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Serological and Molecular Characterization of *Mesoplasma seiffertii* Strains Isolated from Hematophagous Dipterans in France

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Three strains of nonhelical mollicutes previously isolated in France from two different mosquitoes and one tabanid fly were designated strains Ar 2328 (isolated from *Aedes detritus*), Ar 2392 (isolated from *Aedes caspius*), and CP 13 (isolated from *Chrysops pictus*). All of these strains exhibited properties of the genus *Mesoplasma*, a recently described genus of non-sterol-requiring mollicutes isolated from plants and insects. The results of metabolism inhibition and growth inhibition tests revealed that these strains and *Mesoplasma entomophilum* TAC or *Mesoplasma florum* L1 were not serologically related, but all three dipteran strains reacted strongly with *Mesoplasma seiffertii* F7^T (T = type strain) antibodies. Using metabolism inhibition and growth inhibition tests, we found that the dipteran strains were related to each other and to strain F7^T but were not identical. We also found that they were able to multiply and persist in the central nervous systems of suckling mice inoculated intracerebrally, a property that makes their use as biological control agents for pest dipterans inadvisable. Scanning electron microscopy revealed marked differences in the morphologies of the colonies of the different strains on SP4 solid medium. The levels of DNA-DNA homology for strains Ar 2328, Ar 2392, CP 13, and F7^T were more than 70%, indicating that these strains are closely related members of the same species, *M. seiffertii*. In addition, one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that each strain produced about 40 protein bands. This technique also revealed differences between strains. Using the coefficient of Smeath-Jacquart, we constructed a dendrogram that allowed us to estimate of the levels of relatedness of these four strains. The results which we obtained were confirmed by two-dimensional protein electrophoresis results.

During ecological studies performed in 1988 and 1990 on mosquito and tabanid spiroplasmas in Parc Régional de Brière in western France (14, 15), three strains of nonhelical mollicutes were isolated unexpectedly; two of these strains were isolated from the marsh mosquitoes *Aedes detritus* Haliday 1833 and *Aedes caspius* Pallas 1771, which were collected in ancient salt marshes, and one was isolated from the deerfly *Chrypsos pictus* Meigen 1820, which was collected in a nearby forest biotope. As preliminary serological screening revealed that these strains were apparently related to a plant mollicute, *Mesoplasma seiffertii*, they were compared with this organism by using both serological and molecular methods.

An important question about *Mesoplasma seiffertii* is its pathogenicity for plants and animals, including humans. The answer to this question is not known, but the fact that strains of this mollicute were isolated from hematophagous insects suggests that it deserves attention.

Another interesting question is the possible use of dipteran strains of *Mesoplasma seiffertii* as biological control agents for hematophagous insects. In this regard *Aedes caspius* may be a prime target since it is the vector for many arboviruses and filariae. For instance, Tahyna virus, a bunyavirus belonging to the California encephalitis group, was isolated from this mosquito species in the former Czechoslovakia in 1959 (2) and later was isolated in many other countries of Eurasia, including France (10), where it is responsible for a febrile illness, especially in children. Other viruses, including Issik-Kul, Isfahan, and Batken viruses, have also been isolated in the former USSR (12) from this mosquito species, which is also the vector of two canine filariae, *Dirofilaria immitis* and *Dirofilaria repens*; these two helminths may accidentally infect humans. In contrast, no role as a vector has been described for *Aedes detritus* (12) or *Chrypsos pictus* (9, 13), but these organisms are major pests of human beings and animals.

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MATERIALS AND METHODS

Mycoplasma strains. The type strain of *Acholeplasma seiffertii*, strain F7, was isolated in 1980 from the surfaces of *Citrus sinensis* flowers from Morocco (3). Recently, *Acholeplasma seiffertii* was transferred to a new genus of the class *Mollicutes*, the genus *Mesoplasma*, and was renamed *Mesoplasma seiffertii*; the following three other species were also assigned to this genus: *Acholeplasma entomophilium*, *Acholeplasma florum*, and *Mycoplasma lactucae* (22). Strains Ar 2328, Ar 2392, and CP 13 were isolated from the whole bodies of the mosquitoes *Aedes detritus* and *Aedes caspius* (14) and the deerfly *Chysops pictus* (15), respectively.

