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3 August 1977; revised 12 September 1977

Spiroplasmavirus citri 3: Propagation, Purification, Proteins, and Nucleic Acid

Abstract. *SVC3 is a short-tailed polyhedral virus particle morphologically detectable in many spiroplasmas. It was isolated from two different spiroplasmas (*Spiroplasma citri *and the suckling mouse cataract agent) by infecting lawns and broth culture of another strain of* Spiroplasmavirus citri. *Virions from either donor strain had a buoyant density of 1.26 grams per cubic centimeter (metrizamide) or 1.45 grams per cubic centimeter (cesium chloride), and contained five proteins and linear double-stranded DNA with a molecular weight of 14 × 10^6. Other spiroplasmaviruses have not been propagated, and the molecular weights of double-stranded DNA from other mycoplasma* (Acholeplasma) *viruses are unknown.*

Spiroplasmaviruses of three different morphologic types are detectable by electron microscopy in the plant-pathogenic mycoplasma, *Spiroplasma citri*, and some other spiroplasmas (*1, 2*). However, unlike viruses of certain other Mollicutes [such as *Acholeplasma laidlawii* (*3*)], spiroplasmaviruses have not been previously propagated nor characterized. We report here the propagation and some properties of *Spiroplasmavirus citri* 3 (SVC3), a short-tailed polyhedral virus particle (*1*).

To isolate virus, lawns of 21 strains of spiroplasmas (*4*) were inoculated by spotting (*5*) with donor broth cultures of each other. Although, in more than 500 trials, various combinations were occasionally successful, plaques were consistently produced by strain 608 of *S. citri* and by a strain (SMCA) of the suckling mouse cataract agent (*4, 6*), when either was spotted on lawns of *S. citri* strain 750. Samples of these donor cultures in SMT broth (*5*), incubated at 32°C for different durations (2 to 5 days), were tested on SMT agar lawn inoculums of strain 750 that were kept at 32°C for 3 to 24 hours after seeding and before spotting. The best results were found by spotting 48-hour donor cultures on lawns incubated for 6 hours, and then by incubating further at 32°C in an atmosphere of 5 percent CO_2 and 95 percent N_2. Large clear plaques were seen after 5 days of incubation. Washings (*7*) of these plaques examined by electron microscopy showed only SVC3 particles (frequently adsorbed to the few spiroplasmas present). Millipore-filtered (0.22 μm) samples, spotted on fresh lawns of strain 750, reproduced the cycle of plaques and infectious washings repeatedly. Washings from single plates contained 1 × 10^8 to 1 × 10^9 plaque-forming units (PFU) per milliliter, as determined by terminal dilutions showing individual clear plaques about 1.5 mm in diameter (Fig. 1a). Plaques were produced by supernatants of donor cultures (*8*) as well as by whole culture, and by Millipore (0.22 μm) filtrates thereof.

No plaques appeared spontaneously on control (unspotted) lawns or on those spotted with donor cultures heated at 90°C for 30 minutes, although electron microscopy indicates that SVC3-like particles can sometimes be detected in strain 750. The situation is analogous to the use of known virus-carrying strains as indicators for viruses of *A. laidlawii* (*3*).

Both viruses (SVC3/608 and SVC3/SMCA) were propagated in broth cultures of strain 750 to provide better yields for purification, and virions were banded by isopycnic centrifugation in

