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# *Spiroplasma kunkelii* sp. nov.: Characterization of the Etiological Agent of Corn Stunt Disease

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**Nine strains of spiroplasma subgroup I-3, which comprise the etiological agent of corn stunt disease, were similar in their serological properties. Strain E275$^T$ (T = type strain) was studied by using criteria proposed by the International Committee on Systematic Bacteriology Subcommittee on Taxonomy of *Mollicutes* for descriptions of new mollicute species. This strain was shown to belong to the class *Mollicutes* by the ultrastructure of its limiting membrane, its procaryotic organization, its colonial morphology, and its filtration behavior and to the family *Spiroplasmataceae* by its helical morphology and motility. Although some serological cross-reactions with other group I spiroplasma strains was observed, strain E275$^T$ could be readily distinguished from representatives of other group I subgroups. Subgroup I-3 spiroplasmas and other group I strains also differed in their one- and two-dimensional polyacrylamide gel protein patterns, plant and insect host ranges, and pathogenicities. Growth in M1A or M1D medium occurred at 20 to 30°C. Cholesterol was required for growth. Glucose was fermented, and arginine was catabolized. Subgroup I-3 strains, including strain E275$^T$, reacted with considerable homogeneity in deformation tests and were completely separable from strains belonging to subgroup I-1 (*Spiroplasma citri*) and subgroup I-2 (*Spiroplasma melliferum*). Strain E275$^T$ was also serologically distinct from subgroups I-4 through I-8, *Spiroplasma floricola* (group III), *Spiroplasma apis* (group IV), *Spiroplasma mirum* (group V), and representative strains of spiroplasma groups II and VI through XI. The deoxyribonucleic acid of strain E275$^T$ hybridized with the deoxyribonucleic acid of *S. citri* at significant levels (33 to 68%, depending on the technique used). These results demonstrate that strain E275$^T$ and similar strains meet the criteria proposed by the International Committee on Systematic Bacteriology Subcommittee for elevation of spiroplasma subgroups to species. We propose that such strains be named *Spiroplasma kunkelii*. Strain E275$^T$ has been deposited in the American Type Culture Collection as strain ATCC 29320$^T$.**

Corn stunt disease was first reported as a stunting and striping syndrome of maize in Texas (2). Although this disease was studied biologically (53, 54, 65, 66), the procaryotic nature of the etiological agent remained unsuspected until the late 1960s. Then, prompted by reports that certain leafhopper-borne plant diseases thought to be of viral etiology were in fact caused by wall-less procaryotes, R. R. Granados and colleagues demonstrated that wall-less procaryotes were associated with "Louisiana corn stunt disease" in plant and insect vectors (43, 44). These organisms could also be demonstrated in negatively stained preparations of sap from diseased corn (43). Following these morphological studies, Chen and Granados (13) achieved long-term maintenance of the Rio Grande strain of the corn stunt agent in cell-free media and observed filaments similar to those seen in vivo. Subsequently, phase-contrast microscopic examination of sap from plants infected with the Rio Grande corn stunt agent revealed helical microorganisms (32) that were later shown to be consistently associated with the disease (33, 34). Although these organisms could not be continuously cultivated, they were shown to be helical, motile, wall-less procaryotes and were termed "spiroplasmas" (33), a trivial term that was later adopted (85) as a generic name for helical wall-less mollicutes. This designation now applies to the cultivable spiroplasma (*Spiroplasma citri*) that causes citrus stubborn disease and to a plethora of organisms associated with arthropods (18). Previous study of the causal agent of corn stunt disease was hampered by the inability of researchers to cultivate the agent. Actually, some of the "strains" may in fact have represented a nonhelical mollicute, maize bushy stunt mycoplasma (77). Despite early difficulties in continuous propagation of subgroup I-3 organisms (28), the Rio Grande spiroplasma was eventually cultivated simultaneously and independently in two laboratories, and Koch's postulates were fulfilled (14, 110). In these discoveries, two media that were very different in composition and rationale permitted not only primary isolation but also continuous maintenance and demonstration of the etiologic role of the pathogen (14, 110). The taxonomic assignment of subgroup I-3 spiroplasmas was delayed by the discovery of considerable serological cross-reactions with *S. citri* (95) and similar shared antigenicity among all group I strains (6). Corresponding similarities among group I strains were also found by using two-dimensional polyacrylamide gel electrophoresis (PAGE) (71, 73, 74). Extensive studies of group I strains eventually led to a proposal that subdivided the group into eight subgroups (6, 87). Strain E275$^T$ (T = type strain) and similar strains are classified as subgroup I-3 in this classification, which is part of a scheme first proposed by Junca et al. in 1980 (52) and recently revised (98, 100).

