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Use of Immunogold Labelling with Scanning Electron Microscopy To Identify Phytopathogenic Bacteria on Leaf Surfaces†

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Scanning electron microscopy coupled with high-resolution back-scattered electron imaging was used to detect gold-labelled specific immunoglobulins attached to epiphytic bacteria. Strains of *Xanthomonas citri* and *X. campestris* pv. citrumelo were specifically identified on grapefruit leaf surfaces when labelled with homologous gold-labelled immunoglobulins.

Phytopathogenic bacteria can survive and multiply as epiphytes on both host (9, 11, 18, 22, 26) and non-host (5, 10, 12, 20, 26) plant species. A number of researchers have used light microscopy to evaluate epiphytic bacteria on leaf surfaces. Leaves were bleached with chlorine gas or cleared with methanol prior to visualization of bacterial epiphytes with epifluorescence and bright-field microscopy (6, 17). Other methods (3, 7) have utilized acrylic casting resins which are sprayed on leaf surfaces, allowed to polymerize, peeled, and stained for examination with the light microscope. Scanning electron microscopy (SEM) has also been utilized to observe bacterial populations on leaves and buds of plants (2, 21, 25). All of these techniques are useful for obtaining information on spatial distribution and on variations in population, but they do not identify the bacterial organisms present.

More recently, membrane entrapment direct immunofluorescence using specific labelled antibodies has been used to detect the spread of the bacterium *Xanthomonas campestris* pv. citrumelo in simulated greenhouse and field nurseries (4, 10). New methods utilizing SEM and specific gold-labelled antibodies have enabled researchers to label a number of types of cells, including yeasts, erythrocytes, platelets, and cells from a wide variety of tissues (14–16, 27). With the development of highly efficient back-scattered electron detectors (1, 23), the performance of SEM immunocytochemistry has become much more practical. The purpose of our study was to develop a method of labelling bacteria on leaf surfaces with specific colloidal gold-labelled antibodies in order to detect them with SEM and back-scattered electron imaging (BEI).

Strains of *Xanthomonas citri* (XC-62), *X. campestris* pv. citrumelo (F-1), *X. campestris* pv. pruni (XP-1), and *Erwinia herbicola* (EH-1) were obtained from E. L. Civerolo, U.S. Department of Agriculture, Agricultural Research Station, Beltsville, Md.

Cultures were stored at 4°C under sterile oil slants of Wakimoto's semisynthetic potato medium (19). Bacterial inocula were grown in 20 ml of L broth (10 g of casein hydrolysate, 10 g of NaCl, 5 g of yeast extract, 1 liter of distilled water) for 18 to 20 h with gentle rotary shaking at 28°C. The 20-ml cultures were centrifuged (10 min at 10,000 × *g*) and washed two times by resuspending the pellet; the final pellet was then resuspended in sterile distilled water. The final concentration was adjusted to approximately 0.1 A_{620} unit (1-cm light path), which corresponds to 10^8 CFU/ml.

Greenhouse-propagated seedlings of Duncan grapefruit (*Citrus paradisi* Macfadyen), approximately 30 cm tall, were used for inoculations. Bacterial cell suspensions of 0.5×10^8 to 3.0×10^8 CFU/ml were sprayed uniformly onto leaf surfaces until runoff, and inoculated plants were covered with plastic bags for 24 h to maintain a high relative humidity.

Leaf disc samples were collected at 1, 2, 3, 5, and 7 days. Samples were collected from expanding and fully expanded leaves and fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer with 1,500 ppm of ruthenium red, pH 7.4, at 4°C overnight. Samples were rinsed three times for 15 min each in 0.1 M cacodylate buffer containing 5% sucrose, pH 7.4. Samples were stored at 4°C in 0.1 M cacodylate buffer with 5% sucrose, pH 7.4. Polyclonal rabbit antisera to bacterial strains were prepared by previously described methods (4), and the immunoglobulin (IgG) fraction of each antiserum was purified by using the protein A-Sepharose affinity chromatography procedure of Miller and Stone (24). The IgG was dialyzed against 2 mM borate, pH 9.2, prior to conjugation to colloidal gold. Colloidal gold particles approximately 30 to 40 nm in diameter was consistently prepared by reduction of tetrachloroauric acid with sodium citrate as described by Frens (8). Microtitration assays were used to determine the stability of the colloidal gold at different pHs and the optimum amount of protein to be coupled (13). The pH of the colloidal gold was adjusted to approximately 9.5 with 0.1 M potassium carbonate. Colloidal gold (13.95 ml) was placed in a sterile siliconized beaker with a magnetic stir bar, and 1.05 ml of 0.1 M Tris-HCl, pH 9.5, was added. The IgG was gradually added to the colloidal gold and stirred for 15 min at ambient temperature (24°C). Further IgG-gold adsorption was quenched by the addition of 240 μl of 2.5% polyethylene glycol (molecular weight, 20,000). Immediately after conjugation, the pH was adjusted to 8.2 with HCl and bovine serum albumin (BSA) was added from a 10% aqueous stock solution to a concentration of 0.25%. The addition of BSA as a secondary stabilizing agent enhanced the stability of gold-IgG complexes. The IgG-gold solution was centrifuged at 12,000 × *g* for 5 min at 4°C. The supernatant was carefully removed, and the mobile red pellet was washed in 0.01 M Tris-buffered saline–0.25% BSA (0.01 M Tris, 0.15 M NaCl,

