeny from these females were sterile. Similarly, if the extracts injected were of hybrid males that had a mother of a different semispecies than that of the recipient females, the same sterility pattern occurred (Fig. 1*c*).

Electron microscopy has revealed that all semispecies of the *D. paulistorum* complex contain cytoplasmic endosymbionts (2, 4)—i.e., each member harbors a semispecies-specific symbiont with which it coexists. A hybrid fly inherits its chromosomes from both parents. It inherits its cytoplasmic endosymbiont only from its mother. The transmissibility of sterility by injection can be explained as a result of the transfer of an endosymbiont from its native semispecies to a different one. For example, in Fig. 1 *b* and *c*, the endosymbiont α contained in semispecies A (and in the extract of sterile hybrid males), when passed by mating or injection into females of semispecies B (containing symbiont β), causes sterility of the male progeny.

In our earlier studies, the host range of the sterility producing agent had been extended to larvae of *Ephestia kuehniella*, the Mediterranean meal moth (5). Studies in this alternate host suggested that the agent was most likely a cell wall-deficient microbe, perhaps a mycoplasma or similar organism (1).

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In this publication, we report the isolation on artificial media of cell wall-deficient organisms from two of the *D. paulistorum* semispecies. These organisms, commonly called L-forms, demonstrated a semispecies-specific male sterility pattern.

METHODS

Insects. We tested the Mesitas (Andean-Brazilian) and Santa Marta (Transitional) semispecies for the presence of cell wall-less organisms. The origin and history of these two *D. paulistorum* semispecies have been detailed elsewhere (6). Flies were cultured on "Instant Medium" (Carolina Biological, Burlington, NC); methods for handling flies have been described (7). Attempts at isolations of microbes were made from entire Santa Marta flies and from excised Mesitas testes.

Culture Medium for Isolation of Wall-Less Microbes. The primary isolation of L-forms from Mesitas flies was attempted with an SP4 broth formulation (8) supplemented with sorbitol at 7% (wt/vol) final concentration. For the culture of L-forms from Santa Marta flies, we used SP4 medium with a sorbitol concentration at 9%. Subsequently, the sorbitol was replaced with 12% sucrose (wt/vol) and both fetal bovine serum and yeast products were omitted from the SP4 formulation.

A solid medium was prepared by omitting phenol red and adding Noble agar (Difco) at a final 0.8% (wt/vol) concentration. For a semisolid medium, we incorporated agar at 0.05% (wt/vol) final concentration into SP4 broth. Two-milliliter aliquots of liquid or semisolid media were dispensed into 1-dram screw-capped vials (Wheaton Scientific, Millville, NJ). For suppression of contaminating bacterial growth, the SP4 L-form media contained sodium ampicillin injectable (Wyeth) at 1.0 mg/ml final concentration. In some isolation attempts, thallous acetate (Fisher) was added at 500 μg/ml final concentration.

The SP4, Hayflick and co-workers (9), M10B (10), and A7 (11) media formulations were employed in attempts to isolate Mollicutes, including mycoplasmas, acholeplasmas, spiroplasmas, and ureaplasmas. All media formulations were pretested to make certain they would support the growth of appropriate Mollicutes.

L-Forms Used as Controls. *Streptococcus* ADA-L, a stable L-form, was obtained from the National Institutes of Health, Bethesda, MD. This group A streptococcal L-form, used in our injection experiments, was grown on SP4 agar, but with 5% (wt/vol) sodium chloride replacing sorbitol or sucrose as an osmotic stabilizer. An L-form, designated EK1, was isolated in our laboratory from *E. kuehniella* larvae and maintained on SP4 medium with sucrose and ampicillin.

