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Spiroplasma Taxonomy and Identification of the Sex Ratio Organisms: Can They Be Cultivated?

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The spiroplasmas that occur naturally in several species of *Drosophila* were the first spiroplasmas ever observed, even though their discoverers, D.F. Poulson and B. Sakaguchi, in 1961 described them as being "treponema-like spirochetes." These *Drosophila* spiroplasmas are transovarially, or maternally, transmitted by infected females whose progenies are composed entirely of females. A more recently discovered *Drosophila* spiroplasma found in flies originating in Ito, Japan, is also maternally inherited but does not result in the elimination of males from the progeny of infected females. In spite of their early discovery, their high numerical density in the hemolymph of infected females (10^6–$10^7/\mu l$), and numerous attempts at *in vitro* cultivation, they remain prime examples of non-cultivable spiroplasmas. It is the purpose of this paper to recount some of the approaches used in attempts at their cultivation.

INTRODUCTION

In 1961, Poulson and Sakaguchi first reported their observations of a "small treponema-like spirochete" in the hemolymph of female *Drosophila willistoni* and *D. nebulosa* that were displaying an infectious inherited trait called "sex ratio" [1]. This trait, found in natural populations of four closely related neotropical species of *Drosophila, viz., D. equinoxialis, D. nebulosa, D. paulistorum,* and *D. willistoni,* results in the total elimination of males from the progeny of infected females [2]. The sex ratio organisms (SROs), now known to be spiroplasmas, can be transferred through injection of hemolymph to non-infected *Drosophila* species females who will, a day or two after they were injected, begin to produce progeny with a sex ratio distorted in favor of females, with the eventual total elimination of males in the later progeny of the injected females [2]. The female progeny inherit SROs and display the SR trait. Some of the early-brood male progeny will be infected, but neither these nor newly injected males can transmit either the SRO or the SR trait through their sperm [2].

SROs show typical spiroplasma, not spirochete, morphology, being cell wall-free helical filaments 4–8 μm in length and 0.15–0.2 μm in diameter. They are also motile, displaying both rotatory and flexing movements. The numerical density of

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SROs appears to be characteristic of their species of origin, with comparable numbers being obtained in both their natural species and those into which they have been transferred by injection [2]. Estimates of the numbers of SROs in one microliter of hemolymph were made by direct enumeration under darkfield microscopy of dilutions of a measured volume of one microliter of hemolymph pooled from several flies. The numbers obtained for the three species that were examined are the following:

D. equinoxialis ($2.6 \times 10^6/\mu l$), *D. nebulosa* ($2.0 \times 10^6/\mu l$), and *D. willistoni* ($13 \times 10^6/\mu l$). Enumerations have not been made of the SROs in the hemolymph of *D. paulistorum* or of the maternally inherited non-male-killing spiroplasma found in natural populations of *D. hydei* near Ito, Japan [3]. In one-way spiroplasma deformation (DF) [4] tests using antibody prepared against *D. willistoni* SROs, the *D. willistoni* SROs, the *D. nebulosa* and the *D. hydei* non-male-killing spiroplasmas appear to be serologically related, if not identical. However, in spite of their morphological, serological, and host similarities, *Drosophila* spiroplasmas do differ from each other in the cross-lytic abilities of the viruses which they all harbor [5].

The *D. willistoni* SROs have been shown by DF tests to be unrelated to other spiroplasmas [4]. They currently comprise a single serogroup (Group II), awaiting successful cultivation before complete serological characterization can be carried out.

The mechanism of male-killing by SROs is not understood. Studies of various special strains of *D. melanogaster* infected by injection have demonstrated that the presence of a Y-chromosome is not involved in male lethality, that only embryos with two X-chromosomes are able to survive an established SR infection; XXY females survive, but XO males die [2]. The existence of a distinct male lethal factor has been suggested [5] but not demonstrated.