Media, cultivation, and purification. Type strain F7 and the three dipteran strains were isolated previously by using SP4 medium (24), and strains Ar 2328, Ar 2392, and CP 13 were cloned three times before they were characterized. All of the strains were maintained in SP4 medium.

Characterization studies. Cell morphology was determined by dark-field microscopy and electron microscopy. For scanning electron microscopy, colonies on solid SP4 medium were fixed in 2.5% glutaraldehyde in cacodylate buffer, dehydrated through a graded acetone series, critical point dried in liquid carbon dioxide (with a Balzers model CPD 020 apparatus), and coated with gold-palladium (Humer V; Technics). Filterability was determined by using membrane filters having a pore diameter of 220 nm. The ability to metabolize glucose, arginine, and urea was determined in heart infusion broth (Difco) as described previously (1); we used *Spiroplasma taiwanense* CT1^T (T = type strain) (Glu$^+$ Arg$^-$) and *Spiroplasma sabaudiense* Ar 1343^T (Glu$^+$ Arg$^+$) as controls. SP4 media inoculated with each strain were incubated at 30 and 37°C without shaking, and titration values were expressed in color-changing units per milliliter. The sterol and fatty acid requirements of the four strains which we studied were determined by a recently described method (21).

Serological tests. The rabbit antisera prepared against *Mesoplasma seiffertii*,

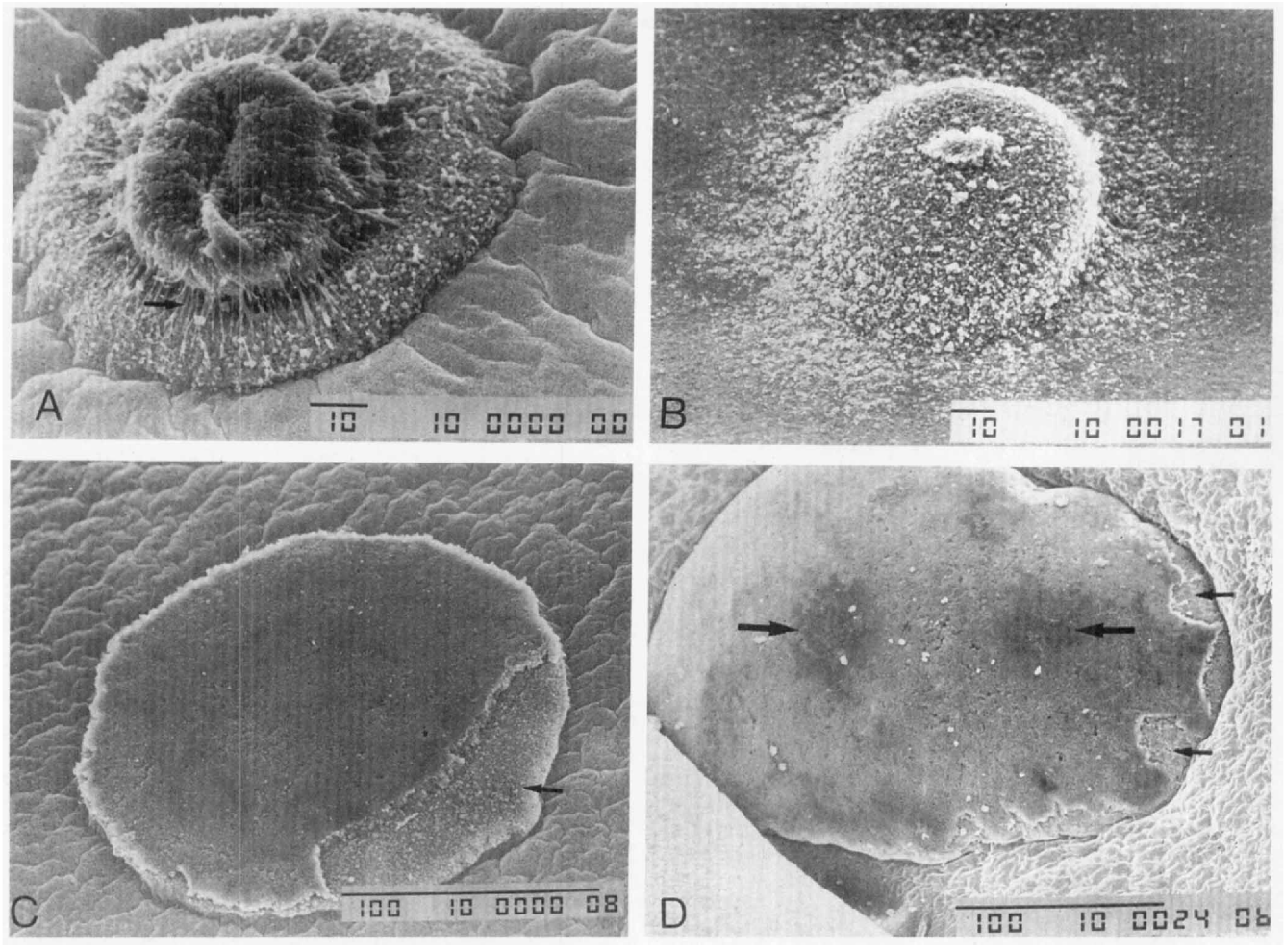

FIG. 1. Scanning electron microscopy of *Mesoplasma seiffertii* colonies after 10 days of incubation at 30°C on solid SP4 medium. (A) Strain F7^T. The arrow indicates filaments that link the two areas of a typical fried-egg colony. Magnification, ×900. (B) Strain CP 13. Magnification, ×450. (C) Strain Ar 2392. The arrow indicates *Mesoplasma* cells in a protruding area. Magnification, ×120. (D) Strain Ar 2328. The large arrows indicate hillocks which can be observed better by light microscopy, - and the small arrow indicate *Mesoplasma* cells in a protruding area. Magnification, ×60.