Reversion of L-Forms to Bacteria. To obtain parent bacterial revertants of our L-form isolates, organisms were cultured on either the modified SP4 agar as described above or on trypticase soy broth medium (Baltimore Biological Laboratory) supplemented with 12% (wt/vol) sucrose and 1%

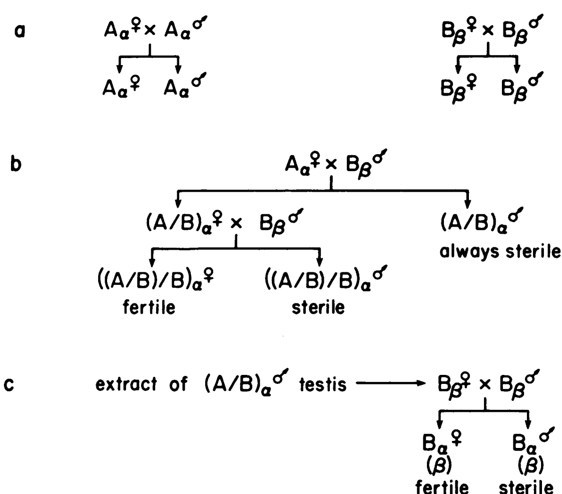

FIG. 1. Possible outcomes of crosses within and between semispecies of *D. paulistorum*. Results indicate the passage of endosymbionts and consequent reproductive capabilities. "A" and "B" represent two semispecies of *D. paulistorum*; "A/B" represents a hybrid from a cross between an A female and a B male; "α" and "β" represent the endosymbiont resident in semispecies "A" and "B," respectively. (*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 the endosymbiont into nonhybrid females of the paternal semispecies (B), as a result of the injection of an α-endosymbiont containing extract of testes of sterile hybrid males. (Cited with permission, from ref. 1.)

(wt/vol) Noble agar. Ampicillin and thallous acetate were omitted. Revertant identifications were based upon morphology, growth in selective media, biochemical reactions, antibiotic sensitivity patterns, and the reaction of isolates with Lancefield serological groups.

Injection of L-Forms into Flies. Inocula for injection into flies were prepared from L-forms. The colonies on agar were covered with 2 ml of 15% (wt/vol) sterile sucrose solution. The surface was rubbed with a glass rod and the aspirated L-form suspension served as "undiluted" inoculum. Serial 1:10 dilutions of inocula were made in 15% sucrose diluent.

The methods of injecting *D. paulistorum* females and evaluating their male offspring for sterility have been described (12). We injected $\approx$0.2–0.4 μl of inoculum into each female. Abdominal distention was evidence that sufficient volume had been inserted. Injections were made between the third and fourth abdominal sternites of the flies. Five to 10 days after injection, the flies were mated with males of their own semispecies. Males produced from these matings were crossed with sibling females to see if larvae would be produced. These same males were then dissected and examined for the presence of motile sperm.

RESULTS

Isolation of Cell Wall-Less Microbes from Mesitas Flies. Although by electron microscopy we saw mycoplasma-like inclusions within the *D. paulistorum* tissues, our initial attempts to isolate Mollicutes were unsuccessful. Subsequently, we used broth and agar formulations that allowed the growth of L-forms present in sterile male flies. With a glass tissue grinder, we homogenized about 20 testes from Mesitas flies in 1 ml of SP4 broth without antimicrobials. Sterile SP4 semisolid culture medium containing ampicillin was added to bring the volume to 5 ml. Approximately 0.1-ml aliquots from this suspension were used as inocula into SP4 broth with 7% sorbitol. This broth culture became acid and turbid 6 days after inoculation. Approximately 0.1 ml of acidic culture fluid was inoculated onto SP4 agar containing sorbitol, ampicillin, and thallous acetate. After 20 days of incubation in a 10% (vol/vol) CO_2-enriched atmosphere, we observed "fried egg" colonies growing on the agar surface. Colonies had a granular, delineated central core and a translucent periphery. The colony surfaces had an overall "lacy" appearance with "vacuolated" peripheral areas. Electron micrographs of colony cross sections revealed membrane-bound prokaryotes with no apparent cell walls or remnant wall material. These colonies resembled L-forms more than mycoplasmas in their morphology and growth on medium of elevated osmolarity. These isolates were designated FM1. No recognizable Mollicutes were detected in mycoplasma broth, on agar media, or on SP4 agar containing 7% sorbitol.