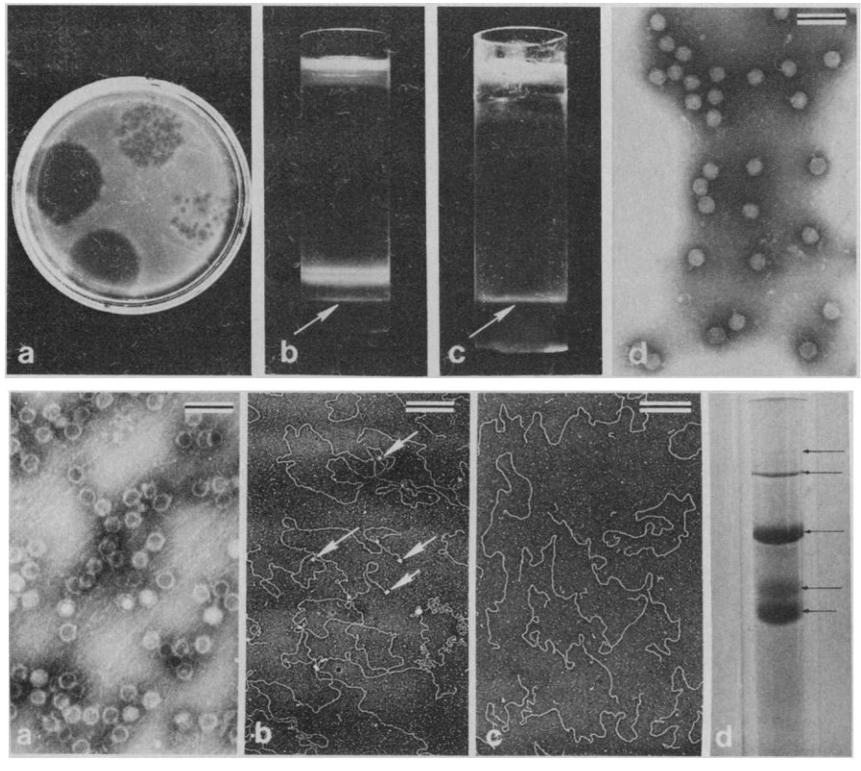

Fig. 1 (top). (a) Plaques produced on *S. citri* 750 lawn by SVC3/608: tenfold dilutions to 10^{-6} of filtered plaque washings. (b) Virus band (arrow) at 1.26 g/cm³, from isopycnic centrifugation in metrizamide (*9*) of PEG-sedimented material from infected broth culture supernatant fluid. (c) some band material, rerun in metrizamide and banding (arrow) at same density. (d) Intact SVC3 virions comprising such bands; electron micrograph (marker = 0.1 μm) of material negatively stained by 2 percent ammonium molybdate. Fig. 2 (bottom). (a) Empty SVC3 virions and stranded material in preparation from isopycnic banding (*10*) in CsCl; electron micrograph (marker = 0.1 μm) of material negatively stained by 2 percent uranyl acetate. (b) Metal-shadowed aqueous Kleinschmidt preparation (*11*) of material from (a), showing strands extruding from virions (arrows): electron micrograph (marker = 1 μm). (c) Similar preparation of purified SVC3 DNA, showing extended linear molecules: magnification same as in (b). (d) Pattern of five structural proteins of SVC3, obtained by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (*11*).

gradients of metrizamide (*9*) (Fig 1, b and c). Fractions taken from the bottom of the tubes were dialyzed extensively against the tris-HCl buffer prior to examination by electron microscopy and titration by plaque assay. Electron microscopy revealed clean, intact SVC3 particles (Fig. 1d) in the lowermost band fraction, which was located at a density of 1.26 g/cm^3 and contained approximately 1×10^{12} PFU/ml.

In an initial alternate method, cesium chloride gradients were used (*10*). A sharp band formed at a density of 1.45 g/cm^3, but electron microscopy of this material after dialysis revealed mostly tail fragments and many empty heads extruding strands presumed to be nucleic acid (Fig. 2, a and b). Both self-generating (*10*) and preformed gradients gave similar results.

Dialyzed material from either CsCl or metrizamide bands was extracted with phenol, purified, and spread for electron microscopy by the Kleinschmidt aqueous technique (*11*). Under aqueous conditions, linear molecules appeared extended (Fig. 2c), but after the material was treated with deoxyribonuclease (once crystallized, beef pancreas; Worthington) they were absent; the viral DNA is therefore considered to be double-stranded (ds) DNA (*12*). Computerized measurements (*11*) of several preparations from both SVC3/608 and SVC3/SMCA viruses showed contour lengths ranging between 6.37 ± 0.24 and 7.74 ± 0.43 μm, and an average of 7.2 ± 0.32 μm. The calculated (*12*) molecular weight (MW) of SVC3 dsDNA is therefore approximately 14×10^6.

Purified SVC3, dissociated and subjected to electrophoresis in 7.5 percent polyacrylamide–sodium dodecyl sulfate gels (*11*), showed a pattern of five bands of structural proteins (Fig. 2d). The pattern was identical for virions of both SVC3/608 and SVC3/SMCA, obtained from either metrizamide or CsCl banding. The MW's of these protein were estimated (*13*) to be 130,000, 110,000, 67,000, 48,000, and 38,000: the total MW of SVC3 proteins per virion is therefore 393,000. If we assume a random unweighted amino acid composition, this would require 7.1×10^6 daltons of dsDNA (*14*), which represents 51 percent of the total viral DNA coding capacity. The total DNA represents approximately 21,000 nucleotide pairs (at 1500 nucleotide pairs per average gene) of approximately 14 genes.

