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Cultivation of the *Drosophila* Sex-Ratio Spiroplasma

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Uncultivable for more than 25 years, the sex-ratio spiroplasma of *Drosophila willistoni* grew in a tissue culture medium (H-2) containing an embryo-derived lepidopteran cell line (IPLB-TN-R^2). After adaptation, it grew in a cell-free H-2 medium. This success demonstrates the usefulness of cell culture systems for cultivation of fastidious microorganisms and facilitates study of the sex-ratio trait in *Drosophila*.

SEX-RATIO DISTORTION CAUSED BY microorganisms and chromosomal and extrachromosomal factors has been documented for a variety of plants and animals (*1*). In 1961 Poulson and Sakaguchi correlated the appearance of "small spirochetes, presumably treponemata" in the hemolymph of adult female *Drosophila* flies to the expression of a sex-ratio trait in the progeny of the flies (*2*). Eventually it was realized (*3*) that the organisms were not spirochetes but belonged instead to a newly recognized microbial group of wall-less prokaryotes (Mollicutes, Spiroplasmataceae, *Spiroplasma*). These spiroplasmas, known as sex-ratio organisms (SRO's), selectively kill the male progeny of infected females of four Neotropical *Drosophila* species—*D. equinoxialis*, *D. nebulosa*, *D. paulistorum*, and *D. willistoni* (*3*, *4*).

Although many attempts have been made to cultivate SRO's, including trials that involved tissue culture media and insect cell or organ culture systems, none were successful (*5*). The media used in these tests contained different types and amounts of serum and serum substitutes, vitamins and cofactors, amino acids and peptones, nucleic acids, hormones, membrane-interacting substances, and fly homogenates. Also, many different temperature and oxygen conditions were used. None of the tested media or culture systems sustained cell division; the best formulations permitted maintenance of motile, helical organisms for no more than 60 days. These failures impeded research on the SRO's and prevented identification of the male-lethal factor and fulfillment of Koch's postulates.

In 1985 a previously uncultivable spiroplasma (LD-1) from the Colorado potato beetle was cultivated in the presence of actively growing insect cells (*6*). We report here that insect cell cultures have also been successfully used for primary isolation of the *D. willistoni* SRO (WSRO) and that cultured WSRO's have been adapted to a cell-free medium.

All *Drosophila* stocks were maintained as described by Williamson *et al.* (*3*). To obtain spiroplasmas, 2.5 μl of hemolymph was collected at Stony Brook by glass needle (*7*) from a total of 100 *D. pseudoobscura* (Piñon Normal strain) females transmitting the B3 strain of WSRO. The hemolymph was transferred to 0.5 ml of M1D medium (*8*) and shipped at ambient temperature to our laboratory. Five days after the hemolymph was collected, 0.2 ml of the suspension was placed in 4 ml of a medium containing equal parts of DCCM (*9*) and M1D media and filtered through a membrane having a pore diameter of 0.45 μm. The filtrate was then distributed in 0.1-ml aliquots to each well of a 24-well microtiter plate (2.5-ml capacity per well) containing either an embryo-derived *Trichoplusia ni* insect cell line (IPLB-TN-R^2) (*10*) cultured in 0.4 ml of modified Grace's (TNM-FH) medium (*11*) or one of six previously described (*6*) lepidopteran or coleopteran cell culture systems (three wells per system). Primary isolation was also attempted (i) in several cell-free tissue culture media [DCCM medium, modified Goodwin's medium (*12*), and TNM-FH medium]; (ii) in commonly used spiroplasma media [SP-4 (*13*) and M1D media]; or (iii) in several "insect cell–conditioned" media [DCCM, modified Goodwin's, or TNM-FH media that had been conditioned for 1 or 3 days by the growth of the respective insect cell lines (three tubes per medium)]. The cell lines were subcultured weekly and maintained at 23°C until used; all WSRO-containing cell cultures were kept at 26°C. Attempts were made to adapt the first, third, fifth, and ninth passages of the cell-cultured WSRO isolate to cell-free media. We removed insect cells by passing the culture through a membrane filter (pore diameter, 0.45 μm), and the WSRO-containing filtrate was transferred at passage ratios of 1:1 to 1:4 into DCCM, modified Goodwin's, TNM-FH, or H-2 (*14*) media. We estimated SRO titers in the wells and tubes by counting the number of organisms in 3-μl samples by dark-field microscopy (*6*, *15*).