In spite of the large numbers of SROs in the hemolymph of infected flies, numerous attempts to cultivate them have failed. In certain media they will maintain their normal helical morphology and display self-generated motility for periods of up to 60 days or more, but without apparent cell division. The purpose of this paper is to present some of the approaches used in the attempts to cultivate these unusual spiroplasmas in order to elicit suggestions for new approaches.

MATERIALS AND METHODS

Flies

The species in which the SRO occurs naturally are difficult to handle and maintain as laboratory stocks. All SROs used in these attempts at their cultivation were transferred by injection to the Piñon Normal strain of *D. pseudoobscura,* an easily reared species, well-adapted to laboratory conditions.The *D. willistoni* B_3SR strain was the primary source of SROs for these studies. Other SR strains were the *D. nebulosa* SR strain and the non-male-killing Ito strain of *D. hydei.* All *Drosophila* stocks were reared on standard cornmeal-agar-syrup medium at 20° or 23°C.

SROs

SROs were obtained from infected flies by hemolymph extraction [6]. The collected hemolymph was placed in the medium to be tested which either contained antibiotics [penicillin (100 U/ml) and streptomycin (100 μg/ml)] or was membrane filtered (450 nm). Estimates of the numbers of spiroplasmas in a diluted suspension were made by darkfield microscopic enumeration of 5 μl samples [2]. Steiner et al.

[7] have studied the ability of spiroplasmas to grow in cell cultures. In the SRO-cell culture studies reported here, SR hemolymph suspensions in M1A [8] were inoculated into wells of multiwell plates (Falcon Plastics) containing 0.9 ml of assay medium with 2×10^5 cells, e.g., Schneider's *Drosophila* cell line, Dm-1 [9], or into 0.9 ml of assay medium without cells. In addition to the medium being assayed, the same hemolymph suspension was also added to cultures of Dm-1 cells in M1A medium. Furthermore, approximately 10^7 colony-forming units (CFUs) of a cultivable spiroplasma, such as *Spiroplasma citri* (R8A2) or the corn stunt spiroplasma (E275) were inoculated into parallel wells with and without Dm-1 cells. The plates were incubated aerobically at 25°C and, after four to seven days, samples were removed for darkfield microscopy, after which the cultures were transferred to fresh medium and incubated for another four to seven days. SRO growth was determined by direct enumeration; the growth of control spiroplasmas was determined by color change of the growth medium along with darkfield microscopy of samples.

Media

The various media employed in the attempts at SRO cultivation are given in Table 1, along with a reference to their formulation. Most of the media were subjected to various modifications; these are listed by category in Table 2.

RESULTS

SROs in Medium Alone

No attempt will be made to describe in detail the results obtained with all the media and their modifications in these endeavors at *in vitro* cultivation of SROs. Our results are summarized in the information presented in Table 3.

TABLE 1
Some of the Media Utilized in Attempts at Sex Ratio Organism Cultivation

Medium	Reference	Number
A. For Sex Ratio Organism Alone		
1. Drosophila tissue culture medium	Echalier and Ohanessian, 1970	[10]
2. Drosophila tissue culture medium	Schneider, 1964	[11]
3. Grace's insect tissue culture medium	Grace, 1962	[12]
4. SMC for *Spiroplasma citri*	Saglio et al., 1971	[13]
5. M1A medium for corn stunt	Jones et al., 1977	[8]
6. C-3 medium for corn stunt	Liao and Chen, 1975	[14]
7. Drosophila tissue culture medium	Wyss and Bachmann, 1976	[15]
8. SP-4 medium for *Spiroplasma mirum*	Tully et al., 1977	[16]
9. IPL medium for Lepidoptera	Goodwin, 1975	[17]
10. Singh's mosquito culture medium	Singh, 1967	[18]
11. Drosophila tissue culture medium	Robb, 1969	[19]
12. Hybridoma medium (Dulbecco MEM)	Kennett, 1980	[20]
B. For SRO-Insect Cell Cultures		
1. M1A medium for corn stunt	Jones et al., 1977	[8]
2. Drosophila tissue culture medium	Schneider, 1964	[11]
3. M3 medium for Drosophila cells	Shields and Sang, 1977	[21]
4. DS medium for Drosophila cells	Sang, 1981	[22]
5. GTC-100[a]	Gardner and Stockdale, 1975	[23]

