Techniques

In Vivo Cultivation of Spiroplasmas in Larvae of the Greater Wax Moth

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ABSTRACT

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Larvae of the greater wax moth, *Galleria mellonella,* supported extensive multiplication of a number of spiroplasma strains after injection with 1-μl doses of spiroplasma broth cultures. Spiroplasma multiplication in the insects was monitored by light-microscopic examination of hemolymph samples. Multiplication rates of the spiroplasmas in larval hemolymph varied with the spiroplasma strain. *Spiroplasma citri* strain G-1 and strain SR3 spiroplasmas reached titers of ca 10^8 helices per milliliter hemolymph after 5–10 days at 30 C but were not detectable in hemolymph for at least 48 hr after injection. However, the flower isolates 23-6 and PPS1 multiplied rapidly with titers approaching 10^7/ml of hemolymph 12 hr after injection and reaching 10^9/ml by 24 hr. Populations remained at these levels for the duration of the larval stage and spiroplasmas were present in adult moths after pupation. Spiroplasma cells observed in hemolymph were short, actively motile helices without blebs or other degenerative signs. We feel that larvae of *G. mellonella* have potential value as laboratory hosts in further studies of the Mollicutes.

Spiroplasmas, which are members of the class Mollicutes, are pathogens of plants (8), and invertebrate (16) and vertebrate (15) animals. They have also been found epiphytically on flower surfaces (2,11). Spiroplasmas are fastidious organisms requiring relatively complex media for axenic culture. Some spiroplasmas of invertebrate origin (6,10,16) and many nonhelical Mollicutes, the mycoplasmalike pathogens of plants and invertebrates, have not been cultivated in vitro despite intensive efforts. In addition to their apparent obligate parasitism, the maintenance of these organisms in their natural hosts often has presented problems. Unusual hosts have proved useful for some experimental purposes. For example, Williamson and Whitcomb (17) first isolated the corn stunt spiroplasma from *Drosophila,* in which it had been maintained for

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months after being injected in extracts from infected corn (*Zea mays* L.). We investigated the potential of cultivating spiroplasmas in an unusual host, the greater wax moth (*Galleria mellonella* L.). This insect is readily reared on an artificial diet and grows over a wide temperature range suitable for the growth of most mycoplasmas. The initial studies reported here show that *G. mellonella* larvae support the growth of several known cultivable spiroplasmas.

MATERIALS AND METHODS

Wax moths were reared in 1.75- and 3.5-L plastic containers held at 25–35 C and filled to one-third capacity with growth medium. Adult moths were allowed to oviposit directly on the medium or on waxed paper that was later transferred to a container of medium. First-instar larvae 2–3 mm in length hatched within 1 wk. Approximately 2 wk were required for completion of the larval stage. Adults emerged approximately 1 wk after pupation. In several instances pupation was delayed by as much as 8 wk by packing the larval containers with excess growth medium (18). The medium consisted of 227 g of Gerber's Mixed Baby Food ® (Gerber Products Co., Fremont, MI 49412), 70 ml glycerin, 140 ml honey, and 9 gm dry yeast, or a similar diet described by Pipa (12). Larval instars were determined by the criteria of Tchang (14).

Fifth-instar larvae approximately 10 mm in length and weighing 0.04–0.06 g were inoculated with 1 μl of a logarithmic-phase broth culture of spiroplasmas by using a microapplicator and a 0.25-ml syringe fitted with a 0.36-mm-diameter (27-gauge) needle (Fig. 1). In several instances spiroplasmas were injected in preparations concentrated ×10 by centrifugation and resuspension of the pellet in fresh culture broth. Either (C3G) (7), SP4 (15), or PPLO (11) broth media were used. Injections were made ventrally in the abdomen or in the core of a proleg with the needle penetrating just beneath the epidermis. Injected larvae were placed in 20× 100-mm plastic petri dishes containing several grams of medium and held at 30 C for observation. Very little mortality was attributable to the injection process.

