The Use of Immuno-Gold Staining Techniques for Detection of a Bacterium Associated with Greening Diseased Citrus

Dvora Ariovich and Helen M. Garnett

Department of Microbiology, University of the Witwatersrand, Johannesburg, P.O. Wits, 2050, South Africa. Present address of second author: Department of Biology, University of Wollongong, Northfields Avenue, Wollongong, NSW 2500, Australia.
Reprint requests should be sent to first author.
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ABSTRACT

Ariovich, D., and Garnett, H. M. 1989. The use of immuno-gold staining techniques for detection of a bacterium associated with greening diseased citrus. Phytopathology 79:382-384.

An immunoelectron microscopic method using immuno-gold staining was developed for the detection of the putative greening organism in greening diseased citrus. This technique had the advantage of being rapid and highly specific and detected bacteria even when they occur in low numbers in infected tissue.

The greening disease of citrus is a major problem to the citrus industries in Africa and Asia. In Africa the disease is transmitted by the citrus psylla, *Trioza erytreae* (Del Gurcio) (9). Moll and Martin (11) suggested that this disease is caused by a prokaryote that infects phloem cells of the host plant. They also reported the presence of bacteria found in phloem cells of greening diseased citrus, in the haemolymph of the vector (10).

Gram negative bacteria were isolated from infected citrus and cultured in a synthetic medium (3). Ariovich and Garnett (1) reported that cultured greening organisms were long, rod-shaped bacteria with the distinctive two-layered cell wall structure, and exhibited pleomorphic characteristics. Similar organisms have been seen in infected plant tissue and psylla (10,11). In addition, a round form was observed in old cultures (1). These pleomorphic properties of the organism make conventional transmission electron microscopy techniques unsatisfactory for detection and identification of this bacterium.

Gold-labeled immunoglobulin (IgG) complexes have been used successfully for detection of plant viruses in post-sectioning, immunostaining at the ultrastructural level (7,8). This technique requires a lengthy preparation procedure and some of the antigenicity is lost due to the fixation of the material. Lin (6) however, described the use of gold-labeled IgG complexes for rapid and highly specific detection of plant viruses in leaf dip preparations.

To specifically identify greening bacteria in infected material, antibodies against the cultured greening organism were raised in rabbits and gold-labeled IgG complexes were prepared for the detection of the greening bacterium both in situ and in crude plant extracts. Positive results were obtained with both preparations; however, crude plant extracts were found to be more satisfactory.

MATERIALS AND METHODS

Gold-IgG preparation. Gold particles (18–20-nm) were prepared according to Geoghegan and Ackerman (5) using chloroauric acid and trisodium citrate. IgG was purified from sera raised in rabbits against greening bacterial cultures by protein A affinity chromatography and was labeled with these particles according to Geoghegan and Ackerman (5).

Fixation and embedding of the plant material. Small blocks of leaf midribs and fruit columella were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate at pH 7.2 for 2 hr at room temperature. The material was dehydrated by immersion in a series of ethanol solutions (30, 50, 70, 80, 90, and 100%) for 1 hr each at −20 C and embedded in Lowicryl K4M at −20 C with ultraviolet irradiation. Sections were picked up on Parlodion-coated grids (3.5% in dry amylacetate) reinforced with a thin carbon coat.

Preparation of crude plant sap. Young and old leaves were collected from infected trees and surface sterilized with 0.35% sodium hypochlorite. Half a gram of midribs was cut out and finely chopped, resuspended in 10 ml of distilled water, and shaken vigorously. The supernatant was collected and centrifuged in a microfuge for 10 min. The pellet was washed three times with phosphate-buffered saline (PBS) (OXOID) pH 7.3 (Na_2HPO_4 1.15 g/L), plus 4% polyvinylpyrrolidone (PVP) (44,000 MW, British Drug House). The final pellet was resuspended in a volume of 0.5 ml of PBS plus 4% PVP.

Immunostaining of sections of plant tissue. For in situ gold-labeled antibody staining, grids were floated for 1 hr on a 10-μl drop of purified anti-greening bacteria-IgG at a concentration of 100 μg/ml, rinsed with glass double distilled water, and then floated on a 10-μl drop of gold-labeled anti-rabbit-immunoglobulin for 1 hr and rinsed with glass double distilled water. Sections were then stained with 1% (w/v) aqueous uranyl acetate at pH 7.2 for 10 min, followed by lead citrate for 2 min, and viewed in a 100S JEOL electron microscope.

Immunostaining of the greening bacteria in crude sap. Parlodion-coated grids were floated on 50-μl drops of crude plant sap for 10 min, excess sap was blotted with filter paper, and the grids floated on 20-μl drops of gold-labeled-anti-greening bacteria IgG. The material was rinsed very gently by passing the grids through five drops of glass distilled water. The material was negatively stained with 1% (w/v) aqueous uranyl acetate for 10 min.