Subculture of the FM1 Isolate. After primary isolation, the L-forms grew at 25°C in ordinary room atmosphere. The FM1 isolate was subcultured for 10 passages on antibiotic-free medium and retained its colonial morphology. Improved growth was obtained when the sorbitol concentration was increased to 9% (wt/vol). We found media containing 12% sucrose instead of sorbitol to be superior. Agar containing sodium chloride at 5% concentration did not support growth of the FM1 isolate. Omission of yeast extract, yeast autolysate, and fetal bovine serum from the SP4 formula resulted in an increase in colony size and numbers. The isolate also grew on a trypticase soy agar medium with added sucrose or sorbitol.

Isolation of an L-Form from Santa Marta Flies. A homogenate was prepared from three Santa Marta males. Utilizing sorbitol- and sucrose-modified SP4 media formulations, we obtained L-forms similar in appearance to the FM1 isolate. This isolate was designated SM1.

Injection of the FM1 Isolates. The L-forms isolated from Mesitas and Santa Marta semispecies were injected into females of the same or other semispecies. Injected females were crossed with males of their same semispecies, and their sons were tested for evidence of fertility.

The FM1 organism, originally isolated from Mesitas flies, was injected into Santa Marta females. As shown in two separate experiments (Table 1, experiments 1 and 2), after matings, all of the male offspring were sterile. They did not possess motile sperm, and larvae were not produced after mating. When the FM1 isolate was injected into Mesitas females (experiment 3), almost all of the male offspring were fertile and females mated to them produced larvae.

Thus, if the L-form isolated from one semispecies was injected into a female of the same semispecies, the sterility rate in the male offspring was relatively low. If the injected L-form had been isolated from a different semispecies, all of the male offspring were sterile.

Reisolation and Injection of Mesitas-Derived L-Form. We recovered an L-phase organism from sterile male offspring of Santa Marta females injected with the FM1 L-form originally isolated from Mesitas flies. For this isolation, $\approx$100 sterile

Table 1. Sterility in F_1 *D. paulistorum* males injected with L-forms

Organism injected	Experiment	Recipient semispecies*	Dissected males, no.	% male sterility	Larvae produced
FM1	1	S	26	100	No
	2	S	75	100	No
	3	M	33	30	Yes
FM1-RA	4	M	100	0	Yes
		S	23	70	Yes
EK1	5	S	40	11	Yes
Streptococcus ADA-L	6	S	100	0	Yes

*S, Santa Marta, Transitional semispecies; M, Mesitas, Andean semispecies.

Table 2. Sterility patterns in male progeny of Santa Marta and Mesitas females injected with suspensions of FM1 and SM1 isolates

Injected material		% sterile sons from injected females*	
Isolate†	Dilution	Santa Marta semispecies	Mesitas semispecies
FM1	10^0	100 (24/24)	10.0 (5/50)
	10^{-5}	87.5 (28/32)	0.0 (0/50)
	10^{-15}	7.1 (3/42)	0.0 (0/50)
SM1	10^0	No male offspring	85.2 (52/61)
	10^{-5}	10.0 (2/20)	100.0 (4/4)
	10^{-15}	0.0 (0/50)	7.7 (2/26)
Sterile 15% sucrose		3.4 (1/29)	ND

ND, not done.
*Numbers in parentheses indicate number of sons found to be sterile/number examined by dissection.
†FM1 is the L-form isolated from Mesitas, Andean semispecies; SM1 is the L-form isolated from Santa Marta, Transitional semispecies.

male progeny were homogenized in SP4 broth and 0.1-ml aliquots were inoculated onto agar. An L-form was isolated 11 days after inoculation and designated FM1-RA.

A suspension of FM1-RA was inoculated into both Santa Marta and Mesitas females (Table 1, experiment 4). When Mesitas females were injected, all of the male offspring were fertile and possessed motile sperm. When Santa Marta females were the recipients, about 70% of the sons were sterile; the same sterility pattern as observed with the FM1 isolate from Mesitas flies (Table 1, experiments 1–3). An L-form cultured from "normal" Santa Marta flies should not cause sterility when injected back into Santa Marta females. Therefore, it appeared that the L-form FM1-RA was derived from organisms of the initial injection of FM1 into Santa Marta females and retained its semispecies specificity.