Mesoplasma entomophilum, and *Mesoplasma florum* that were used for preliminary screening were kindly supplied by J. G. Tully, National Institute of Allergy and Infectious Diseases, Frederick, Md. Antibody to *Mesoplasma lactucae* was not available.

Mouse immune ascitic fluid for strain F7^T and each dipteran strain was prepared by using the protocol of the Yale Arbovirus Research Unit, New Haven, Conn. Briefly, 6-week-old female Swiss EOPS mice (Etalb. Janvier, Le Genest-Saint-Isle, France) were immunized against each of the four strains. The mice were inoculated intraperitoneally four times at 10-day intervals and then received Ehrlich ascitic tumor fluid intraperitoneally. Antibodies were collected at the peak of ascite formation (generally 12 to 15 days later).

Growth inhibition and metabolism inhibition tests (7, 19) were performed concurrently in order to compare the four strains serologically.

Pathogenesis studies. The multiplication and persistence of each strain were studied by using the suckling mouse model that was developed to determine the pathogenicity of bee and tabanid spiroplasmas for the central nervous system (4, 5).

Electrophoresis of proteins. A 100-ml portion of a culture of each strain was centrifuged for 30 min at 9,000 × *g* and 4°C and then washed three times with 10 ml of sterile water, and the pellet was resuspended in 8 to 10 volumes of lysis buffer (2% Triton X-100, 2% mercaptoethanol, 2% ampholyte solution [pH 3 to 10; Pharmacia, Uppsala, Sweden], 9 M urea, 8 mM phenylmethylsulfonyl fluoride). The resulting preparation was subjected to four cycles consisting of 5 min on ice and 1 min of bead beating in the presence of 0.5 g of 0.1-mm-diameter zirconium beads. The sample was then centrifuged at 30,000 × *g* for 45 min at 4°C. The resulting supernatant was considered the protein fraction. The protein concentration was determined by using a protein assay kit (Sigma Chemical Co., St. Louis, Mo.) that was based on a modification of the method of Lowry et al. (16).

