Division of Group XVI Spiroplasmas into Subgroups

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Some group XVI spiroplasmas, such as strains CC-1 (*Spiroplasma cantharicola*) and CB-1, are associated with cantharid beetles. Fifteen related but heterogeneous strains have been isolated from mosquitoes, other insects, and a flower in France and the United States. In the present study, these seventeen strains have been compared by deformation and metabolism inhibition serological tests, by one-dimensional protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and by determination of the guanine-plus-cytosine content of their DNA. Five of the 17 strains were further compared by DNA-DNA hybridization and by restriction enzyme (*EcoRI* and *HindIII*) analysis of their DNA. On the basis of the resulting data, we propose that group XVI be subdivided into three subgroups. Subgroup XVI-I is represented by strain CC-1 (ATCC 43207) from a cantharid beetle in the United States, and strain MQ-6 from a wasp; subgroup XVI-2 is represented by strain CB-1 (ATCC 43208) from a cantharid beetle and two strains from mosquitoes, all in the United States; and subgroup XVI-3 is represented by strain Ar-1357 (ATCC 51126) and contains 11 strains from mosquitoes and 1 strain from a flower, all from the Savoy region of France.

Since the first isolation of spiroplasmas in 1971 (16), the genus *Spiroplasma* has continued to grow and currently contains many strains isolated from various insects, ticks, and plants. Serological techniques were first used for spiroplasma classification (26). Genomic and other molecular techniques were subsequently applied to this group of microorganisms (12). These tests include the guanine plus cytosine (G+C) content of DNA, genome size, DNA-DNA hybridization, DNA restriction endonuclease patterns, and polyacrylamide gel electrophoresis (PAGE) patterns of cell proteins (2). The current classification recognizes 24 groups of spiroplasmas (9, 18). A group (12) is a cluster of spiroplasma strains that are serologically related, share 10 to 100% DNA-DNA homology, and possess a significant number of similar proteins by PAGE. Usually, but not always (11), all strains of groups have an identical G+C content of their DNA (24).

Many groups are represented by one strain or by closely related strains (24). However, some groups are heterogeneous. For example, group I has been subdivided into eight subgroups on the basis of serological data, cell protein analysis, and genomic analysis (2, 18). Strains of some other heterogeneous groups (e.g., group IV) (1) are too closely related to warrant a subgroup designation.

Group XVI contains strains isolated in the United States (Maryland and Alabama) and in France (Savoy) from various insect species and a flower (4-6, 17, 18). Preliminary serological studies (18) showed that group XVI was heterogeneous. We chose 17 group XVI strains for the present study. To define the taxonomic heterogeneity of these strains, reciprocal deformation (DF) and metabolism inhibition (MI) tests were performed, protein patterns of all strains were analyzed by one-dimensional PAGE, and five strains were

selected for determination of the DNA restriction endonuclease patterns and the amount of DNA-DNA homology among them.

In this report, we present the results of the comparative serological and molecular studies of group XVI strains and propose that the group be subdivided into three subgroups.

MATERIALS AND METHODS

Origin of strains. Seventeen strains were studied. Five strains (CC-1, CB-1, MQ-6, ANP, and AEF-2) were collected in the United States, and the 12 other strains were collected in France. The hosts and geographic origins of the strains are given in Table 1. Strain CC-1 was designated as the type strain of *Spiroplasma cantharicola* (21).

Culture media. The CC-1, CB-1, and MQ-6 strains were isolated in SM-1 medium (19). Strains ANP and AEF-2 were isolated in M₁M₂M₃ media (17), and the strains collected in France were isolated in SP-4 medium (19). All the strains were triply cloned on M1D agar medium and then maintained at 30°C by passing a 10% inoculum every day into fresh medium. To provide a uniform source of inoculum, a 10-ml batch culture of each strain was prepared and aliquoted in mid-logarithmic-growth phase into 0.5-ml volumes in microtubes, which were stored at -70°C.

Serological tests. Antisera against the CC-1, CB-1, MQ-6, ANP, and Ar-1357 strains were prepared in rabbits by previously described techniques (26). Sera were inactivated at 56°C for 30 min and stored at -20°C. The 17 strains were compared serologically by using DF and MI tests, which have been described elsewhere (25).

Genomic analysis. The G+C content of the DNA of the 17 strains was calculated from the melting temperature and the buoyant density (ρ) as described previously (3).