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Taxonomic problems presented by the group of partially related group I strains have been discussed at length by the International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Mollicutes* (48–50); although a conservative approach was recommended pending further studies, Davis and Lee proposed (29) that spiroplasma subgroup strains could be promoted to species status if they were serologically distinguishable. While disagreeing strongly with some aspects of this proposal, the Subcommittee in 1984 proposed (50) that subgroups might be elevated to species status under certain conditions. These were (i) all requirements proposed in the minimal standards document (47) must be fulfilled; (ii) the level of deoxyribonucleic acid (DNA)-DNA homology between the candidate subgroup and all other subgroups must be demonstrated to be less than 70%; (iii) the ecology of the organism must be studied, and principal hosts or alternate hosts or both must be identified; (iv) the subgroup should be of importance to agriculture or public health or as a basic microbiological model; and (v) the subgroup should be shown to consist of a cluster of homogeneous strains isolated from as many different circumstances as possible. Strain homogeneity should be assessed by techniques such as serology, PAGE, or the cleavage patterns of DNAs digested by restriction endonucleases. This recommendation was prompted by demonstration of clusters of relative genotypic homogeneity in several mollicute species (84). Whether taxonomic proposals fulfilled these conditions was left to the judgment of referees and editors.

In this report, we summarize evidence that the species requirements have been fulfilled for strain E275$^T$ and related subgroup I-3 strains with respect to both the Subcommittee proposal for elevation of subgroups to species (50) and the minimal standards for mollicute species descriptions proposed in 1979 by the Subcommittee (47). Because we believe that the requirements have been met, we propose that strain E275$^T$ and related strains be recognized as a new species in the genus *Spiroplasma*.

## MATERIALS AND METHODS

**Origin of isolates.** Nine representatives of subgroup I-3 were examined serologically. Strain E275$^T$ (from maize plants infected with the Rio Grande corn stunt spiroplasma) was derived from an isolate cultured in M1 medium (110). The isolate was triply cloned, and strain E275$^T$ was chosen to represent the subgroup I-3 cluster. The following other subgroup I-3 strains were used: strain I-747, from corn with the Rio Grande syndrome (14); strains B647, B652, and B655, from corn in Jamaica (36); strains CSEE1 and CSEE2, from *Exitianus* leafhoppers (Williamson, unpublished data); strain Miss E, from corn in Mississippi (unpublished data); and strain CRL, from corn in Florida (R. E. McCoy, unpublished data). Other spiroplasmas were compared with spiroplasma strain E275$^T$. These included *S. citri* R8A2$^T$ (= ATCC 33219$^T$), *Spiroplasma melliferum* BC-3$^T$ (= ATCC 33219$^T$) (16, 20), tick spiroplasma strain 277F (= ATCC 29761) (subgroup I-4 [10]), strain LB-12 (= ATCC 33649) (subgroup I-5 [58]), Maryland flower spiroplasma strain M55 (= ATCC 33502) (subgroup I-6 [102]), *Cocos* spiroplasma strain N525 (= ATCC 33287) (subgroup I-7 [37]), strain P40 (subgroup I-8) from Syrian *Catharanthus* (= *Vinca*) (87), uncultivated strain WSRO of the *Drosophila* sex ratio organism (group II [106]), *Spiroplasma floricola* OBMG (= ATCC 33221) (group III [17]), *Spiroplasma apis* B31$^T$ (= ATCC 33834) (group IV [71, 72]), *S. apis* PPS1 (= ATCC 33450) (group IV [69]), *Spiroplasma mirum* SMCA$^T$ (= ATCC 29335$^T$) (group V [97]), *Ixodes* spiroplasma strain Y32 (= ATCC 33835) (group VI [94]), *Monobia* spiroplasma strain MQ-1 (= ATCC 33825) (group VII [18]), syrphid spiroplasma strain EA-1 (= ATCC 33826) (group VIII [18]), *Cotinus* spiroplasma strain CN-5 (= ATCC 33827) (group IX [19]), *Aedes* strain AES-1 (= ATCC 35112) (group X [98]), and *Monobia* spiroplasma strain MQ-4 (= ATCC 35262) (group XI [98]).

