Spiroplasma Citri and the Organism Associated with Likubin: Comparison of Their Envelope Systems

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The first paper to document the nonviral etiology of citrus greening disease was that of Laflèche and Bové (1970*a*) in which these authors, early in 1970, described the presence of "structures" within the sieve tubes of sweet orange seedlings with South Africa greening. Soon thereafter, they reported the occurrence of the same structures in citrus affected either by Reunion greening or India citrus decline (Laflèche and Bové, 1970*b*). In these first two papers the structures seen in the phloem of affected plants were referred to as mycoplasma or mycoplasmalike. At the same time, mycoplasmalike structures were found by Igwegbe and Calavan (1970) to be associated with citrus stubborn disease in California and their findings were confirmed by Laflèche and Bové (1970*b*) in France. Thus it appeared that mycoplasmalike structures were involved in both greening and stubborn. Since the similarities between these two diseases were often emphasized (Calavan, 1968; Schwarz, 1972), it became of great interest to compare the structures associated with greening with those related to stubborn. It was immediately clear that they were not alike. The greening structures seemed to be more rigid, longer, and more filamentous than those of stubborn which, on the contrary, were more dumbbell-shaped, rounder, more pleomorphic, and more sinusoidal (Laflèche and Bové, 1970*b*). It was even suggested that these morphological differences could be of diagnostic value to distinguish greening from stubborn (Laflèche and Bové, 1970*b*). In the summer of 1970 Laflèche and Bové, aided by R. L. Steere, discovered an even more basic difference. While the cell envelope surrounding the stubborn structures was 10 nm thick and resembled a true unit membrane, the envelope system of the greening structures was at least twice as thick and did not fit the definition of a unit membrane (Bové, 1971; Bové and Saglio, 1974; Bové and Vogel, 1973; Saglio *et al.*, 1971; Saglio *et al.*, 1972). This discovery made the mycoplasma nature of the greening structures questionable for the reasons which follow.

The Subcommittee on the Taxonomy of Mycoplasmatales (1972), recognized by the International Committee on Nomenclature of Bacteria, gave long and careful consideration to its "Proposal for minimal standards for description of new species of the order Mycoplasmatales." Among the properties an organism must have to be recognized as a mycoplasma are listed those relative to its envelope: the organism should be surrounded by a triple-layered unit membrance, sometimes covered by a thin layer of electron-dense material, it should be devoid of a cell wall and have no cell wall precursors. Do the stubborn and greening structures fit these minimal standards; The structures associated with stubborn have been isolated, obtained in pure culture, and extensively characterized (Saglio *et al.*, 1973). The cultured organism complies with the minimal standards for description as a new species of the class Mollicutes (Edward and Freundt, 1967) and has been accepted in that class by the Subcommittee on the Taxonomy of Myco-

plasmatales (1974) under the name *Spiroplasma citri* (Saglio *et al.*, 1973). The structures associated with greening have not yet been obtained in culture. They have only been examined by electron microscopy of ultrathin sections prepared from infected plant material or from insect vectors. These observations have revealed that the greening structures represent probably a procaryotic type of organism, but one with an envelope system more complex than a simple unit membrane. Accordingly, the greening organism cannot be strictly mycoplasmalike, and we have suggested that it might represent a new phytopathogen (Bové and Saglio, 1974; Saglio *et al.*, 1971). Moll and Martin (1974) in South Africa have confirmed and extended our observations.

All forms of greening that we have studied previously (South Africa and Reunion greening, India citrus decline, Philippines leaf mottling) showed the same procaryote-like structures, with a 25 nm thick envelope system (Bové and Saglio, 1974). Mycoplasmalike bodies have also been described independently by Chen *et al.* (1971) in the case of likubin, a form of greening occuring in Taiwan. These bodies were found "similar to the mycoplasmalike bodies observed in greening-, stubborn-, and decline-diseased citrus plants." Tanaka and Doi (1973) also observed "mycoplasmalike organisms" in likubin-infected leaves.

Since distinguishing between the greening organsim and *S. citri* by examining the envelope system, we have also studied likubin-affected material to see whether the likubin structures were similar to those of greening (25-nm envelope system) or to those of stubborn (10-nm unit-membrane envelope). As expected, we found that the likubin-associated structures are of the greening type, not of the stubborn type.