Of DNA's of other mycoplasma viruses, the MW (2×10^6) is known for the single-stranded circular molecule of the group 1 viruses (*15*). Although the group 3 virus MVL3 is morphologically similar to SVC3, contains five structural polypeptides having an aggregate MW of 436,000, and contains dsDNA (*16*), the MW of this DNA is unknown—as is that of the dsDNA of the enveloped *Acholeplasma* virus MVL2 (*1, 3*). The few other bacterial viruses containing dsDNA of MW similar to, or less than, that of SVC3, are ϕ15 (MW, 17×10^6), ϕ29 (MW, 11.5×10^6), and PBSH defective (MW, 9×10^6) of *Bacillus subtilis*; and PM-2 (MW, 5.3×10^6) of *Pseudomonas* (*17*).

Spiroplasmaviruses, as examples of phagelike entities attacking sterol-requiring new mycoplasmas (*18*) instead of sterol-nonrequiring Mollicutes (*19*), are of interest in relation to absorption to wall-less prokaryotes, infectious cycles and growth characteristics, state of carriage, characterization of their nucleic acids, and taxonomy and evolution of their hosts. Propagation and characterization of SVC3 makes available a new virus with a dsDNA of low MW. The virus should prove useful in studying the above-mentioned questions, as well as serving as a probe of molecular mechanisms of infection, replication, and transcription.

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5. Lawns were prepared by spreading 0.2 ml of 72-hour broth culture in SMT medium on SMT solidified by 1.5 percent Bacto-Agar (Difco), previously distributed in 8-ml amounts into plastic petri dishes, 60 by 15 mm (Lux Scientific Corp., Thousand Oaks, Calif.). For plaque tests, 0.02-ml amounts of donor material were spotted on the lawns. SMT is a modification [M. J. Daniels, P. G. Markham, B. M. Meddins, A. K. Plaskitt, M. Bar-Joseph, *Nature (London)* **244**, 523 (1973)] of the original SMC medium [P. Saglio, D. Laflèche, C. Bonissol, J. M. Bové, *Physiol. Veg.* **9**, 569 (1971)]. As used in our laboratory, it lacks tryptone, contains 0.5 percent Difco yeast extract instead of 10 percent Oxoid, contains 0.4 percent L-arginine HCl, contains a 1 to 50 solution of thallous acetate (10 ml/liter), and contains 250,000 units of penicillin per milliliter rather than 1000 units per milliliter.
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7. Plates were washed for 4 to 6 hours or overnight, at room temperature, with 2 ml of 0.01*M* tris-HCl buffer, *p*H 8.3.
8. Supernatants were obtained by centrifuging 1.0 ml of broth cultures in conical plastic tubes in the Brinkmann (Eppendorf) microcentrifuge 3200, at 12,000 rev/min for 2.5 minutes.
9. SMT (200 ml) was inoculated with 20 ml of a 2- to 3-day broth culture of strain 750, incubated 6 hours at 32°C, infected by adding 2 ml of filtered (0.22-μm Millipore) plaque wash, and incubated at 32°C. Daily monitoring by electron microscopy indicated that the optimum time for harvest was 4 to 5 days. The culture was filtered (0.22-μm Millipore), and polyethylene glycol (PEG) [K. R. Yamamoto, B. M. Alberts, R. Benzinger, L. Laurhome, G. Treiber, *Virology* **40**, 734 (1970)] at 6 percent by volume was added to the filtrate. After refrigeration overnight at 4°C, the dense precipitate was sedimented (45 minutes, 9000 rev/min, 4°C, GSA rotor of Sorvall model RC2-B) and the pellet was suspended in 3 ml of 0.01*M* tris-HCl buffer, *p*H 8.3. Nonidet P40 (Gallard-Schlesinger, New York) was added to a concentration of 0.04 percent by volume, and 0.5 ml of the suspension was layered onto a preformed linear gradient (15 to 60 percent) of metrizamide (Gallard-Schlesinger) [D. Rickwood and G. D. Birnie, *FEBS Lett.* **50**, 102 (1975); W. H. Wunner, R. M. L. Buller, C. R. Pringle, in *Iodinated Density Gradient Media for Biological Separations*, D. Rickwood, Ed. (Information Retrieval Ltd., London, 1976), p. 159]. Centrifugation was for 6 hours at 189,000*g*.
10. The PEG pellet was suspended in the tris-HCl buffer containing 0.6 g/ml of solid CsCl, followed by centrifugation (150,000*g*) for 48 hours.
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11 August 1977