**Culture media and cultivation procedures.** Strain E275$^T$ and all subgroup I-3 strains could be cultivated in M1A (51) or M1D medium. The M1D formulation was identical to M1A medium, except that fresh yeast extract was deleted. The techniques used for primary isolation of corn stunt spiroplasmas from plants and insects have been described previously (8, 14, 67, 110). For maintenance of the organisms, cultures were passed at intervals of 3 or 4 days by using 10% inocula added to fresh media. Most spiroplasmas used in our studies were grown in M1D medium. However, *S. mirum* and spiroplasma strain Y32 were cultivated in SP-4 broth (96).

**Filtration studies.** An M1A broth culture of strain E275$^T$ that had been incubated for 3 days at 30°C was passed by means of a hypodermic syringe with minimum hand pressure through a series of membrane filters with graded pore diameters (650, 450, 300, 220, and 100 nm). Each filtrate was diluted in a series of half-$\log_{10}$ dilutions in M1A medium, and all of the resulting tubes were incubated at 30°C. After 14 days, the tubes were examined for growth and color change, and turbidity was recorded. The passage of strain E275$^T$ through various filters was assessed by identifying the last dilution tube that showed growth of helical cells and a color change. The results were expressed in color-changing units (CCU) per milliliter.

**Morphology.** Cultures of strain E275$^T$ were routinely monitored by dark-field microscopy. For electron microscopy, all cells were fixed for 2 h in 3.0% glutaraldehyde in M1A medium, postfixed in 1% osmium tetroxide for 1 h, dehydrated in acetone, embedded in Epon, and stained with uranyl acetate.

**Sterol requirement.** The growth response to cholesterol was determined by the direct broth culture method (83).

**Biochemical tests.** Phosphatase activity, tetrazolium reduction, hemadsorption, and digitonin sensitivity tests were performed on solid medium (serum fraction broth containing 2.25% Noble agar) by using previously described techniques (3, 9, 39, 40, 64). Substrate tests were performed in M1B medium (97).

**Serological tests.** Hyperimmune spiroplasma strain E275$^T$ antiserum was prepared as described previously (95). Disk growth inhibition tests (21, 101) were performed with spiroplasmas grown in M1A medium. Antigens grown in broth were usually diluted to concentrations of about $10^5$ CCU/ml. The techniques used for deformation tests, metabolism inhibition tests, and enzyme-linked immunosorbent assays have been described previously (86, 108, 111). The metabolism inhibition test antigens (except subgroup I-3 strains, which were grown in M1A medium) were prepared from cultures that were grown for 72 h at 30°C in SP-4 broth, and most of the test procedures were performed in SP-4 broth. The metabolism inhibition tests with subgroup I-3 strains were always performed in M1A broth.

**PAGE of cell proteins.** The techniques used to determine patterns of cell proteins by one- and two-dimensional PAGE have been described previously (70).

**Genomic analysis.** The techniques used for DNA extraction and to determine (by buoyant density and melting

temperature methods) guanine-plus-cytosine contents of the subgroup I-3 spiroplasma DNAs have been described previously (11, 12, 52).

## RESULTS

**Morphological and cultural properties.** Primary isolation of strain E275$^T$ was accomplished by expressing juice from maize tissue, suspending it in M1 medium (110), and maintaining the culture at 30°C. In M1 medium, strain E275$^T$ initially grew slowly but eventually could be subcultured 3 to 4 days after transfer. Cultured organisms produced turbidity and acidified the medium. The presence of penicillin (500 U/ml) had no influence on growth rates or yields. When strain E275$^T$ was adapted, it grew to a titer of about $10^8$ CCU/ml in 72 h at 30°C. The terminal pH was about 5.3. The morphological features of strain E275$^T$ and related strains were essentially those of the type strain of the genus (22). Cultures examined by dark-field microscopy contained numerous helical cells, which frequently flexed and twitched and occasionally appeared to exhibit rotatory motility. In stationary growth (cultures kept at 30°C for more than 5 days), the organisms became deformed, but some evidence of helicity persisted for many days. Cells grown in M1A medium were examined by electron microscopy. When negative staining or sectioning procedures were used, no evidence of periplasmic fibrils or cell wall material was observed. Thin sections revealed only a typical single membrane surrounding the cells (110) (Fig. 1). As with other spiroplasmas (41, 109), one end of the helix appeared to be more pointed than the other.

Strain E275$^T$ and other subgroup I-3 spiroplasmas formed diffuse colonies on solid medium containing 0.8% Noble agar. Classical "fried egg" colonies were formed on C3-G agar supplemented with 5% horse serum (46) or on M1A medium formulations containing 2.25% Noble agar. Colonial growth was best when plates were maintained in an anaerobic environment (GasPak; BBL Microbiology Systems, Cockeysville Md.).