MATERIALS AND METHODS

Hamlin sweet orange seedlings were graft inoculated on December 30, 1971 with budwood from a Ponkan tree affected by likubin (ref. Pd TY 10). The budwood was collected in Taiwan and carried to Bordeaux by Dr. D. Catling. The infected seedlings were grown under greenhouse conditions at 20 to 27°C. Typical symptoms of greening appeared 6 months after inoculation and have remained conspicuous.

Stubborn-infected Madam Vinous sweet orange seedlings were grown at 27 to 32° C.

Leaf specimens were fixed 6 hours in 4 percent glutaraldehyde with 0.1 M phosphate buffer, pH 7.4. They were rinsed three times for 1 hour in the same buffer. One per cent osmium tetroxide in the above buffer was used for post fixation. After dehydration in ethanol, specimens were embedded in Epon and thin sectioned. The ultrathin sections were examined in a Siemens Elmiskop 101 electron microscope.

RESULTS

Figure 1A shows the structures associated with likubin in an ultrathin section through a sieve tube of an affected Hamlin sweet orange seedling. Two main forms are present: electron dense filaments and relatively large, electron-clear, round structures. These two forms probably represent different aspects of the same procaryote-like organism since the round structures and filaments can be shown to be connected (fig. 1B and C).

The envelope system of the likubin organism has an electron-dense outer layer, an electron-clear middle layer, and an electron-dense inner layer (fig. 1B and C). Sometimes the inner layer appears less distinct than the outer one (fig. 1B). The three layers together are approximately 25 nm thick. Figure 1E and F represent sections through *S. citri*, for comparison with the greening structures of fig. 1B and C at the same magnifications, X 50,000 and X 100,000, respectively. It is clear from the comparison between Fig.