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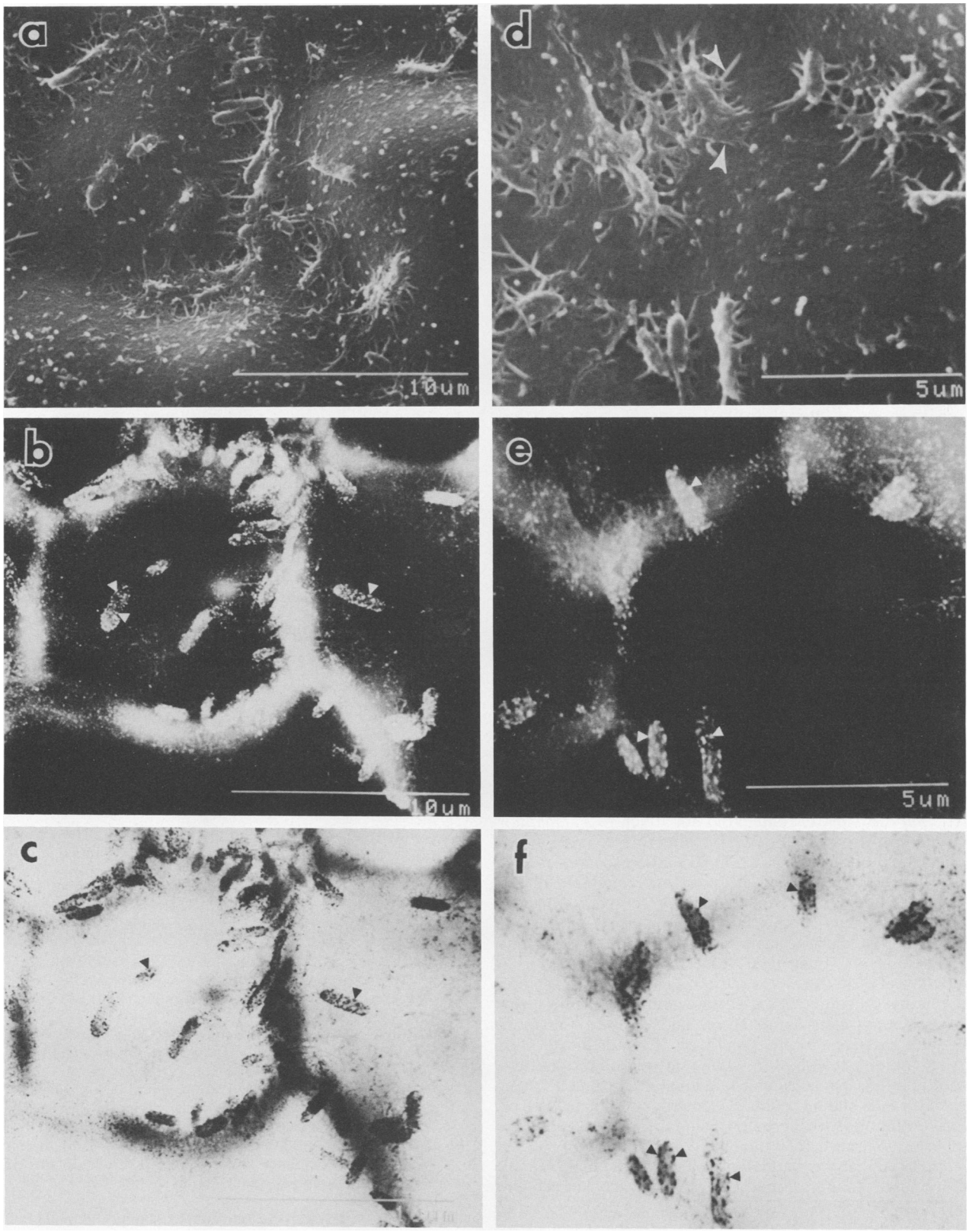