We found that the best results in primary isolation of WSRO were obtained by using the *T. ni*–derived cell lines. Primary isolation was accomplished only in the IPLB-TN-R^2 cell line cultured in TNM-FH medium. The other *T. ni* cell line, IAL-TND1 (*16*), cultured in modified Goodwin's medium, supported WSRO growth and multiplication for about 1 month; continuously cultivable strains were not obtained in this cell line. In the previously described formulations, the organisms became elongate and

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nonhelical and they did not divide. Cultivation was accomplished by the following procedure: (i) A microtiter well containing 0.4 ml of TNM-FH medium was seeded with the IPLB-TN-R^2 insect cell line at 10^5 insect cells per well. (ii) After 3 days, 0.1 ml of WSRO suspension containing 10^7 organisms per milliliter of culture was inoculated into the well. (iii) We made initial transfers at 1- to 3-week intervals by replacing 0.2 ml of medium from a 3- to 7-day-old cell culture with 0.2 ml of spiroplasma culture and insect cells that had been gently resuspended from the bottom of the well. (iv) Culture medium (0.1 ml) was removed every 2 days and replaced with 0.1 ml of one of the fresh media noted above. Since we found that spiroplasma cell morphology was best and growth titers were highest when the cell culture contained a mixture of TNM-FH, DCCM, and M1D media, we used this mixture (H-2 medium) (*14*) in the fifth and all subsequent passages. (v) Suspensions (0.1 ml) of insect cells were added to spiroplasma-containing wells every 5 to 10 days to maintain a confluent insect cell monolayer.

SRO titers in wells of the first passage declined to about 7×10^5 organisms per milliliter, a concentration that approaches the practical limit of the microscopic method of enumeration. Establishment of the WSRO in cell culture was indicated 1 month after inoculation by the consistent presence of short, three- to six-turn helical organisms in the media; at that time WSRO titers had increased to about 1.5×10^6 organisms per milliliter. WSRO's from cell-containing and cell-free cultures and from the hemolymph of infected females reacted against homologous antisera (*17*) with titers of 1:5120 in the spiroplasma deformation test (*18*), which demonstrated that the cultured organisms were indistinguishable from WSRO's found in vivo. The cultured spiroplasmas maintained their characteristic motility in vitro, although some spiroplasma cells were two or three times the typical length (4 to 5 μm) of spiroplasma cells observed in freshly drawn hemolymph from SRO-infected flies. Although WSRO's were frequently attached to the cultured insect cells, most appeared to move freely in the medium.

We do not know what critical growth factors or conditions, if any, are supplied by the growing insect cells to the spiroplasmas during the course of primary isolation. The most important element in our success in culturing the WSRO, other than the use of insect cells, may have been the development of an optimal passage regimen and maintenance of monitored replicates of each culture system. Ability to evaluate culture conditions by microscopic observation of the number and morphology of helical organisms was also important.

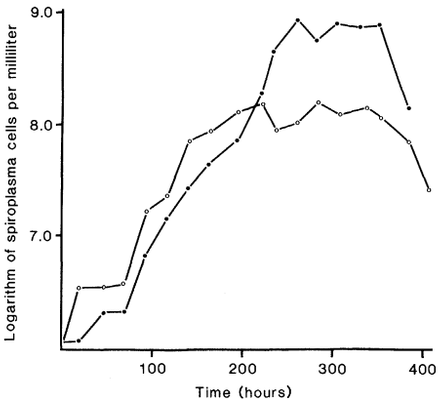

Fig. 1. Growth of *D. willistoni* SRO strain DW-1 (that had grown for 34 passages overall, including 9 passages in cell culture followed by 25 passages in cell-free medium) at 26°C in (○) H-2 medium containing insect cell line IPLB-TN-R^2, or in (●) cell-free H-2 medium.

The WSRO isolate had adapted to the cell-free H-2 medium by passage 9 and was triply cloned in liquid media in microtiter plates by passage 20. At passage 23, the triply cloned isolate (strain DW-1) cultured in cell-free H-2 medium was deposited in the American Type Culture Collection (ATCC 43153).