Injections of Other L-Forms. To show that other L-forms would not induce the same sterility pattern as demonstrated with the FM1 isolate, we injected EK1, the L-phase isolate from *E. kuehniella,* into Santa Marta females (Table 1, experiment 5). Only 11% of the male offspring were sterile, a value slightly above that considered to be "background" level (2). Also, we injected *Streptococcus* ADA-L, a stable L-form, into Santa Marta females (Table 1, experiment 6). There were no sterile male progeny.

Dose–Response of Flies to Injection of Mesitas-Derived FM1 Isolate. We determined the extent to which Santa Marta and Mesitas flies would yield sterile offspring when the concentrations of FM1 and SM1 injected were varied. The isolates were grown on SP4 agar medium containing 12% sucrose but with yeast components and serum omitted. After 5 days of incubation, the organisms were harvested and suspended in 15% sucrose solution, and dilutions of the suspension were injected into Santa Marta and Mesitas flies.

The initial suspension of FM1 organisms injected into Santa Marta females caused sterility in all male offspring (Table 2). In contrast, injection of Mesitas females resulted in sterility in only 10% of the offspring. The data in Table 2 show clear evidence of dosage-dependent sterility with injections of FM1 isolate into Santa Marta flies. There was a progressive diminution in the percentage of sterile male offspring as higher dilutions were used. As expected, in either semispecies, the 10^{-15} dilution had little or no effect above background level.

To determine if our L-phase isolates cause sterility only in other semispecies, dilutions of the SM1 isolate were injected into Santa Marta and Mesitas females. Our results with the Santa Marta isolate should be opposite to those obtained with the Mesitas-derived L-phase organism. Overall, injections of the SM1 isolate caused 85–100% sterility in male Mesitas progeny but only 10% in its own semispecies. The only exception to the pattern was the observation that the undiluted suspension of SM1 organisms caused Santa Marta flies to produce only female offspring. Perhaps the high potency of the undiluted suspension was responsible for this effect.

Identification of Revertants. Bacterial revertants developed incidentally within L-form colonies grown on media without antimicrobials. All of the bacterial forms derived from L-forms were streptococci; these bacterial forms were obtained from the FM1, SM1, and FM1-RA isolates. The FM1-derived bacterial form was identified as a group D *Streptococcus faecalis* (Table 3). This organism hydrolyzed esculin and grew in medium containing 6.5% (wt/vol) sodium chloride. The FM1-RA bacterial form showed the same antibiotic sensitivities and serological relationships as did the FM1 bacteria. The SM1-derived bacterial form had characteristics of a streptococcus but did not hydrolyze esculin, did not grow in 6.5% sodium chloride, and did not react with Lancefield groups A–G antisera.

DISCUSSION

The microbial endosymbionts of the *D. paulistorum* complex are apparently involved in the male sterility that results from crosses between semispecies. We have cultured, on artificial media, L-forms that, when injected into nonhybrid females, are able to induce the sterility patterns observed in the hybrid state. These microbes made it possible to perform the experiments on host-symbiont recognition and incompatibilities described herein. The L-form isolates either from Mesitas or Santa Marta flies, when injected into the other semispecies, resulted in sterility of male progeny. The L-form isolate from *E. kuehniella* and the group A streptococcal L-form did not induce sterility above background levels in Santa Marta flies, further demonstrating the specificity of our L-phase isolates from *Drosophila.*

Such results support our concept concerning a normally benign relationship of an L-form symbiont with its own host; entrance of the symbiont into a closely related, but different, host produces consequences revealed in subsequent generations. It was not the unusual sensitivity of the Santa Marta flies or the uniqueness alone of the L-form isolated from Mesitas that produced the sterility pattern illustrated in Table 1. Dilution series experiments with both Mesitas and Santa