Evaluations of spiroplasma growth in the wax moth larvae were determined by phase-contrast or dark-field microscopic counts of helices in hemolymph samples collected at intervals from the larvae by inserting a 0.454-mm-diameter (25-gauge) needle just through the epidermis and collecting a droplet (~10μl) of hemolymph on a cover slip. Estimation of the number of spiroplasmas per milliliter of hemolymph was determined by counting helices in 10 representative microscope fields and dividing by the area per 10 fields times the depth of the liquid layer between the slide and cover slip.

Spiroplasmas used for injection of the larvae came from four defined serogroups (Table 1) (5). These were: serogroup I, represented by *Spiroplasma citri*-related strains; and serogroups III, IV, and V, defined by strains 23-6, SR3/PPS1, and SMCA/GT48, respectively. Serogroup I-1, *S. citri*, was represented by strains SC-27, SC 189, SC W762, SC MC909, SCC, and SC R8-A2; serogroup I-2, the honeybee spiroplasma, by strains AS 576 and G-1; and serogroup I-3, the corn stunt spiroplasma, by strains Miss E and E275.

TABLE 1. Sources of spiroplasma strains tested for reproduction by injection into wax moth larvae

Serotype[a]	Strain	ATCC[b] no.	Source
I-1	SC-27		Stubborn diseased citrus
	SC 189	27563	Stubborn diseased citrus
	SC W762		Stubborn diseased citrus
	SC MC909		Stubborn diseased citrus
	SCC		Stubborn diseased citrus
	SC R8-A2	27556	Little-leaf diseased citrus
I-2	AS 576	29416	Honeybee spiroplasma
	G-1		Spanish needle flower
I-3	Miss E	27954	Corn stunt disease
	E275	29320	Corn stunt disease
III	23-6	29989	Tulip tree flower
IV	SR3	33095	Tulip tree flower
	PPS1	33450	Powder puff flower
V	SMCA	29335	Rabbit tick isolate, suckling mouse cataract agent
	GT48	29334	Rabbit tick isolate

[a] Junca et al (5).
[b] American Type Culture Committee classification.

RESULTS

Spiroplasmas of all serogroups tested multiplied to populations ranging from 10^8 to 10^{10} helices per milliliter in wax moth larvae. Rates of multiplication of the spiroplasmas in the insects varied among the different strains. Strain PPS1 grew most rapidly, reaching maximum titers less than 24 hr after inoculation. *Spiroplasma citri* grew more slowly, requiring 5–7 days to reach peak populations (Fig. 2). Multiplication of the corn stunt spiroplasma strains to

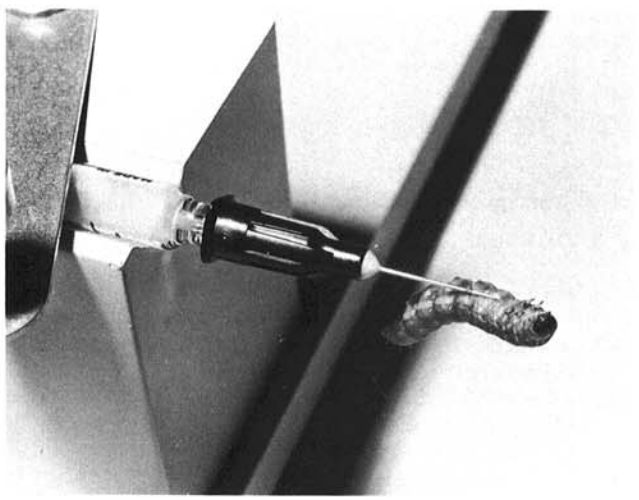

Fig. 1. Injection of *Galleria* larva with spiroplasma suspension.