FIG. 1. (a) Scanning electron micrograph of *X. campestris* pv. citrumelo on a leaf disc sample which has been incubated on a homologous 30- to 40-nm gold-IgG complex. (b) Back-scattered electron (BSE) image (normal polarity) of panel a. Arrows show that gold particles are evident and well distributed over the bacterial cells. (c) BSE image (reverse polarity) of panel a. Arrows indicate gold particles. (d) Scanning electron micrograph of *X. citri* on a leaf disc sample which has been incubated on a homologous 30- to 40-nm gold-IgG complex. Arrows indicate the EPS matrix. (e) BSE image (normal polarity) of panel d. Arrows indicate gold-IgG particles. (f) BSE image (reverse polarity) of panel d. A uniform distribution of particles can be seen on the bacterial cell surfaces. Arrows indicate gold particles.

0.25% BSA, 0.02% NaN_3, pH 8.2) and centrifuged at 12,000 × *g* for 5 min. The resulting mobile red pellet was resuspended in 1.5 ml of 0.01 M TBS–0.25% BSA and stored at 4°C. The sizes of gold-IgG complexes were determined by transmission electron microscopy.

Previously fixed and washed leaf discs were incubated for 15 min on 0.10-ml drops of BSA-Tris (0.1% BSA, 20 mM Tris, 0.9% NaCl, 20 mM NaN_3) buffer containing 1% normal goat serum, pH 8.2. The leaf discs were blotted on filter paper and placed immediately on a 0.10-ml drop of a strain-specific gold-IgG complex diluted 1/10 in a BSA-Tris buffer and incubated overnight at 4°C. Control leaf disc samples were prepared by placing leaf discs on a 0.10-ml drop of a 30-nm gold-labelled goat anti-rabbit IgG diluted 1/10 in a BSA-Tris buffer overnight at 4°C. After incubation, the leaf discs were rinsed three times on 0.10-ml drops of BSA-Tris buffer for 5 min each and rinsed three times on 0.10-ml drops of filtered, double-distilled water for 5 min each. Leaf discs were optionally fixed for 10 min on drops of 1% glutaraldehyde in 0.06 M phosphate buffer, pH 6.8, and rinsed on drops of filtered distilled water. Discs were dehydrated in an ethanol series (10 to 100%) and critical-point dried. Dried samples were mounted on carbon planchets attached to specimen mounts and given three coats of carbon using a Ladd 30802 carbon fiber evaporation unit attached to a Ladd 30800 sputter coater (Ladd Research Industries, Inc., Burlington, Vt.). Specimens were examined with a Hitachi S-530 scanning electron microscope operating at 20 kV and equipped with a GW Electronics 113A back-scattered electron detector (GW Electronics, Inc., Norcross, Ga.).

With the use of SEM and BEI, bacterial cells of *X. citri* and *X. campestris* pv. citrumelo could be identified on leaf discs when incubated with their homologous gold-labelled IgG (Fig. 1). With the use of high-resolution BEI with positive and negative polarity, individual gold-IgG particles were visualized and well distributed over the bacterial cell surfaces (Fig. 1b, c, e, and f). No particles were observed attached to *E. herbicola* or *X. campestris* pv. pruni when incubated with either the *X. citri* or *X. campestris* pv. citrumelo gold-IgG complexes. Gold-IgG complexes specific to *X. campestris* pv. citrumelo did not attach to *X. citri*, nor did complexes specific for *X. citri* attach to *X. campestris* pv. citrumelo on leaf surfaces. Gold particles also were not visible when *X. citri*- and *X. campestris* pv. citrumelo-inoculated leaf discs were incubated on a control 30-nm goat anti-rabbit gold-IgG complex.

Incubation of leaf disc samples on strain-specific gold-IgG complexes at 4°C overnight was found to be superior to incubation at 37°C for 30 to 60 min. The lower incubation temperature prevented the gold-IgG complexes from forming patches or caps on bacteria and allowed for more individual particles to be visualized. For the most part, gold-IgG complexes of 30 to 40 nm showed better distribution of gold-IgG particles and remained stable much longer than did 60- to 100-nm gold-IgG complexes. There was no indication that postfixation after gold labelling increased the number of gold particles attached to bacteria.

It appeared that the population of bacteria declined rapidly after removal of the humidity bags which surrounded the plants for 24 h. However, *X. citri* and *X. campestris* pv. citrumelo could be positively identified on leaf surfaces by homologous gold-IgG labels at 7 days postinoculation. *X. citri* and *X. campestris* pv. citrumelo were mostly observed in anticlinal wall areas of the abaxial side of the leaf and were embedded in an extracellular polysaccharide (EPS) matrix (Fig. 1d). Gold-IgG labels did not show an affinity for the EPS matrix, and the back-scattered image showed that most gold-IgG particles attached directly to the bacteria (Fig. 1f).