For one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) we used SDS-polyacrylamide gels (16 cm by 14 cm by 1 mm; the running gel contained 12% T and 2.6% C, and the stacking gel contained 4% T and 2.6% C). Samples of each preparation were diluted in SDS sample buffer (1.52 g of Tris base, 2 g of SDS, 1 mg of bromophenol blue, 20 ml of glycerol, 40 ml of distilled water) to a concentration of 25 μg/10 μl and then boiled for 3 min and loaded into wells. Electrophoresis was performed in a Protean II electrophoresis tank (Bio-Rad, Richmond, Calif.) until the migration front had migrated 14 cm at a limiting power of 1 W per plate (stacking gel) or 5 W per plate (running gel).

For two-dimensional gel electrophoresis (NEPHGE) we used a technique derived from the original method of O'Farrell et al. (18). Samples containing 100 μg of proteins were focused at 4,000 V/h and then for 1 h at 400 V and for 4 h at 800 V in glass tubes (diameter, 1.2 mm), which produced 13-cm-long gels which contained 9.6% T and 12% C. The ampholytes used were pH 5 to 8, pH 3 to 10, and pH 8 to 10.5 ampholytes (Pharmacia) at a ratio of 5:3:1. The gels were extruded and loaded onto a square SDS-polyacrylamide gel (the running gel contained 12% T and 2.6% C, and the stacking gel contained 4% T and 2.6% C). Electrophoresis in the second dimension was performed in a Protean II electrophoresis tank as described above for one-dimensional electrophoresis.

The one- and two-dimensional electrophoresis gels were silver stained as described by Heukeshoven and Dernick (11).

Genomic comparison. DNA-DNA hybridization was performed by previously described methods (8).

RESULTS

Cultural and morphological properties. The four strains which we studied grew rapidly and extremely well in SP4 medium, usually reaching the peak logarithmic phase in 8 to 10 h

TABLE 1. Comparison of *Mesoplasma seiffertii* CP 13, Ar 2392, Ar 2328, and F7^T by metabolism inhibition and growth inhibition tests

Antigen	Metabolism inhibition test results (growth inhibition test results)			
	Strain CP 13 antibody	Strain Ar 2328 antibody	Strain Ar 2392 antibody	Strain F7^T antibody
CP 13	1/128 (2)	1/32 (0)	1/256 (0)	1/512 (3)
Ar 2328	1/64 (0)	1/256 (1)	1/64 (1)	1/64 (0)
Ar 2392	1/64 (0)	1/64 (0)	1/512 (2)	1/256 (0)
F7^T	1/64 (2)	1/32 (0)	1/256 (1)	1/512 (4)

at 30 and 37°C. On solid SP4 medium, growth was observed after 10 days; only strains F7^T and CP 13 produced typical fried-egg colonies. Moreover, scanning electron micrographs revealed specific morphological characteristics of each strain (Fig. 1). Individual cells in the colonies were spherical (mean diameter, about 500 nm; range, 300 to 1,000 nm), whereas cells in liquid SP4 medium exhibited typical mollicute pleomorphism. The cells of each strain passed through a 220-nm-pore-size filter.

Biochemical properties. All four strains which we studied fermented glucose, hydrolyzed arginine but not urea, and were able to grow in serum-free mycoplasma broth containing Tween 80; thus, they exhibited biochemical properties consistent with inclusion in the genus *Mesoplasma*, family *Entomoplasmataceae*, and order *Entoplasmatales* (22).