PAGE of proteins. We used one-dimensional PAGE analysis in the presence of sodium dodecyl sulfate (SDS) to

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TABLE 1. Geographical and host origin of group XVI spiroplasmas

Strain ^a	Host	Binomial name	Geographical origin
CC-1 (ATCC 43207)	Beetle	<i>Cantharis carolinus</i>	Maryland
CB-1 (ATCC 43208)	Beetle	<i>Cantharis bilineatus</i>	Maryland
MQ-6	Wasp	<i>Monobia quadridens</i>	Maryland
ANP	Mosquito	<i>Anopheles punctipennis</i>	Alabama
AEF-2	Mosquito	<i>Aedes fulvus/annulipes</i>	Alabama
Ar-1357 (ATCC 51126)	Mosquito	<i>Aedes cantans/vexans</i>	Savoy, France
Ar-1531	Mosquito	<i>Aedes cantans/vexans</i>	Savoy, France
Ar-1569	Mosquito	<i>Aedes cantans/vexans</i>	Savoy, France
Ar-1528	Mosquito	<i>Aedes sticticus/vexans</i>	Savoy, France
Ar-1548	Mosquito	<i>Aedes sticticus/vexans</i>	Savoy, France
Ar-1549	Mosquito	<i>Aedes sticticus/vexans</i>	Savoy, France
Ar-1550	Mosquito	<i>Aedes sticticus/vexans</i>	Savoy, France
Ar-1567	Mosquito	<i>Aedes sticticus/vexans</i>	Savoy, France
Ar-1537	Mosquito	<i>Coquillettidia richiardii</i>	Savoy, France
Ar-1546	Mosquito	<i>Aedes cinereus/geminus</i>	Savoy, France
Ar-1570	Mosquito	<i>Aedes cinereus/geminus</i>	Savoy, France
PI-30L	Plant (thistle)	<i>Cirsium</i> sp.	Savoy, France

^a Accession numbers from the American Type Culture Collection are given in parentheses.

examine the patterns of cellular proteins from the 17 isolates. The SDS-PAGE system consisted of a separating gel containing an exponential polyacrylamide gradient (15 to 20%) with a 5% stacking gel prepared according to the methods of Daniels et al. (7) and Mouches et al. (15).

DNA-DNA hybridization. DNA of strain CC-1, the representative strain of group XVI, was labeled by nick translation by using the nick translation kit (N 5000) from Amersham Corp. (Arlington Heights, Ill.) and [³H]dCTP and hybridized with unlabeled DNA from strains CB-1, MQ-6, ANP, and Ar-1357. The percentages of hybridization were determined by the S₁ nuclease technique (8).

DNA hydrolysis by restriction enzymes. DNA of strains CC-1, CB-1, MQ-6, ANP, and Ar-1357 were hydrolyzed by *Eco*RI and *Hind*III restriction endonucleases purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). The fragments were separated by agarose gel electrophoresis (1%), revealed by ethidium bromide staining, and photographed in UV light with a Polaroid camera.

Temperature requirements. The temperature requirements of the group XVI spiroplasmas were studied in M1D medium. Twelve 10-fold dilution series of late-logarithmic-phase cultures were prepared by using preincubated medium for each temperature. The growth was recorded daily for more than 40 days. Doubling times at 5, 10, 15, 20, 25, 30, 32, 37, 41, and 43°C were determined by fitting the temperature-time curves to logistic growth equations (13).

RESULTS

Serological tests. The results of DF and MI tests of the 17 strains (Table 2) permit comparison of group XVI strains. Strains AEF-2 and ANP from Alabama were similar or identical. Strains CB-1 and ANP were very closely related, since the twofold difference in their DF titers is within experimental error. Strains CC-1 and MQ-6 were relatively closely related, since their threefold MI separation was also within experimental error. On the other hand, strain Ar-1357 and other strains from Savoy were distinct from strains CC-1, CB-1, MQ-6, and ANP (differences of two to four dilutions in their DF and MI endpoints).

PAGE protein patterns. The banding patterns of cell proteins from the 17 strains are shown in Fig. 1. All the strains

had approximately 30 visible protein bands. The molecular mass of the majority of proteins was between 18 and 67 kDa. Comparative analysis of the one-dimensional PAGE protein patterns indicated that all strains have many proteins in common. Nevertheless, protein differences among the strains could be identified. For example, a 21-kDa protein was present in all strains, except for strains CC-1 and MQ-6; a 26-kDa protein was absent in strains CC-1, MQ-6, and PI-30L; a 35-kDa protein was present only in strains CB-1, AEF-2, and ANP; and a 17-kDa protein was missing in all strains from Savoy.

Genomic analysis. The G+C content of the DNA of all spiroplasma group XVI strains was (26 ± 1) mol% (Table 3).

DNA-DNA hybridization. Table 4 gives the percentage of hybridization between labeled CC-1 DNA and unlabeled DNAs of strains CB-1, MQ-6, ANP, and Ar-1357, as determined by analysis with S₁ nuclease. The percentage of homology between strains CC-1 and MQ-6 DNA was 100%. The percentage of homology between strain CC-1 DNA and strains CB-1, ANP, and Ar-1357 DNAs was 61, 63, and 69%, respectively.