[a]Modified by R. Granados, Boyce Thompson Institute, Cornell University, Ithaca, New York

Although no medium or its modification led to actual growth of SROs, certain media and conditions promoted long-term maintenance—up to 66 days for the M1A medium developed for the corn stunt spiroplasma. Certain media, such as Singh's mosquito culture medium, with a survival time of four days, obviously do not provide suitable conditions for long-term maintenance. Such media were not usually subjected to modifications in attempts to improve their maintenance characteristics. Robb's *Drosophila* cell culture medium [19] in its complete formulation does not contain serum. SROs in this medium were very sticky, adhering to membrane filters and glass slides and cover glasses, and became beaded within four days. When the medium was supplemented with 10–15 percent fetal bovine serum (FBS), the stickiness disappeared and survival increased to 10–14 days.

It should be noted that Schneider's Drosophila Medium [11], with a survival time of 20–30 days, and SMC [13], the first medium successfully used for *S. citri* cultivation, with a survival time of 15–32 days, were combined and used in the successful

TABLE 2
Modifications of Growth Media

A. Serum
   1. Fetal bovine serum (FBS) unheated and heated (56°C, 60 minutes)
   2. FBS dextran-charcoal filtered
   3. FBS dialyzed
   4. Horse serum heated (56°C, 60 minutes)

B. Serum substitutes
   1. Bovine serum fraction (5%)
   2. Cholesterol (10 and 18 μg/ml) plus FBS (0, 2, 8, 16%)
   3. Bovine serum albumin (0.5%) plus lecithin (0.8 and 1.6 ng/ml) plus cholesterol (10 μg/ml) plus FBS (0 and 16%)
   4. Filtered whole milk (2, 5, 8, 10%) plus FBS (8, 5, 2, 0%) totaling 10%
   5. Filtered skim milk (8%) plus FBS (2%)
   6. Lobster hemolymph (5, 10, 20%)
   7. Drosophila hemolymph (5 and 10%)

C. Vitamins and amino acids
   1. NCTC vitamins (0.5, 1, 2, 5%)
   2. BME vitamins (0.01, 0.1, 0.5, 1.0, 2.0 × stock solution
   3. MEM amino acids (5 μl of 50 × stock)
   4. L-Arginine (4.2 mg/ml)
   5. L-Glutamine (0.04 g/ml)

D. Nucleic acids and related compounds
   1. DNA from calf thymus (0.05 mg/ml)
   2. DPN (10 mg/ml)
   3. GMP (0.2, 1.0, 5.0, 10.0 mM)

E. Hormones
   1. α-ecdysone
   2. β-ecdysone (100 ng/ml)
   3. Insulin (.5–10 μg/ml)
   4. Transferrin (5–10 μg/ml)
   5. Prostaglandin E_1 (25 ng/ml) plus Triiodothyronine (5 pM) ± FBS
   6. Mouse epidermal growth factor (EGF) 50 ng/ml plus bovine fibroblast growth factor (FGF) 100 ng/ml
      a. EGF + FGF ± FBS
      b. EGF + FGF + dextran-charcoal filtered FBS
      c. EGF + FGF without FBS

TABLE 2—*Continued*

F. Membrane-interacting substances
   1. Phytohemagglutin (0.005–0.02 μg/ml)
   2. Concanavalin A (5 μg/ml)
   3. Ionophore (0.5–16 μg/ml)
   4. Glutathione (0.0001–0.1 mM)