Serological tests. During preliminary screening each dipteran strain reacted strongly with *Mesoplasma seiffertii* antibody; the titers were 1:96 for strain CP 13,1:384 for strain Ar 2328, and 1:192 for strain Ar 2392. Tests in which *Mesoplasma entomophilum* and *Mesoplasma florum* antibodies were used were negative (titers, <1:12).

The results of growth inhibition and metabolism inhibition tests performed with F7^T and the three other strains which we studied are shown in Table 1. All of the strains cross-reacted with each other, but the levels of the cross-reactions were variable. Our results showed that there was antigenic heterogeneity, especially in the growth inhibition tests.

Electrophoretic analysis. We observed about 40 protein bands for each strain after one-dimensional electrophoresis

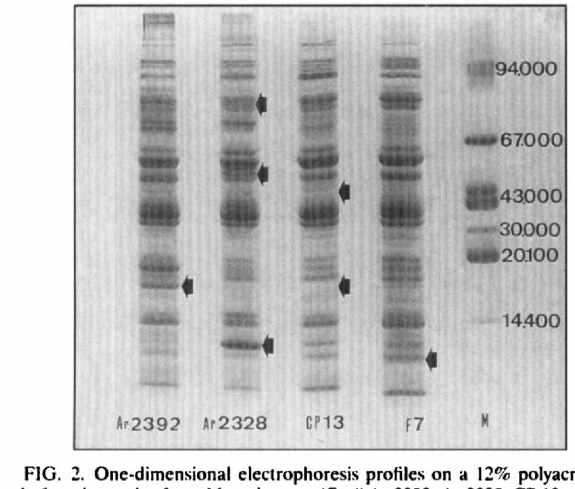

FIG. 2. One-dimensional electrophoresis profiles on a 12% polyacrylamide gel of total proteins from *Mesoplasma seiffertii* Ar 2392, Ar 2328, CP 13, and F7^T. The arrows indicate some striking differences in the profiles.

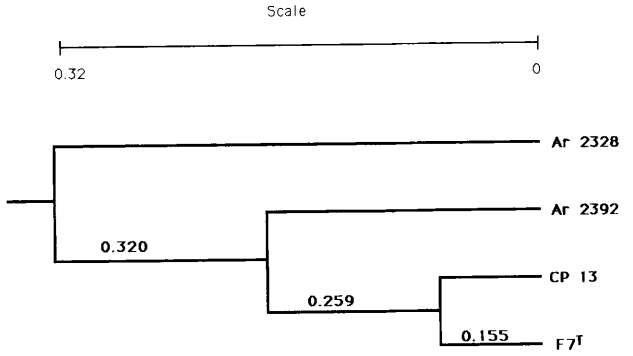

FIG. 3. Dendrogram constructed by using the Smeath-Jacquard coefficient after an analysis of the profiles of *Mesoplasma seiffertii* Ar 2392, Ar 2328, CP 13, and F7^T (see Fig. 2).

(Fig. 2), and there were differences in the migration profiles of the strains studied. Using the Smeath-Jacquart coefficient, we constructed a dendrogram which allowed us to estimate of the levels of relatedness of the strains (Fig. 3). In fact, deerfly strain CP 13 appeared to be more closely related to strain F7^T than the mosquito strains were; the most distantly related organism was apparently strain Ar 2328 (the strain isolated from *Aedes detritus*). These results were confirmed by the results of two-dimensional protein electrophoresis (data not shown).

DNA-DNA hybridization. The DNA-DNA hybridization results are shown in Table 2. As the levels of homology for strains F7^T, Ar 2328, Ar 2392, and CP 13 were always more than 70% (the lowest level of homology was 73% when high-stringency conditions [39°C; melting temperature −12°C), it is clear that the four strains are closely related members of the same species, *Mesoplasma seiffertii*.