Restriction enzyme patterns. The restriction enzyme patterns are shown in Fig. 2. DNA from strains CC-1, MQ-6, and Ar-1357 was each hydrolyzed by both *Eco*RI and *Hind*III; DNA from strains CB-1 and ANP was hydrolyzed by *Eco*RI but not by *Hind*III. In addition to their insensitivity to *Hind*III hydrolysis, strains CB-1 and ANP showed very similar *Eco*RI restriction patterns of DNA, providing further evidence that these two strains are closely related. Finally, the restriction patterns produced by *Eco*RI and *Hind*III digestion of strains CC-1, MQ-6, and Ar-1357 DNAs were very different.

Temperature ranges. Strain CC-1 grew at 10 to 37°C, with an optimum at 32°C. The optimal doubling time was 2.6 h. Strain CB-1, on the other hand, grew at 10 to 32°C. The optimum was 30°C, at which temperature the doubling time was 2.6 h. Strain Ar-1357 grew at a range of 10 to 32°C. The optimum temperature of growth was 30°C, and the doubling time at that temperature was 3.4 h. Strain MQ-6 had a temperature range of 10 to 37°C, with an optimum at 32°C. The optimal doubling time was found to be 2.5 h.

TABLE 2. Serological comparison of group XVI spiroplasmas by DF and MI tests

Antigens	Antiserum to:									
	CC-1		CB-1		MQ-6		ANP		Ar-1357	
	DF ^a	MI ^b	DF	MI	DF	MI	DF	MI	DF	MI
CC-1	1,280	39,000	N ^c	162	320	13,000	N	162	80	162
MQ-6	320	4,374	40	1,458	1,280	118,000	N	1,458	160	1,458
CB-1	40	162	640	39,000	40	4,374	320	13,000	80	486
ANP	40	162	640	13,000	20	486	640	39,000	320	486
AEF-2	40	162	640	13,000	20	486	640	39,000	320	486
Ar-1357	80	486	80	486	160	4,374	40	486	1280	118,000
Ar-1528	40	4,374	20	4,374	N	1,458	N	486	2560	4,374
Ar-1531	40	162	40	4,374	N	4,374	N	486	2560	13,000
Ar-1537	40	486	N	39,000	160	39,000	40	39,000	2560	13,000
Ar-1546	20	1,458	N	39,000	40	1,458	20	486	1280	13,000
Ar-1548	40	168	40	1,458	80	1,458	N	162	1280	13,000
Ar-1549	N	4,374	20	39,000	80	13,000	N	4,374	1280	118,000
Ar-1550	40	1,458	N	4374	20	486	N	1,458	1280	39,000
Ar-1567	N	486	N	13,000	20	4,374	N	486	1280	13,000
Ar-1569	N	162	N	162	N	118,000	N	1,458	1280	39,000
Ar-1570	N	13,000	20	13,000	80	39,000	20	13,000	1280	39,000
PI-30L	40	162	80	4,374	320	4,374	20	162	1280	39,000

^a Titers are expressed as the reciprocal of the highest antiserum dilution at which one-half of the spiroplasmas are free and motile and one-half are deformed.

^b Reciprocal of the highest antiserum dilution inhibiting glucose fermentation.

^c N, DF titer of less than 1:20.

DISCUSSION

In taxonomic studies of spiroplasmas, serological tests should be performed first. The DF test is very convenient and provides reliable information about interstrain relationships. MI tests usually confirm the data of DF tests but also provide additional sensitivity. In our study, these tests showed that the strains from Savoy are much more closely related to each other than to the strains from other insects in the United States and could represent a single subgroup. Among the New World strains, ANP and AEF-2 were similar or identical. Strains CC-1, MQ-6, CB-1 and ANP could not be subdivided clearly into subgroups on the basis of serological tests.

PAGE analysis of proteins is also an important technique in spiroplasma classification. Analyses of protein profiles of the strains of the subgroups of group I spiroplasmas agree well with serological analyses (15). In our study, also, the data obtained by PAGE analysis agreed with those obtained from serological studies.

Although serological techniques and one-dimensional PAGE of proteins were in good agreement, it was not possible to subdivide group XVI unequivocally into subgroups by these tests alone. We therefore selected five strains that appeared to have major differences in their serological and PAGE patterns and compared their genomes by three additional procedures: (i) determination of G+C

FIG. 1. SDS-PAGE of spiroplasma proteins of group XVI. Lanes: A, Protein standard (Bio-Rad) containing phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); 1, *S. cantharicola* CC-1 (ATCC 43207) (reference strain); 2, CB-1; 3, MQ-6; 4, AEF-2; 5, ANP; 6, Ar-1357; 7, Ar-1528; 8, Ar-1531; 9, Ar-1537; 10, Ar-1546; 11, Ar-1548; 12, Ar-1549; 13, Ar-1569; 14, Ar-1550; 15, Ar-1566; 16, Ar-1567; 17, Ar-1570; 18, PI-30L.