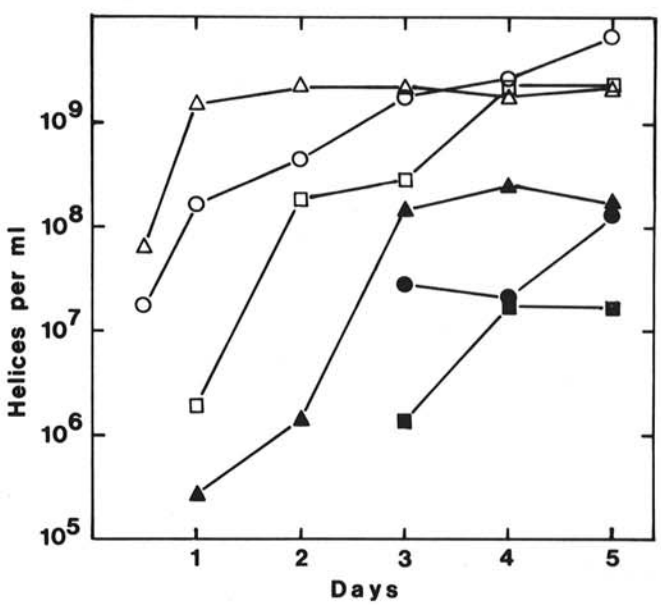

Fig. 2. Population densities of six spiroplasma strains in *Galleria* hemolymph at intervals after inoculation. Limit of detection is population level defined by observation of one spiroplasma cell in 10 microscope fields. Legend: open triangles = PPS1, open circles = 23-6, open squares = AS 576, solid triangles = G-1, solid circles = SR3, and solid squares = SC-27.