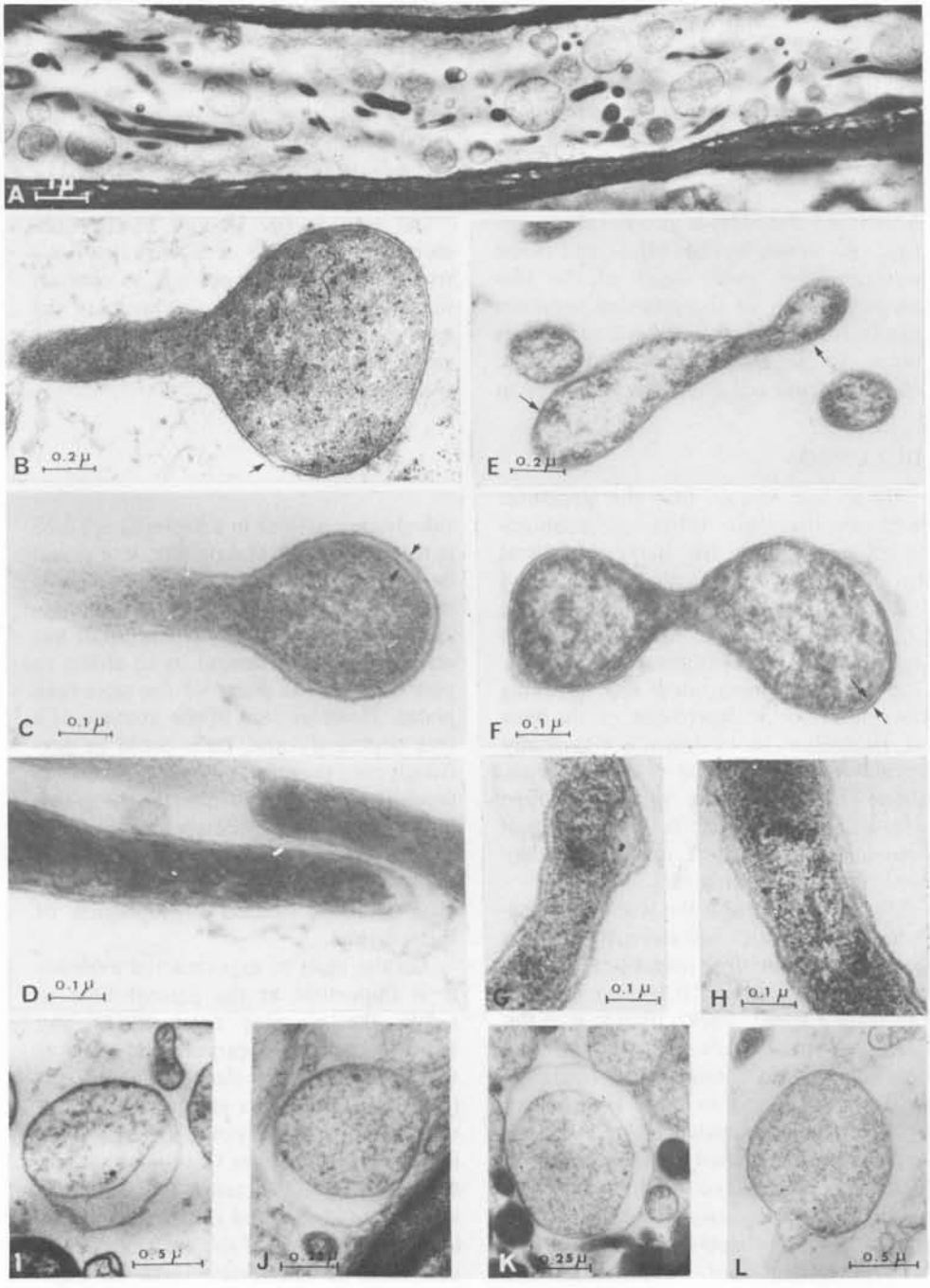

Fig. 1. (A) "Structures" associated with likubin within a sieve tube of a likubin-infected Hamlin sweet orange seedling; (B, C, D, G, H, I, J, K, and L) various aspects of the structures associated with likubin; (E and F) thin sections through *Spiroplasma citri*, the stubborn mycoplasma; E and F are at the same magnification as B and C, respectively.

1B and E or Fig. 1C and F that the envelope of the likubin structure is approximately twice as thick as the 10-nm unit membrane which surrounds *S. citri.*

Besides their difference in thickness, the envelope system of the greening organism and the unit-membrane envelope of *S. citri* are dissimilar in another characteristic. As shown by fig. 1D, G and H the electron-dense outer layer of the filamentous form of the greening organism can be somewhat wrinkled and sometimes seems to be separated from the inner, electron-dense cell contents. Moreover, in the round forms of the organism the inner and outer electron-dense layers, which are normally associated to form the 25-nm envelope, can become separated from each other at Y-shaped bifurcation points, suggesting plasmolytic effects (fig. 1I to L).

As seen in fig. 1E and F, the unit-membrane envelope of *S. citri* also has a triple-layered appearance but, in contrast to the above situation, the layers of the unit membrane always remain in close parallel association; they never separate from each other.

DISCUSSION

It is now known that the structures seen in the sieve tubes of stubborn-infected material are living organisms because they have been isolated and cultured. The organism belongs to the class Mollicutes and has been named *Spiroplasma citri* (Saglio *et al.*, 1973). The structures associated with greening have never been cultured but, on the basis of circumstantial evidence, are generally considered to be living cells too. If so, these cells are of the procaryote type because their nuclear zone is not surrounded by a nuclear membrane, they have no mitochondria, etc.

Knowing then that the stubborn structures are mollicutes and assuming that the greening structures represent a procaryote-like organism, it is clear that the organism associated with likubin is of the greening type, not the stubborn type. Its envelope system consists in particular of an outer layer and an inner layer which under certain circumstances behave, each by itself, as individual envelopes (fig. 1I to L). The inner layer could represent a true cytoplasmic membrane. If so, it should show the triple-layered aspect of a unit membrane, but so far, with the techniques used, we have been unable to detect this. The nature of the outer layer is more intriguing. Is it a coating of some type? Does it contain a peptidoglycane network? In so far as the role of the peptidoglycane present in a bacterial cell wall is to confer shape and rigidity, one would be tempted to suggest that the greening organism contains no such peptidoglycane since it is not only pleomorphic but also flexible as evidenced by its ability to pass through the pores of the sieve-tube plates. However even in the absence of a true peptidoglycane, there could be peptidoglycane precursors, and their presence would be enough to eliminate the greening organism from the class Mollicutes. In any case the existence of the outer layer or "coat" distinguishes the greening organism from recognized mycoplasmas or spiroplasmas.

On the basis of experimental evidence it is impossible at the present time to relate the greening organism to any known types of procaryotes. It could as well belong to the class Schizomycetes (bacteria), in case its envelope turns out to contain peptidoglycane sybstances, as to the class Mollicutes (mycoplasmas and spiroplasmas) if bacterial cell wall substances are absent and if other properties (genome size, etc.) of the organism fit the definition of Mollicutes. Until the organism has been further characterized it seems premature to relate it to one class or the other. We suggest that for the time being it be called procaryote-like rather than mycoplasmalike.

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