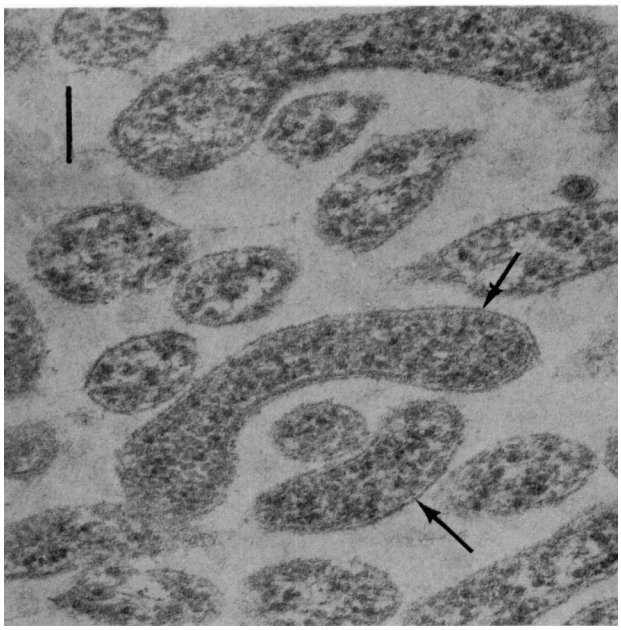

FIG. 1. Electron micrograph of spiroplasma strain E275$^T$. Thin-section preparation of a cell pellet obtained from a 4-day broth culture grown in M1A medium, fixed in 2% glutaraldehyde, postfixed in 1% $OsO_4$, and stained with 2% uranyl acetate for 40 min and with Reynold's lead citrate for 1.5 min. Note the single membrane (arrows) on the cells. Bar = 100 μm.

TABLE 1. Sterol requirement test for strain E275$^T$

| Cholesterol concn in medium (μg/ml) | Cell protein (mg)[a] |
|---|---|
| O[b] | <0.32 |
| O[c] | <0.59 |
| O[d] | <0.67 |
| 1.0 | 1.17 |
| 5.0 | 1.13 |
| 10.0 | 1.08 |
| 20.0 | 1.16 |
| Control[e] | 1.67 |

[a] Amount of protein in cell pellet obtained from 100 ml of growth medium.
[b] Serum-free medium.
[c] Serum-free medium supplemented with 0.5% albumin and 10 μg of palmitic acid per ml.
[d] Serum-free medium supplemented with 0.5% albumin, 10 μg of palmitic acid per ml, and 0.01% Tween 80.
[e] Medium containing 1% bovine serum fraction.

**Reversion studies.** Prior to the cloning procedures with which strain E275$^T$ was selected, cultures of spiroplasma subgroup I-3 strain E were maintained in antibiotic-free broth medium for five passages without reversion to bacterial forms. Actively growing cultures examined by dark-field microscopy contained typical uniformly helical organisms. When such organisms were examined with an electron microscope, they showed no evidence of cell walls. Although strain E275$^T$ and all subgroup I-3 strains which we studied showed absolute resistance to penicillin, the organisms could be routinely obtained in primary isolation cultures from surface-sterilized corn in media without antibiotics.

**Filtration studies.** A 72-h broth culture of strain E275$^T$ contained $10^{7.5}$ CCU/ml before filtration through a graded series of membrane filters. After filtration through membrane filters having average pore diameters of 650, 450, 300, and 220 nm, the preparations contained $10^{7.5}$, $10^6$, $10^5$, and $10^4$ CCU/ml, respectively. The organism did not pass through membrane filters with average pore diameters of 100 nm.

**Biochemical and biological properties.** Strain E275$^T$ fermented glucose and hydrolyzed arginine; the latter response occurred whether or not glucose was present in the medium. As with *S. citri* (92), when both substrates were present, the medium first became acid, but eventually, as the arginine was catabolized, became basic. Strain E275$^T$ fermented fructose but did not hydrolyze urea. Other properties of strain E275$^T$ are summarized in the taxonomic description given below.

**Sterol requirement.** Small amounts of growth of strain E275$^T$ occurred in the serum-free base formulation or when supplements of albumin, palmitic acid, and Tween 80 were added (Table 1). However, addition of 1 to 20 mg of cholesterol per ml to the base medium containing fatty acid supplements resulted in at least twofold growth stimulation of spiroplasma strain E275$^T$.