Table 3. Characterization of L-form revertant bacteria

Test	Organism		
	FM1-REV	FM1-RA-REV	SM1-REV
Antibiotic sensitivity, μg/ml			
Ampicillin	1.0	1.0	0.5
Penicillin	2.0	2.0	0.5
Nafcillin	>4	>4	0.1
Clindamycin	>4	>4	0.1
Erythromycin	1.0	1.0	0.1
Tetracycline	0.5	0.5	0.5
Biochemical reaction*			
Esculin	+	0	0
Pyruvate	+	0	+
Sorbitol	+	0	0
Lactose	+	0	0
Arabinose	0	0	0
Arginine	+	+	0
Serotype group	D	D	(not in A–G)

*+ indicates utilization, hydrolysis, or acid production by fermentation.

Marta isolates produced substantial evidence of a dosage-dependent sterility.

Usually, L-forms do not thrive in broth cultures and are not easily adapted to liquid media (13). We did not obtain uniform suspensions of L-form organisms in broth cultures; there was a tendency for these organisms to aggregate and to appear as small granular colonies that settled to the bottom of the broth. For this reason, inocula for subcultures or for injections were obtained from organisms scraped from the surface of agar medium. Even so, aggregates of L-form organisms are difficult to disperse. In all probability, organisms were unevenly distributed through the dilution series and injected flies received uneven amounts of organisms.

The isolate called FM1-RA had been obtained from sterile offspring of Santa Marta females injected with FM1. Because no aposymbiotic semispecies is available, the reisolation and subsequent experiment was part of an attempt to show the etiologic role of these L-forms in hybrid sterility. First, an L-phase organism, FM1, had been isolated and grown in pure culture. Second, sterile male progeny had been produced when the females injected were genetically different from the L-form's host but not when injected into females of the same semispecies. Third, we reisolated from the sterile Santa Marta males, an L-phase variant, FM1-RA, which produced sterility similar to the FM1 L-form. We simply recovered what had been injected into the previous generation. Symbionts belonging to one semispecies proliferate in unimpeded fashion when transferred to an alien semispecies (3).

The FM1 and FM1-RA streptococcal parents were both classified by antibiotic sensitivity pattern and serology as *S. faecalis*. In biochemical tests the bacterial form of FM1 gave many more positive results than did the revertant of FM1-RA. Perhaps repeated isolations and prolonged exposure of FM1-RA to antibiotics caused these changes. The SM1 bacterial parent did not serotype in Lancefield groups A–G nor were its antibiotic sensitivity pattern or biochemical reactions similar to FM1 (see Table 3).

Although it is possible that the three L-form isolates were generated from the bacterial flora of the flies, the specificity of induced male sterility suggests that this is not the case. Indeed, *Streptococcus faecium* and other streptococci are present on the flies. Also the results in our initial experiments (Table 1) might be explained by variations in inocula, but the dosage experiments employed simultaneous injections of a single inoculum preparation into two semispecies, minimizing these problems. Nevertheless, the fact remains that we have obtained cell wall-free prokaryotes that show semispecies specificity.

Recently we have isolated an L-form from the Centroamerican semispecies (from our Lancetilla, Honduras strain). It conforms to the previous pattern—i.e., this L-form induces sterility in the Mesitas and Santa Marta semispecies but not in its own (unpublished data).

The host–symbiont relationship in the *D. paulistorum* complex certainly fostered fragmentation of a presumably once-unified species into semispecies. More important for current purposes, the presence of endosymbionts confers certain properties on their hosts and may still play a role in their evolution. Our possession of L-forms that show the host specificity observed in the hybrid state offers opportunities to further characterize the microbes and to correlate these features with their effects in *Drosophila*, their natural host, and in other insects.

Streptococcal revertants were characterized with the aid of Jean Barnishan of The Ohio State University Hospital Laboratories under the direction of Dr. Leona W. Ayers. The Cornell Medical Center (New York, NY) laboratories under direction of Dr. Laurence B. Senterfit confirmed these identifications. We gratefully acknowledge the expertise and willing assistance of these scientists. This work was supported by Grant PCM 79-102062 from the National Science Foundation.

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