Strain DW-1 exhibited consistently different growth patterns in cell-free media compared to cell culture systems (Fig. 1). Exponential growth of the WSRO isolate began after 2 to 3 days in both systems, with doubling times of about 19.2 hours. (i) Strain DW-1 typically doubled in titer within 24 hours after inoculation into cell culture, compared to 48 hours in the cell-free medium. (ii) Growth yields of DW-1 were invariably higher in the cell-free medium (maximum of 8.7×10^8 organisms per milliliter) than in cell cultures containing the same medium (maximum of 1.5×10^8 organisms per milliliter). The ability of DW-1 to attain higher titers in cell-free media suggests either that there may be competitive depletion of nutrients by the insect and spiroplasma cells or that inhibitory substrates or conditions may be produced by the insect cells.

Spiroplasmas from passage 5 in cell culture and passage 23 of strain DW-1 growing in cell-free H-2 medium have maintained their ability to eliminate males from the progenies of injected females (*19*).

Two fastidious spiroplasmas have now been isolated by the use of insect cell cultures. One inhabits the midgut lumen of the Colorado potato beetle (*6*); the other, WSRO (strain DW-1), is an ovarially transmitted mutualist that inhabits the hemolymph and cells of *Drosophila* flies (*3*). Compared to the Colorado potato beetle spiroplasma, which can be readily isolated and has a doubling time of 7.5 hours in cell-supplemented media, DW-1 is difficult to isolate and grows much more slowly in vitro. This difference is not surprising, since the habitat of the WSRO is much less variable than that of the Colorado potato beetle spiroplasma. Although both are highly fastidious in their growth requirements, isolates of the Colorado potato beetle (*6*) and WSRO spiroplasmas maintain pathogenicity for their hosts when isolated by methods that use cell cultures. WSRO remains pathogenic when subsequently cultured in the cell-free H-2 medium.

Transovarial transmission and the homeostatic habitat of hemolymph protect SRO's from the vagaries of their environment and suggest a long-term adaptation of these organisms to their *Drosophila* hosts. This protection led Williamson *et al.* (*3*, *5*) to postulate that a similar system might be necessary for the successful cultivation of SRO's. They suggested that a tissue or organ culture system might provide the proper environment for growth. Our success in finding a tissue culture system that facilitates adaptation of a *Drosophila* spiroplasma to in vitro conditions supports their suggestion. These results and other recent successes in the use of tissue culture systems to cultivate previously refractory spiroplasmas (*6*, *20*) demonstrate the usefulness of these systems for the cultivation of fastidious mollicutes.

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Non–Watson-Crick G · C and A · T Base Pairs in a DNA-Antibiotic Complex

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The structure of a DNA octamer d(GCGTACGC) cocrystallized with the bis-intercalator antibiotic triostin A has been solved. The DNA forms an unwound right-handed double helix. Four base pairs are of the Watson-Crick type while four are Hoogsteen base pairs, including two A · T and two G · C base pairs. This is the first observation in an oligonucleotide of Hoogsteen G · C base pairs where the cystosine is protonated. It is likely that these also occur in solutions of DNA complexed to this antibiotic.

IN RECENT YEARS WE HAVE BECOME aware of the large number of conformations that the DNA double helix can adopt. It is now clear that specialized nucleotide sequences in DNA may facilitate alternative DNA conformations. In addition to right-handed conformations, a left-handed double-helical conformation has been found, especially in sequences with alternating purines and pyrimidines (*1*). Furthermore, DNA sequences containing long stretches of purines on one strand and pyrimidines on the other may also adopt alternative conformations (*2*). An additional mode of conformational variability is found in the hydrogen bonding interaction between the bases. It has been known for some time that types of base pairing other than the Watson-Crick type are also possible. Recent work suggested that when quinoxaline antibiotics interact with DNA they can alter the type of hydrogen bonding found in the base pairs (*3, 4*). We now show that both A · T and G · C base pairs can exist in the same segment of DNA in both Watson-Crick and non–Watson-Crick conformations when the DNA is bound to antibiotics of this type.