**Serological tests.** Sera prepared against strain E275$^T$ resulted in zones of homologous growth inhibition with diameters ranging from 12 to 18 mm (Table 2). Growth inhibition zones having smaller diameters were observed in reciprocal tests of strain E275$^T$ with other group I strains. Antiserum prepared against the *Drosophila* sex ratio spiroplasma

TABLE 2. Results of serological tests with spiroplasma strain E275$^T$

| Group | Strain | Sizes of inhibition zones (mm)[a] | | | Results of deformation tests[b] | | | Results of metabolism inhibition tests[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Strain E275$^T$ antigen vs heterologous antiserum | Strain E275$^T$ antiserum vs heterologous antigen | Homologous reaction | Strain E275$^T$ antigen vs heterologous antiserum | Strain E275$^T$ antiserum vs heterologous antigen | Homologous reaction | Strain E275$^T$ antigen vs heterologous antiserum | Strain E275$^T$ antigen vs heterologous antigen | Homologous reaction |
| I-1 | *S. citri* R8A2$^T$ | 12 | 12 | 16 | 80 | 80 | 2,560 | 162 | 54 | >117,000 |
| I-2 | *S. melliferum* BC-3$^T$ | 14 | 2 | 12 | 640 | 160 | 10,240 | 54 | 486 | 117,000 |
| I-3 | E275$^T$ | | | 18 | | | 20,240 | | | 39,000 |
| I-4 | 277F | 3 | 4 | 14 | 320 | 160 | 5,120 | 18 | 1,458 | 39,000 |
| I-5 | LB-12 | 4 | n[d] | 18 | 80 | n | 5,120 | 18 | 54 | >117,000 |
| I-6 | M55 | 4 | n | 13 | 20 | 20 | 1,280 | 18 | n | 13,000 |
| I-7 | N525 | n | 1 | 11 | 20 | 20 | 10,240 | n | 54 | 39,000 |
| I-8 | P40 | 6 | 12 | 17 | 640 | 640 | 10,240 | 486 | 486 | >117,000 |
| II | WSRO | n | ND[e] | ND | n | n | 4,000 | n | ND | ND |
| III | *S. floricola* OBMG | n | n | 6 | n | n | 10,240 | n | n | 13,000 |
| IV | *S. apis* B31$^T$ | n | n | 5 | n | n | 1,280 | n | n | 13,000 |
| V | *S. mirum* SMCA$^T$ | n | n | 13 | n | n | 5,120 | n | n | 13,000 |
| VI | Y32 | n | n | 16 | n | n | 2,560 | n | n | 4,324 |
| VII | MQ-1 | n | n | 6 | n | n | 1,280 | n | n | 117,000 |
| VIII | EA-1 | n | n | 10 | n | n | 1,280 | n | n | 39,000 |
| IX | CN-5 | n | n | 8 | n | n | 1,280 | n | n | 4,374 |
| X | AES-1 | n | n | 5 | n | n | 640 | n | n | 4,374 |
| XI | MQ-4 | n | n | 6 | n | n | 640 | n | n | 4,374 |

[a] Results of growth inhibition tests (sizes of zones of inhibition around disks saturated with antiserum).
[b] Reciprocal of the highest antiserum dilution that deformed at least 50% of the helices.
[c] Reciprocal of the highest antiserum dilution that resulted in inhibition of metabolism.
[d] n, No zone of inhibition in growth inhibition tests, a deformation titer of less than 1:20, or a metabolism inhibition test titer of less than 1:18.
[e] ND, Not done.

(group II) or sera directed against representatives of groups III through XI failed to inhibit the growth of strain E275$^T$. The results of reciprocal deformation and metabolism inhibition tests (Table 2) confirmed the unique serological status of spiroplasma strain E275$^T$. Thus, our serological data demonstrated that strain E275$^T$ represents a subgroup that is distinguishable from other group I spiroplasma subgroups, including *S. citri* (subgroup I-1) and *S. melliferum* (subgroup I-2), from groups given species designations (*S. floricola* [31]), *S. apis* [72], and *S. mirum* [97]), and from all other currently designated groups.

**PAGE.** The patterns obtained from one-dimensional and two-dimensional PAGE of cell proteins confirmed the patterns of partial relationship between strain E275$^T$ and strains that represent other group I subgroups, as shown by the sharing of a number of comigrating proteins (6). Also, the sharing of a few comigrating proteins (6) with other groups confirmed the existence of a deep separation between group I spiroplasmas and other groups.