G. Miscellaneous substances and modifications
   1. Whole fly homogenate (2.5–20%)
   2. Larval homogenate (10–20%)
   3. Larval lipid
   4. Chicken chorio-allantoic fluid (5, 10, 15, 20%)
   5. Chicken amniotic fluid (5, 10, 15, 20%)
   6. Yeastolate (0.4%)
   7. Tryptone (0.3%)
   8. Peptone (0.3–0.6%)
   9. Fresh yeast extract (3%)
   10. Urea (1%)
   11. HEPES buffer (1.4%)
   12. Thioglycolic acid (0.5%)
   13. Trehalose (0.2%)
   14. Putrescine (0.1 and 1 mM)
   15. Spermidine (0.1 and 1 mM)
   16. Spermine (0.1 and 1 mM)
   17. Mammalian hybridoma medium [Dulbecco's MEM(60%), FBS(20%), NCTC(20%)]

H. Conditioned media
   1. Corn stunt spiroplasma (E275) in MIA (2–4 days)
   2. Honeybee spiroplasma (BC3) in MIA (1 day)
   3. Schneider's Drosophila cell line (Dm-1) in MIA (1–4 days)
   4. Rabbit cell culture AG-4676

TABLE 3
Survival Times For Sex Ratio Organisms in Various Media

Medium	Survival Times[a] (in days)
1. Drosophila tissue culture medium (Echalier and Ohanessian)	6–12
2. Drosophila tissue culture medium (Schneider)	20–30
3. Grace's insect tissue culture medium	15–22
4. SMC for *S. citri*	15–32
5. M1A medium for corn stunt	30–66
6. C-3 medium for corn stunt	14
7. Drosophila tissue culture medium (Wyss and Bachmann)	15
8. SP-4 medium for tick spiroplasma, *S. mirum*	4–21
9. IPL medium for Lepidoptera (Various formulations)	6–26
10. Singh's mosquito culture medium	4
11. Drosophila tissue culture medium (Robb):	
Without fetal bovine serum	3
With fetal bovine serum	10–14

[a]Survival times based on normal morphology and motility

cultivation of the corn stunt spiroplasma [24]. Since its development for corn stunt spiroplasmas, originally called M1 [24], and later modified, and called M1A by Jones et al. [8], this has been the medium most extensively used, along with its modifications, in our attempts at SRO cultivation. Other medium combinations, e.g., Grace's insect tissue culture medium and SMC, were not as successful.

The temperature at which cultures were maintained was a factor in their survival. Several media were tested at 20°, 23° or 25°, and 30°C. Uniformly, cultures at 20°C survived longer than those at the higher temperatures. The longer survival at 20°C probably does not necessarily indicate an optimum temperature but may only retard the deterioration that occurred more rapidly at the higher temperatures.

For most of the media tested, the basis for evaluating the survival of SROs was continued helical filamentous morphology and the display of self-generated movement, primarily the bending or flexing of filaments. However, helicity and motility are not necessarily reliable criteria for viability. In one experiment, SROs from one-day *in vitro* cultures were injected into *Drosophila* females. The SR trait was not induced. This was dramatically demonstrated in the following experiment: Hemolymph from non-infected females was pooled (10 μl) under sterile mineral oil in a depression plate, after which 0.2 μl of SR hemolymph was added. Following mixing of the suspension, six normal, non-infected females were injected, each with 0.1 μl of the suspension. Each female was mated and their progeny collected daily for 14 days. This procedure was repeated on days 1, 2, 6, 10, and 13, following the establishment of the hemolymph culture. Samples of the suspension examined under darkfield microscopy showed the SROs in the hemolymph to be in excellent condition throughout the time span of the experiment. Nevertheless, only flies injected with the initial suspension produced the SR trait (absence of males) 12 days following injection. Females injected with SROs that had been in culture for one and two days all became infected but did not develop the SR trait during the time they were observed (14 days). After six days in culture, none of the injected flies became infected, though there were normal-appearing SROs present in the culture, even on day 13. Similar results were obtained in bioassays of cultures of SROs in SMC and M1A media.