RESULTS

Within phloem cells of leaf midrib and fruit columella sections, structures that resembled the greening bacteria were found. The gold-labeled antibodies raised against the cultured greening bacteria reacted positively with the cell wall of the bacterium within the phloem cells of the infected citrus (Fig. 1). Low numbers of bacterial cells were found within the leaf midrib phloem cells; these bacterial cells were unevenly distributed. The omission of postfixation with OsO_4 and the use of Lowicryl K4M for low temperature embedding, necessary for improving the preservation

of the antigenicity, resulted in reduced resolution of the plant material.

An indirect labeling procedure was applied for the in situ preparation of plant sections. It was found that by using an indirect labeling procedure the level of labeling is higher. This has an advantage when using sections of embedded material.

Crude plant sap from 15–20 leaf midribs was concentrated into a volume of 0.5 ml. Aliquots of 50 μl of this crude sap were used for each assay in which whole bacterial cells were found. These unfixed whole bacteria reacted very well with the gold-IgG complex (Figs. 2 and 3). Here, a more simplified direct labeling procedure was used since there was no reduced antigenicity due to fixation or sectioning.

Both long, thin rods (Fig. 3) and round forms (Fig. 2) of the greening bacteria in plant sap reacted positively with the gold-labeled IgG probe. Similar reaction was obtained when using the gold-IgG probe against the cultured bacteria.

This assay was used for detection of the greening bacteria in greening-infected citrus leaves, supplied from various regions with infected leaves, resulting with positive labeling. As a control, healthy material, which was supplied from a region free of disease, was tested, resulting with negative labeling.

The gold-IgG probe was tested against three bacterial species: *Bacillus subtilis, Erwinia liquefasciens*, and *Escherichia coli*. No labeling occurred. In addition, preimmune IgG from rabbits labeled with colloidal gold particles was used as a control. It failed to label greening bacteria cultures (Fig. 4). Furthermore, contaminants found in the crude plant sap did not react with the anti-greening gold-labeled-IgG (Fig. 5). These were cells 20 times larger than the greening organism's average size, and of different shape.

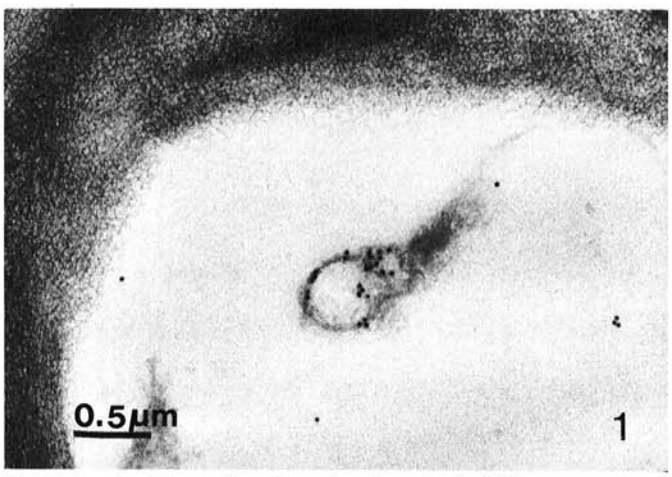

Fig. 1. An ultrathin section through a citrus leaf midrib processed and immunostained with gold particles. The greening bacterium within a phloem cell reacted positively with the purified IgG raised against the cultured greening organism.

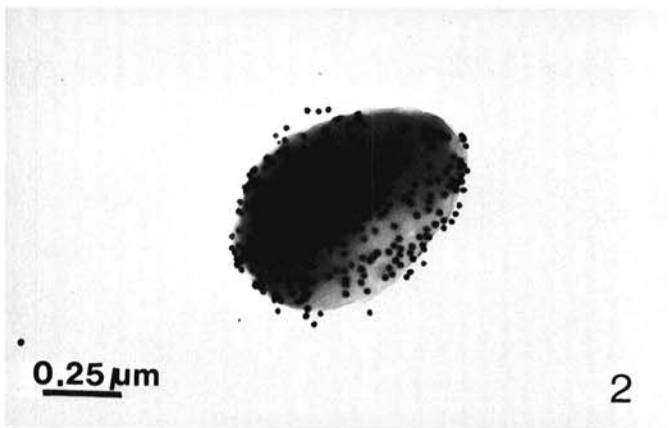

Fig. 2. The greening organism (round form) found in crude plant sap immunostained with gold particles.