**Strain homogeneity.** The serological homogeneity of the subgroup I-3 cluster was assessed by testing nine subgroup I-3 strains against antisera to *S. citri* R8A2$^T$ (subgroup I-1), *S. melliferum* BC-3$^T$ (subgroup I-2), and strain E275$^T$ (subgroup I-3) in the deformation test. The results, reported previously (20), showed that strain E275$^T$ and other subgroup I-3 strains form a coherent subgroup that can be distinguished from all other strains in the group I cluster. Strain homogeneity was also assessed by comparing nine subgroup I-3 strains by one-dimensional PAGE (Fig. 2). The electrophoretic protein profiles of the strains tested were almost identical. Thus, as determined by both serological and electrophoretic criteria, all of the subgroup I-3 strains tested could be grouped into a single homogeneous taxon.

**Genomic analysis.** The guanine-plus-cytosine content of the DNA of strain E275$^T$ was determined (7) from the melting temperature (assayed spectrophotometrically in three independent analyses) and from the buoyant density (obtained by equilibrium centrifugation in CsCl in six independent analyses). The guanine-plus-cytosine content was about 26 ± 1 mol%. This value is generally observed with group I spiroplasmas (6, 7, 56).

## DISCUSSION

The serological results obtained in this study are in good agreement with the results obtained by other workers using a variety of serological techniques. Deformation (14, 30, 98–100, 103, 107, 108, 110, 111), metabolism inhibition (14, 30, 98, 100, 107, 108), growth inhibition (14, 95, 101, 103), crossed-immunoelectrophoresis (4, 5), enzyme-linked immunosorbent (4, 86), precipitin ring (95), and Ouchterlony double diffusion (23) tests all indicate that there is a significant degree of antigenic sharing between strains of the E275$^T$ cluster and representatives of other group I subgroups. The sharing of protein determinants that underlies these serological cross-reactions is also reflected by the significant numbers of comigrating or homologous proteins revealed by one-dimensional (24, 80) or two-dimensional (73–75) PAGE of spiroplasma proteins. The lipid compositions of corn stunt spiroplasmas and *S. citri* have also been shown to be identical (81), and the organisms share a common fibril protein (93). On the other hand, the existence of a significant number of unique proteins in strain E275$^T$ (73–75) is in accord with our view that the corn stunt spiroplasma should be regarded as a distinct spiroplasma species.