M1A medium was modified in a multitude of ways (refer to Table 2), most of which either shortened survival time or did not noticeably increase it. Certain of the modifications merit an accounting of their effects.

The standard M1A medium contains 16 percent FBS. In an experiment in which the final concentration of FBS was 0 percent, 5 percent, 10 percent, 15 percent, and 20 percent, survival times in days at 20°C in the respective FBS concentrations were 0, less than 12, 23, 23, and 35.

There was considerable variation in the survival times observed from experiment to experiment utilizing the same medium and the same culture conditions. This may have been due simply to the variation in the number of SROs placed in culture in different experiments. No special effort was made to standardize the numbers of SROs placed in the various media tested, although this was done within an experiment, whenever modifications of the medium were being tested.

The longest survival time was obtained in an experiment in which the supernatant from a homogenate of non-infected flies was used to supplement M1A. Supplements of 0 percent, 2.5 percent, 5 percent, 10 percent, and 20 percent fly extract were added to M1A. In this experiment, it was the unsupplemented (0 percent) M1A, i.e., containing no homogenate, that produced a survival time of 66 days. During this time span, the culture was transferred to an equal volume of fresh M1A on days 5

and 13. The SROs appeared normal but there was no apparent increase in numbers. On day 58 the culture was in an obvious state of deterioration. To eliminate accumulated toxins that might have been inhibiting growth, the culture was centrifuged (12,000 *g*) and the pelleted SROs were resuspended in fresh M1A. Ten normal female flies were injected with this suspension. SROs were never observed in the hemolymph of these injected flies. After an additional eight days of culture (total time = 66 days), the number of typical SROs had declined to a level at which no further observations were made. Survival times for the cultures containing fly extract were 35 days (2.5 percent), 38 days (5 percent), 25 days (10 percent), and 23 days (20 percent).

In some media modifications SROs grew to lengths that were three to six times their usual length (ca. 6 μm). Such length increases were observed in Schneider's Drosophila Medium containing 0.5 percent thioglycolic acid (for anaerobiosis), in SMC containing DPN (1 mg/ml), in M1A with DPN (0.1 mg/ml), and in SMC containing DNA (50 μg/ml). The growth in length was not accompanied by an increase in number. No other modification produced any dramatic change in numbers, morphology, or longevity of SROs.

Hemolymph from flies transmitting the *D. nebulosa* SRO and from flies transmitting the non-male-killing spiroplasmas from *D. hydei* were also placed in M1A medium and neither of these showed any indication of growth.

We have varied the culture conditions by placing suspensions under sterile mineral oil, pure N_2 atmosphere in an anaerobic Gas Pak (BBL) containing H_2 and CO_2, and in a mixture of 95 percent N_2, 5 percent CO_2. The longest survival time in all situations occurred under standard atmospheric conditions.

SROs were also injected into embryonated chicken eggs, and both chorioallantoic and amniotic fluids were used as supplements to M1A. Survival times in the fluids were 11 days for amniotic fluid and 24 days for chorioallantoic fluid. No SROs were recovered from any of the chicken embryo fluids after 11 days incubation at 37°C, but SROs rarely survive even at 30°C for more than a few days.

Cell Cultures

The rationale for using cell cultures is simple: If a medium can support the growth of *Drosophila* cells in culture, the cells might condition the medium to permit the growth of SROs. The current results of our experiments using various established *Drosophila* cell lines are presented in Table 4.

In these experiments we intentionally kept the number of SROs inoculated into a culture at a low level in order to evaluate any increase in number more easily. Although both Schneider's Drosophila Medium and M1A permitted the growth of both *Drosophila* cells and control cultivable spiroplasmas, neither medium promoted the growth of SROs in the cell cultures tested. Neither of the other two media tested (MS and DS) permitted growth of either SROs or control spiroplasmas, although the cell lines adapted to these media grew well. None of the modifications listed in Table 2 was effective in promoting SRO growth.