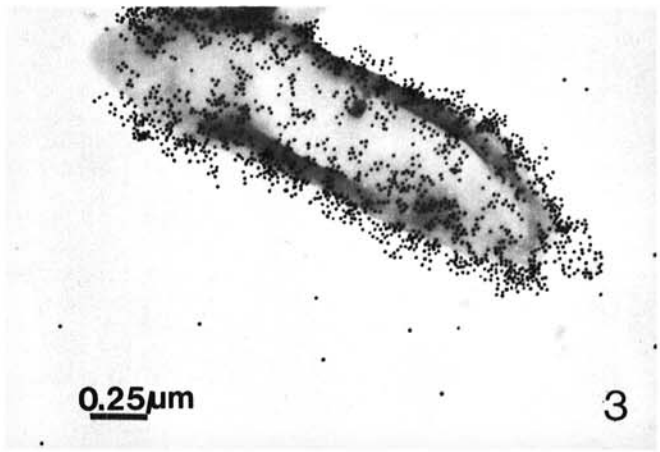

Fig. 3. As in Figure 2 but showing a long rod form.

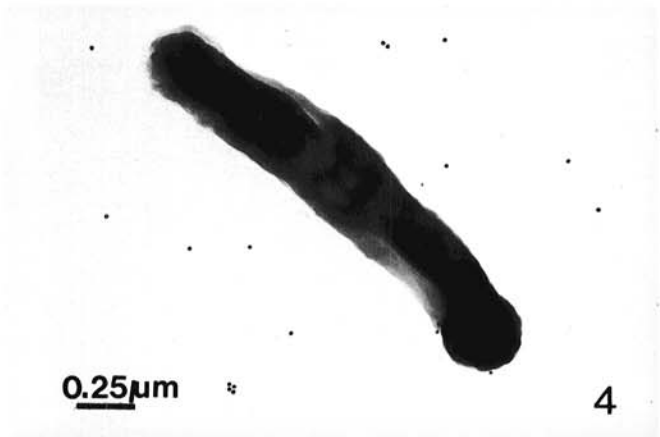

Fig. 4. Gold-labeled IgG from a preimmune rabbit, failing to label a long form of the cultured greening organism.

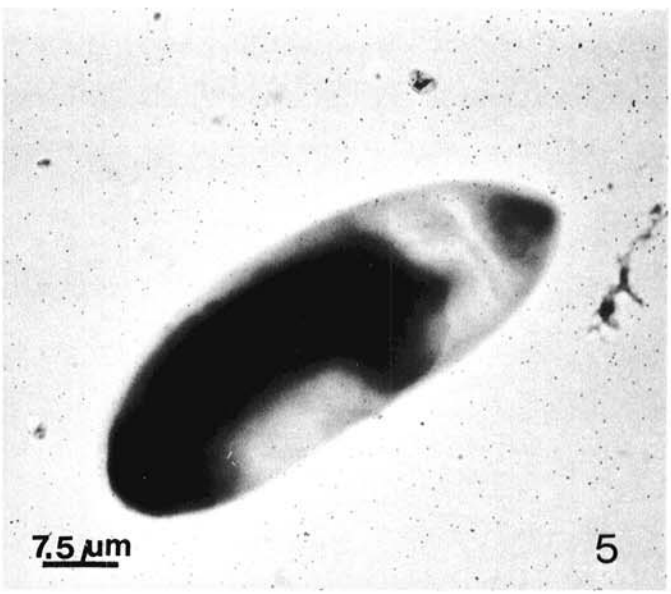

Fig. 5. A contaminant found in the crude plant sap from greening infected material. No specific IgG binding occurred. Note the unbound gold colloid. The cell is ×20 larger then the average greening bacteria size, and of a different shape.

DISCUSSION

Polyclonal antisera were raised against the cultured greening bacteria isolated from greening-infected citrus (2). IgG fractions of these sera were absorbed onto colloidal gold particles (18–20 nm) and used as a probe for detection of these bacteria in infected citrus plants. Positive results were obtained for the bacterium in situ. Figure 1 shows the gold particles attached to the cell wall of the bacteria within the phloem cell of greening-infected leaf. The number of gold particles is low due to partial loss of antigenicity while fixing the material as well as the effects of sectioning of the material which exposes only limited antigenic sites. The use of Lowicryl K4M as an embedding resin for low temperature polimerzation help to perserve antigenicity and give very low background levels of nonspecific labeling (12).

Much heavier labeling was obtained when using unfixed crude sap (Figs. 2 and 3). When applying this approach, there is no loss of antigenicity due to fixation. After concentrating the crude sap of 15–20 leaf midribs the bacteria can be easily found on the grid.

Our results suggest that the organism present in greening-infected citrus, and the cultured organism are serologically similar. This supports the contention that the cultured organism is in fact the causal agent of citrus greening.

The immunogold staining technique was found to be most convincing, since the serological reaction can be visualized. Extracted crude plant sap treated with immunogold-labeled conjugates followed by uranyl acetate negative staining provides a rapid, easy, and inexpensive detection method for plant pathogens.

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