The most refined arbiter of interstrain relatedness in

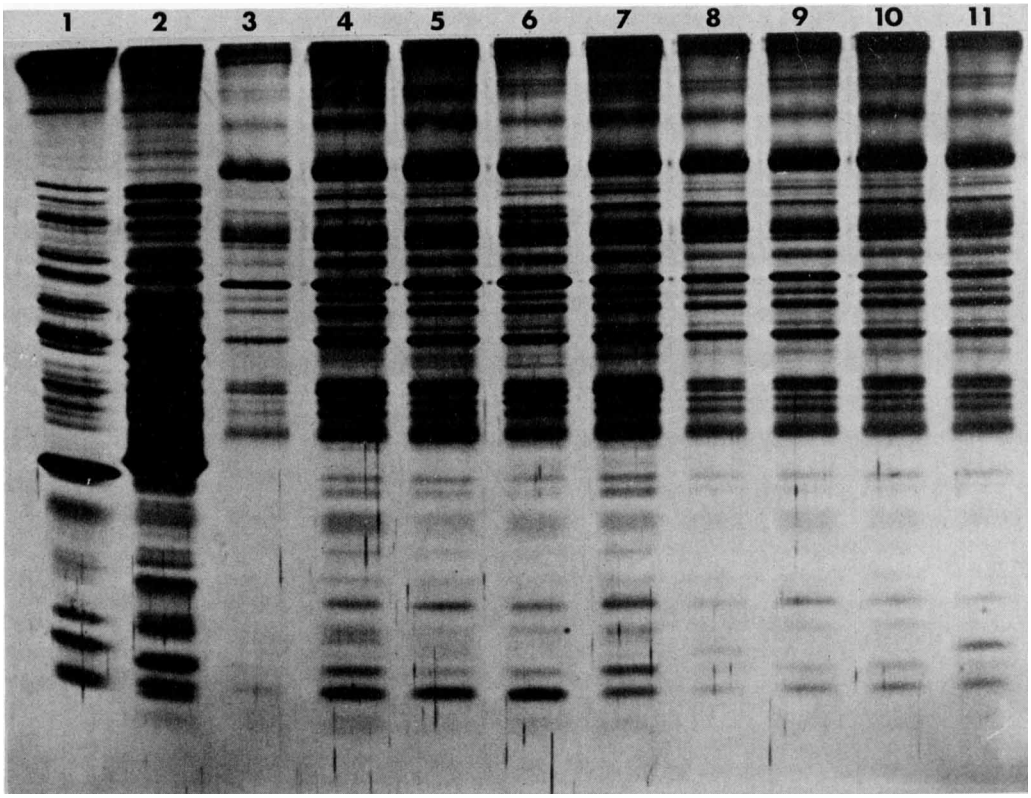


FIG. 2. One-dimensional PAGE protein profiles of group I spiroplasmas. Track 1, *S. citri* R8A2$^T$; track 2, *S. melliferum* BC-3$^T$; tracks 3 through 11, subgroup I-3 strains (track 3, strain E275$^T$; track 4, strain I-747; track 5, strain Miss E; track 6, strain CRL; track 7, strain B655; track 8, strain B652; track 9, strain B647; track 10, strain CSEE1; track 11, strain CSEE2).

general use today is DNA-DNA hybridization. Several variations of this technique have been used with strain E275$^T$ and other members of subgroup I-3, and hybridization values between 33 and 68% have been obtained (7, 15, 52, 56, 82). The differences probably reflect differences in techniques. However, it is significant that none of the values obtained was more than 70%, a value suggested by the Subcommittee on the Taxonomy of *Mollicutes* (50) as an upper limit for the amount of hybridization between spiroplasmal species.

Certain conditions, other than a restricted amount of DNA-DNA hybridization, were proposed by the Subcommittee as requirements for elevation of spiroplasma subgroups to species. For example, it was proposed that a number of strains with similar properties should be studied. In this study we examined nine strains from diverse geographical locations and found them to be serologically homogeneous. In addition, nine subgroup I-3 strains were shown to possess almost identical protein profiles by PAGE. Thus, the subgroup I-3 cluster has a specific and unique serological and electrophoretic identity that permits its unequivocal identification. Finally, the Subcommittee proposed that the ecology of the organisms should be studied and that some important feature of the organism should be identified. Strain E275$^T$ and its allies, as etiological agents of a serious disease of maize in the neotropics (27), readily qualify under these criteria. The major natural plant host of subgroup I-3 spiroplasmas appears to be maize (*Zea mays*), although other *Zea* species are susceptible (78). Species of *Dalbulus* leafhoppers appear to be natural vectors of the agent in the neotropics and in the southern United States; however, experimentally at least, leafhoppers in the genera *Graminella*, *Exitianus*, *Stirellus*, and *Euscelidius* have also been shown to transmit subgroup I-3 spiroplasmas from diseased to healthy plants (68, 76). One of the vectors has even been used (1, 68) to infect dicotyledonous plants (*Catharanthus roseus* and *Vicia faba*). Maize infected with subgroup I-3 spiroplasmas becomes stunted and develops chlorotic striping. Tillering and tassel sterility also commonly occur. In the neotropics, the subgroup I-3 spiroplasma is actually only one of a complex of leafhopper-borne corn pathogens that includes viruses and mycoplasmalike organisms (76, 77).

Corn stunt spiroplasma is experimentally pathogenic to some of its vectors (45, 63, 79). Elaborate studies have established that pathogenicity of subgroup I-3 spiroplasmas to vectors is associated with unusual leafhopper-spiroplasma associations (63). In fact, recent evolution within the genus *Dalbulus* may have been affected to some extent by the complex of pathogens that these leafhoppers transmit to *Zea*, *Tripsacum*, and related plants in the neotropics (63, 79).

Subgroup I-3 spiroplasmas yielded to cultivation attempts (14, 110) only after many failures. Simplification of media for their cultivation culminated in the discovery that a medium consisting of horse serum, PPLO broth base, and sucrose permitted primary isolation and continuous maintenance (60). On the other hand, a comparison of media suitable for subgroup I-3 spiroplasmas and *S. citri* showed (51) that a number of components (e.g., amino acids or α-ketoglutaric acid) present in the rich M1A medium stimulated the growth of subgroup I-3 spiroplasmas and improved primary isolation results. Other medium formulations have also been reported to support primary isolation from plants (57). Recently, a role played by amino acid-carbohydrate balance in the early

cultural history of group I spiroplasmas was indicated (20). Strains of *S. melliferum* that had been isolated and maintained in carbohydrate-rich media with little supplementation with amino acids failed to catabolize arginine, a metabolic ability possessed by all *S. melliferum* strains that were cultivated in media with rich amino acid contents. Variations in the concentration of arginine have been found to have critical effects on the growth and helicity of subgroup I-3 spiroplasmas (91).