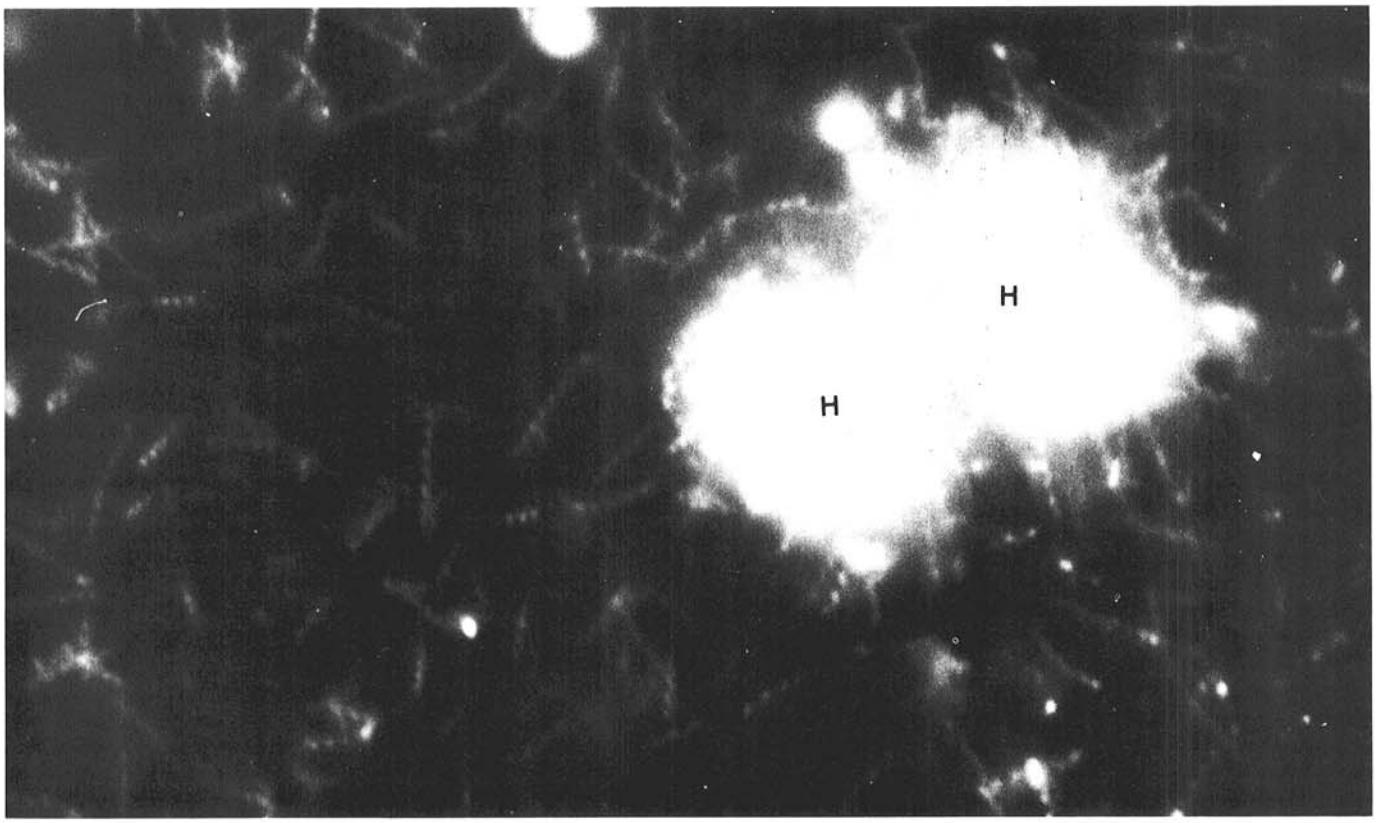

Fig. 3. Dark-field photomicrograph of spiroplasmas in *Galleria* hemolymph. H = *Galleria* hemocytes.

high titers was recorded only in insects for which the larval stage had been extended 4–6 weeks. All spiroplasmas in hemolymph were short helices (Fig. 3). Blebs or other degenerative signs were virtually absent. Reisolation of the spiroplasmas from hemolymph was readily accomplished by placing a droplet of hemolymph collected from a larva (after surface sterilization for 5 sec in 70% ethanol) into an appropriate culture broth. Larval pathogenicity was induced in several cases, particularly by the epiphytic spiroplasmas PPS1, SR3, and 23-6 (4). Larvae infected with these spiroplasmas were stunted and increased mortality was evident. *S. citri* and G-1 spiroplasma had little effect on growth and development of the larvae. When pupation was delayed by packing the larval containers with excess growth medium, spiroplasmas were reisolated from larvae after 7 wk of incubation. Spiroplasmas were readily transferred from one larva to another by injection of hemolymph from infected larvae. All adults developing from infected larvae retained large numbers of spiroplasmas in their hemolymph. In no case did infected adults give rise to infected progeny. Equal numbers of noninjected larvae or larvae injected with sterile media contained no spiroplasmas.

DISCUSSION

Cell wall-free prokaryotes of the class Mollicutes (the mycoplasmas) have been known as animal inhabitants since early in the century. However, it has only been in the past 10 yr that they have been identified as insect-vectored causal agents of a large variety of plant yellows diseases (3,9), as epiphytes (surface residents) on a wide variety of plants (1,11), and as parasites of a number of arthropods (2,6,10,16). The importance of these newly recognized habitats for mycoplasmas is only beginning to be understood, but a new taxon of the class Mollicutes, the genus *Spiroplasma,* already has been established as a result of these studies (13).

A number of new culture media have been devised for the spiroplasmas (1); however, several insect-dwelling organisms have defied all culturing attempts to date. Also numerous noncultivable mycoplasmalike organisms (MLO) of the plant yellows disease group that are vectored by homopteran insects have been described. These agents multiply in their insect vector hosts; however, their diminutive size, together with the attendant handling difficulties, limit their usefulness for generating large numbers of MLO.

G. mellonella, an unusual spiroplasma host, supports high titers of spiroplasmas. Thus, this insect may be an ideal vehicle for the production of large numbers of mycoplasma cells in an in vivo system. Future studies will explore the potential of using this system for fastidious mycoplasmas and spiroplasmas that have not been cultivated in vitro. *G. mellonella* has been a principal insect host for the study of immune metabolism in invertebrates. The ability of spiroplasmas to escape an immune system that effectively clears Gram-negative bacteria is of interest. We feel that the *Galleria*-mycoplasma system also may contribute to an understanding of the ecology of some of the mycoplasmas recently isolated from plant surfaces and arthropods.

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