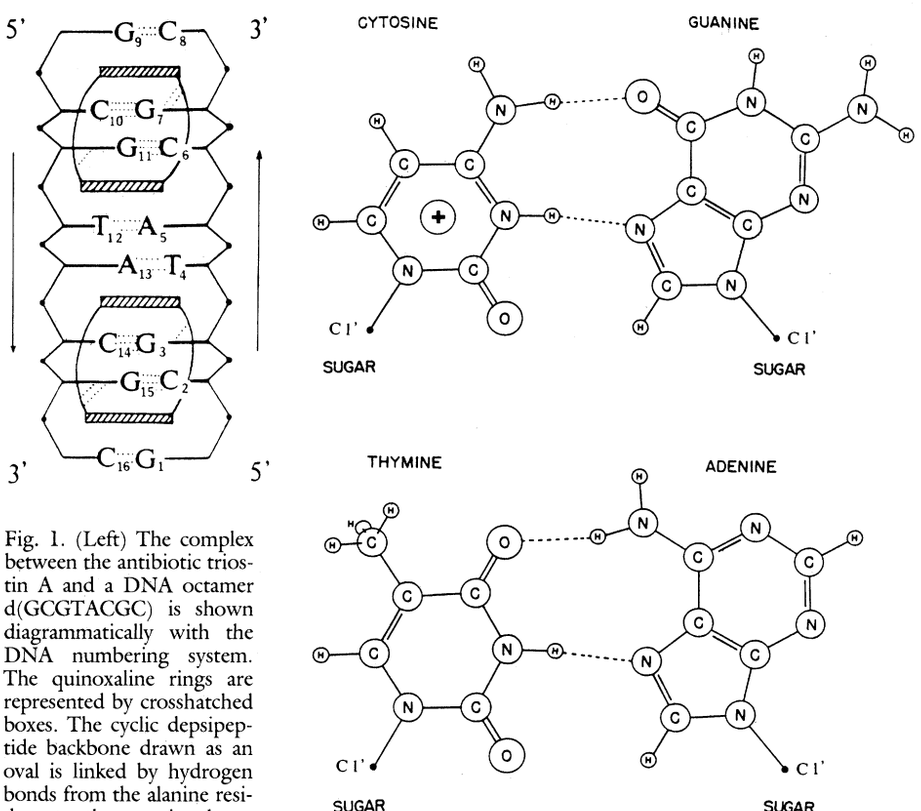

Fig. 1. (Left) The complex between the antibiotic triostin A and a DNA octamer d(GCGTACGC) is shown diagrammatically with the DNA numbering system. The quinoxaline rings are represented by crosshatched boxes. The cyclic depsipeptide backbone drawn as an oval is linked by hydrogen bonds from the alanine residues to the guanine bases (dotted lines). (Right) Schematic drawings show the G · C as well as the A · T Hoogsteen base pairs. The G · C Hoogsteen base pairing requires a protonated cytosine.

Triostin A, a cyclic octadepsipeptide antitumor antibiotic containing two quinoxaline rings, has been cocrystallized with the DNA octamer d(GCGTACGC) and its structure has been solved at near-atomic resolution by x-ray analysis. Two triostin A molecules bind to the DNA octamer with the quinoxaline rings intercalating as shown (Fig. 1, left). The central A · T base pairs in the complex are held together by Hoogsteen rather than Watson-Crick hydrogen bonds, as when triostin A was complexed with the DNA hexamer d(CGTACG) (*3, 4*). The cyclic depsipeptide of the drug molecule lies in the minor groove of the double helix, where it hydrogen bonds in a sequence-specific manner to two CpG base pairs that are surrounded by the bis-intercalating quinoxaline rings. In the octamer complex the outer G · C base pairs are also held together by Hoogsteen hydrogen bonds even though these require protonation of the cytosine residues (Fig. 1, right). Nevertheless, these crystals form readily at *p*H 6.5. This structure indicates that DNA is able to accommodate both Watson-Crick and Hoogsteen base pairs in a right-handed double helical structure at the same time. Conformational changes of this type, in which purine residues are in the *syn* conformation, may occur in a wide variety of alternative DNA forms.

Footprinting experiments of triostin A and its close relative echinomycin have been carried out on plasmid DNA fragments (*5–7*). These experiments showed that the

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