Pathogenesis studies. Alternate passages in vitro at 37°C and in vivo revealed that each of the four strains which we studied was able to multiply and persist in the central nervous systems of suckling mice after intracerebral inoculation. However, strains F7^T and Ar 2392, which persisted after the third intracerebral passage, appeared to be more aggressive than strains CP 13 and Ar 2328, which were lost after the second intracerebral passage (data not shown).

DISCUSSION

The results of serological tests and DNA-DNA hybridization experiments obviously demonstrated that all four strains which we studied (one strain isolated from the surfaces of plants and three strains isolated from the bodies of different hematophagous dipterans) belong to the same species, *Mesoplasma seiffertii* (formerly *Acholeplasma seiffertii*). For the cluster of strains which we studied, genomic analysis is the best method for identifying an individual strain at the species level. However, phenotypic studies performed with the same strains were more informative since the results of a combination of morphological, serological, and pathological tests revealed a notable level of biodiversity in *Mesoplasma seiffertii*. In fact, variations in the serological properties, pathogenic effects on mice, and morphologies of the colonies as determined by scanning electron microscopy may be interrelated phenomena that are linked, for instance, by the way of a surface antigen(s) is lost or acquired and the presence of a plasmid, phage, etc. Another important problem concerns the pathogenicity of *Mesoplasma seiffertii* for plants or animals. We are not aware of pathoge-

TABLE 2. Results of DNA-DNA hybridization experiments performed with *Mesoplasma seiffertii* F7^T, CP 13, Ar 2392, and Ar 2328

Organism	% DNA-DNA hybridization[a]							
	Strain F7^T		Strain CP 13		Strain Ar 2392		Strain Ar 2328	
	27°C	39°C	27°C	39°C	27°C	39°C	27°C	39°C
F7^T	100 (82)[b]	100 (93.4)	89.3	78	85	96.7	89.6	89.5
CP 13	80.9	83.9	100 (91.6)	100 (86)	84.2	94.9	91.4	73
Ar 2392	98.2	90.4	87.7	97.7	100 (96)	100 (80.2)	95.1	88.6
Ar 2328	97.7	82.9	89.6	95.3	85.4	91.5	100 (94.6)	100 (95.4)
Escherichia coli	6.8	7.4	7.6	7.2	ND[c]	9.9	10.5	8.9
Spiroplasma citri	8.6	7.8	7.4	6.9	ND	6.4	8.6	8.6

[a] Averages of three experiments. Hybridization experiments were performed under low-stringency conditions (27°C) and high-stringency conditions (39°C).
[b] The values in parentheses are the experimental homologous hybridization values, which were normalized to 100%.
[c] ND, not determined.

nicity for plants, but it is important to note that strain F7^T was isolated from the surfaces of flowers and could have been deposited there accidentally by scavenging insects. In contrast, strains Ar 2328, Ar 2392, and CP 13 were isolated from the whole bodies and not the surfaces of hematophagous insects. The presence of these microorganisms in insects may represent an opportunity for *Mesoplasma seiffertii* to be transferred, even by accident (mechanical transmission), to domestic animals and humans. In addition, the four strains are able to multiply in vitro at 37°C (the approximate body temperature of mammals) and to multiply and persist in the central nervous systems of inoculated mice.

For the reasons given above, it is obvious that *Mesoplasma seiffertii* is not a good candidate for a biological control agent for pest dipterans, either mosquitoes or tabanid flies. In fact, mosquito spiroplasmas are better potential biological control agents (4).

Finally, more isolations of *Mesoplasma seiffertii* strains are needed in order to clarify the general ecology of this organism and its biological cycle in nature, which may involve other hosts. This is also true for the other members of the genus *Mesoplasma*, including *Mesoplasma florum* (17), *Mesoplasma entomophilum* (23), and *Mesoplasma lactucae* (20), all of which were previously classified as *Acholeplasma* or *Mycoplasma* species and were isolated from plant surfaces or insects. Very little is known about the general biology and ecology of these mollicutes.

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