Primary isolation of subgroup I-3 spiroplasmas may be complicated by the presence of spiroplasmastatic substances in maize extracts (59). However, this problem can be easily overcome by standard methods of preparing serial dilutions of primary extracts, blind passage of extracts (59), or pelleting the isolated organisms and suspending them in fresh media (110). Although anaerobic conditions may assist in primary isolation (25), they are usually unnecessary.

Extended cultural passage of subgroup I-3 strains in all known media results in loss of the ability of the spiroplasmas to complete the biological cycle in plants and insects (104, 105); the exact site of blockage is unknown. However, this loss of natural pathogenicity may be accompanied by an increase in the experimental pathogenicity of the organism for leafhoppers inoculated with cultured spiroplasmas (104). Because the process of triple cloning involves numerous cultural passages, strain E275$^T$ and all strains similarly handled (so far as known) do not induce disease in maize. Therefore, it is important to preserve pathogenic isolates from early cultural passages. We have deposited a pathogenic isolate, strain E (passage level 6), with the American Type Culture Collection as strain ATCC 27954.

A number of other studies, which were performed with strain I-747, provided additional information that contributes to our knowledge of the subgroup I-3 spiroplasmas. For example, the genome molecular weight of the organism was reported to be $10^9$ (56). Also, subgroup I-3 organisms grew at a wide range of osmolalities (from 360 to 1,120 mOsm), and optimal growth occurred in media containing 0.25 to 0.35 M sucrose (61). Optimal growth also occurred at pH 7.2, and the organisms lost helicity and motility when they were transferred to medium with a pH of 4.5 (61). Subgroup I-3 spiroplasmas grow at 20 to 32°C; optimum growth occurs at 30 to 32°C. Susceptibility and resistance to a wide range of antibiotics have also been determined (26, 62).

Finally, the taxonomic status of subgroup I-3 spiroplasmas has been somewhat confused by the publication of the inappropriate binomials "*Spiroplasma zeae*" (80) and "*Spiroplasma maidis*" (35) and by the equally invalid designation of "*Spiroplasma kunkelii*" (A. H. McIntosh, K. Maramorosch, and F. Kondo, Proc. Am. Phytopathol. Soc. **4**:193, 1977) and "*S. kunkelii* var. *callistephi*" (K. Maramorosch and F. Kondo, Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. 1 Abt. 1 **241**:196, 1978) for other group I spiroplasmas. Since the organisms in these studies were not characterized and the descriptions were not validly published, neither "*Spiroplasma kunkelii* McIntosh, Maramorosch and Kondo 1977" nor "*Spiroplasma kunkelii* Maramorosch and Kondo 1978" was included on the Approved Lists of Bacterial Names (88), and the designations have no taxonomic standing (55). Therefore, the specific epithet *kunkelii* remains available.

Strain E275$^T$ and related spiroplasmas from maize and associated leafhoppers have been shown to be members of the class *Mollicutes* (38, 42) and to be referrable to the family *Spiroplasmataceae* (89, 90) and to the genus *Spiroplasma* (85). We propose that these organisms be designated *Spiroplasma kunkelii* sp. nov. The taxonomic description given below summarizes the properties of the organism.

***Spiroplasma kunkelii* sp. nov.** *Spiroplasma kunkelii* (kun. kel' i.i. L. gen. n. *kunkelii* of Kunkel, after L. O. Kunkel, to honor his major and fundamental contributions to the study of plant mollicutes) cells are pleomorphic, varying from helical filaments 100 to 150 nm in diameter and 3 to 10 μm long to nonhelical filaments or spherical cells 300 to 800 nm in diameter. The cells lack true cell walls and periplasmic fibrils. Motile. Colonies on solid medium containing 0.8% Noble agar are usually diffuse, rarely exhibiting central zones of growth into agar. Colonies on solid C-3G medium containing 5% horse serum or on media containing 2.25% Noble agar frequently have a fried egg morphology. Chemoorganotroph. Acid produced from glucose. Hydrolyzes arginine. Does not hydrolyze urea. Reduction to tetrazolium variable. Phosphatase negative. Film and spot reaction positive. No liquefaction of coagulated serum. Agar colonies do not hemadsorb guinea pig erythrocytes.

Cholesterol is required for growth.

Facultative anaerobe.

Temperature range, 20 to 32°C; optimum temperature, 30 to 32°C.

Isolated from maize displaying symptoms of corn stunt disease and from leafhoppers associated with diseased maize, largely in the neotropics. Pathogenicity for plants and insects has been experimentally verified.

The guanine-plus-cytosine content of the DNA is 26 ± 1 mol%.

Genome molecular weight, $10^9$.

The type strain is strain E275$^T$ (= ATCC 29320)

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