SROs in Organ Cultures

The use of organ cultures is the most recent approach to SRO cultivation. Since *Drosophila* fat bodies and ovaries are responsible for the formation of many hemolymph proteins [25], experiments to take advantage of the possibility of their providing critical substances for SRO growth have been undertaken. *Drosophila* fat body tissue lines the interior of the abdomen. The entire abdomen can be removed,

TABLE 4
Assays of Growth of SROs With Different Insect Cell Lines

Insect Species	Cell Strain	Medium[a]	Growth[b] SRO	Growth[b] Control Spiroplasma
DIPTERA				
Drosophila melanogaster	Dm-1	M1A	–	+
	Dm-1	Schneider's	–	+
	I-XII	Schneider's	–	+
	75B	M3	–	–
	75F	M3	–	–
	75M	M3	–	–
	75e7	DS	–	–
	75L	DS	–	–
LEPIDOPTERA				
Spodoptera frugiperda	–	GTC-100	–	NT[c]
Trichoplusia ni	TN-368	GTC-100	–	NT
	TNP	GTC-100	–	NT
Mamestra brassica	MBP	GTC-100	–	NT
Estigmene acrea	BTI-EAA	GTC-1–	–	NT
Heliothis zea	IPBL-1075	GTC-100	–	NT

[a]Refer to Table 1.
[b]Aerobic incubation at 25°C (Dm-1 and I-XII also incubated at 30°C)
[c]Not tested

dissected free of the gut, and placed in culture. At the same time, the ovaries can be disconnected from the uterus and placed in culture. In our experiments we have placed the ovaries and fat bodies (entire abdominal wall) from five females in 100–200 μl of culture medium in multiwell plates. The media used in these cultures were M1A and Robb's Drosophila cell culture medium containing 15 percent FBS. Abdomens and ovaries from SR females and from normal females were used. The former provided cultures that were naturally infected; the latter were inoculated with SRO suspensions in the culture medium used for the organs. Organ cultures from non-infected females were either inoculated immediately with SRO suspension or after allowing a period of time for the culture medium to be conditioned. Cultures were fed by adding 100 μl of fresh medium and removing 50 μl after mixing. After four weeks in culture, the *Drosophila* ovarian tissues were still living, as evidenced by contractions of ovariolar sheath muscle, but the SROs had become beaded, branched, and very elongated, yet still capable of some flexing movements. Although the organs survived for longer times, the SROs continued to deteriorate and the cultures were discarded after five weeks.

DISCUSSION

Successful cultivation of the SRO appears to be as elusive now as it was in the mid-1960's when the first attempts at its cultivation were made. In spite of great efforts, we actually know very little about the growth requirements of *Drosophila* spiroplasmas. What we do know is that there are many substances that have been added to various media that are apparently not critical for SRO growth. But perhaps they were added in the wrong concentration or combination, or both.

The special mode of transmission of *Drosophila* spiroplasmas, i.e., through the

egg, may be a major factor in our inability to find a suitable medium for *in vitro* cultivation. Transovarial transmission must reflect a long-term association of the spiroplasma with its host, the fly. Moreover, the fact that the SRO is found in a group of closely related species of flies restricted to the neotropics suggests that these spiroplasmas have co-evolved with their hosts. Establishing transovarial transmission as a normal mode for its continued existence would seem to mean that the spiroplasma was not subjected to the rigors of the environment outside the fly. Consequently, those characteristics that would have allowed it to survive such exigencies lost their selective value as the spiroplasma became more adapted to living within the fly. The hemolymph environment is like a chemostat. To be able to cultivate *Drosophila* spiroplasmas outside the fly may require creating a similar kind of enviroment. Unfortunately, very little is known about adult *Drosophila* hemolymph.It is hoped that the organ culture approach may be a means of approximating that environment, although it is apparent from our initial studies that some modifications of both the medium and the culture conditions will be required to achieve SRO growth.

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