TABLE 3. G+C content of DNA of group XVI spiroplasmas

Strain	G+C content (mol%) determined from:	
	Buoyant density (mean)	Melting temperature (mean)
CC-1	26.6	25.4
MQ-6	ND ^a	26.5
CB-1	25.8	25.3
ANP	ND	26.3
AEF-2	ND	ND
Ar-1357	26.4	25.9
Ar-1528	27.4	27.0
Ar-1531	26.7	25.0
Ar-1537	26.3	26.0
Ar-1546	26.5	25.6
Ar-1548	26.0	25.3
Ar-1549	25.8	26.0
Ar-1550	26.4	25.0
Ar-1567	26.6	26.0

^a ND, not done.

content, (ii) DNA-DNA hybridization, and (iii) hydrolysis by restriction endonucleases.

The determination of the G+C content of DNA is a recommended procedure for group classification (18, 20, 22). Spiroplasma strains belonging to the same group almost always have identical G+C contents of their DNAs (24). In our study, we found that the DNAs of 13 group XVI strains had a G+C content of (26 ± 1) mol%.

DNA-DNA hybridization, a technique that is routinely used to evaluate homologous sequences among related DNAs, has become a useful procedure for spiroplasma classification (22). DNA-DNA hybridizations between or among intergroup strains should show no homology (23). Clusters of strains that form subgroups show high levels of intragroup homology (approximately 90%), whereas inter-subgroup homology may range between 10 and 80%. Subgroups that have 70% or less DNA-DNA homology with other subgroups within the same group are eligible for elevation to species status (10).

DNA-DNA hybridization has been used, in part, to subdivide group I spiroplasmas into eight subgroups. In this grouping, the hybridization data were in close accord with the serological data (2). On the other hand, DNA-DNA hybridization between strains of group IV revealed (1) that all strains had DNA homologies in excess of 75% and that this group could not be subdivided into different subgroups as had been previously proposed by using serological techniques (14). With group XVI, DNA-DNA hybridization data have led us to conclude that CC-1 and MQ-6 strains are very

TABLE 4. Percentages of hybridization between labeled DNA from strain CC-1 and nonlabeled DNA of strains CB-1, MQ-6, ANP, and AR-1357

Strain	% Hybridization
CC-1-[³ H]	100 (76) ^a
CB-1	61
MQ-6	100
ANP	63
Ar-1357	69
Salmon sperm ^b	3.3

^a The number in parentheses represents the actual hybridization in the homologous reaction, which was normalized to 100%.

^b Negative control.

FIG. 2. DNA patterns of strains CC-1, MQ-6, CB-1, ANP, and Ar-1357 hydrolyzed by *Eco*RI (lanes 1 to 5) and *Hind*III (lanes 6 to 10) restriction enzymes by 1% agarose gel electrophoresis. Lanes: A, 1-kbp ladder (GIBCO-Bethesda Research Laboratories); 1, CC-1; 2, MQ-6; 3, CB-1; 4, ANP; 5, Ar-1357; 6, CC-1; 7, MQ-6; 8, CB-1; 9, ANP; 10, Ar-1357.

similar (the homology of their DNA is 100%) and, on the basis of similar PAGE protein patterns and restriction enzyme profiles, can be placed in the same subgroup. Strains CB-1, ANP, and Ar-1357 would constitute another subgroup because the homology between their DNA and CC-1 DNA is less than 70% (61, 63, and 69%, respectively). However, a number of factors served to differentiate Ar-1357 and the other strains isolated in France from the four strains isolated from insects in the United States. (i) They are geographically separated. (ii) With the exception of one plant-derived strain (PI-30L), all the French strains were isolated from various species of mosquitoes, suggesting a commonality in their ecology. (iii) We observed close uniformity in the PAGE protein patterns of the French strains versus the four U.S. strains. (iv) The *Eco*RI- and *Hind*III-restricted DNA banding patterns of Ar-1357 are strikingly different from those produced by the four U.S. strains. The New World strains formed two related clusters: CC-1 and MQ-6 and CB-1 and ANP.

Comparative analysis of all our data suggests a classification of spiroplasma group XVI into the following three subgroups: (i) subgroup XVI-1, comprising strains CC-1 and MQ-6; (ii) subgroup XVI-2, comprising strains CB-1, ANP, and AEF-2; and (iii) subgroup XVI-3, comprising strains Ar-1357 and all other strains from Savoy.

Strain CC-1 (ATCC 43207), the type species of *S. cantharicola*, is the representative strain of subgroup XVI-1, strain CB-1 (ATCC 43208) represents subgroup XVI-2, and strain Ar-1357 (ATCC 51126) represents subgroup XVI-3.

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