Immunogold labelling coupled with SEM and BEI was effectively used to detect bacteria on leaf surfaces. The high levels of contrast obtainable with high-resolution BEI proved useful in detecting attachment of gold particles to the bacterial cells. The BEI negative-polarity mode allowed for the best observation of the gold particles attached to the bacterial cell surfaces. BEI was also useful in showing outlines of the bacterial cells, even when a large number of bacterial cells were encapsulated in EPS.

Phytopathogenic bacteria may multiply and persist as epiphytes for long periods without producing symptoms. Weeds can be asymptomatic and overwintering hosts of several plant-pathogenic xanthomonads (5, 10). Evidence suggests that these bacteria are part of the natural bacterial flora. Most previous techniques to locate bacteria on leaf surfaces have not distinguished among the bacterial organisms present. The results from our study indicate that the use of direct or indirect immunogold labelling and SEM with BEI for observing epiphytic phytopathogenic bacteria on weeds or host plants is practical if specific antisera are available. At present, we are continuing studies to identify specific pathovars on leaf surfaces in mixed inoculations and to determine the detection limits of this technique. This immunolabelling technique may also prove useful in early detection of bacteria in the xylem or phloem of infected plants and, possibly, in studies of the ecology of bacteria and their possible effectiveness as biological control agents on plant surfaces.

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REFERENCES

1. **Abraham, J. L., and P. B. DeNee.** 1974. Biomedical applications of backscattered electron images. Scanning Electron Microsc. **1974**:251–258.
2. **Andrews, J. H., and C. M. Kenerley.** 1978. The effects of a pesticide program on non-target epiphytic microbial populations of apple leaves. Can. J. Microbiol. **24**:1058–1072.
3. **Birkby, K. H., and T. F. Preece.** 1981. Differentiation of gram positive and gram negative bacteria in transparent acrylic resin emulsion replicas of surfaces of plants. J. Appl. Bacteriol. **50**:59–63.
4. **Brlansky, R. H., R. F. Lee, and E. L. Civerolo.** 1990. Detection of *Xanthomonas campestris* pv. *citrumelo* and *X. citri* from citrus using membrane entrapment immunofluorescence. Plant Dis. **74**:863–868.
5. **Cafati, C. R., and A. W. Saettler.** 1980. Role of nonhost species as alternate inoculum sources of *Xanthomonas phaseoli*. Plant Dis. **64**:194–196.
6. **Daft, G. C., and C. Leben.** 1966. A method for bleaching leaves for microscope investigation of microflora on the leaf surface. Plant Dis. Rep. **50**:493.
7. **Dickinson, C. H., J. Watson, and B. Wallace.** 1974. An impression method for examining epiphytic microorganisms and its application to phylloplane studies. Trans. Br. Mycol. Soc. **63**:616–619.
8. **Frens, G.** 1972. Particle size and sol stability in metal colloids. Kolloid Z. Z. Polym. **250**:736–741.
9. **Fryda, S. J., and J. D. Otta.** 1978. Epiphytic movement and survival of *Pseudomonas syringae* on spring wheat. Phytopathology **68**:1064–1067.
10. **Goto, M.** 1972. The significance of the vegetation for survival of plant pathogenic bacteria, p. 39–53. *In* H. P. Maas Geesteramus

(ed.), Proceedings of the International Conference on Plant Pathogenic Bacteria, 3rd. ed. Cent. Agric. Publ. Doc., Wageningen, The Netherlands.
11. **Gottwald, T. R., E. L. Civerolo, S. M. Garnsey, R. H. Brlansky, J. H. Graham, and D. W. Gabriel.** 1988. Dynamics and spatial distribution of *Xanthomonas campestris* pv. *citri* group E strains in simulated nursery and new grove situations. Plant Dis. **72**:781–787.
12. **Hirano, S. S., and C. D. Upper.** 1983. Ecology and epidemiology of foliar bacterial plant pathogens. Annu. Rev. Phytopathol. **21**:243–269.
13. **Hodges, G. M., M. A. Smolira, and D. C. Livingston.** 1984. Scanning electron microscope immunocytochemistry in practice, p. 189–234. *In* J. M. Polak and I. M. Varndell (ed.), Immunolabelling for electron microscopy, 1st ed. Elsevier Scientific Publishers, New York.
14. **Horisberger, M., and J. Rosset.** 1977. Colloidal gold, a useful marker for transmission and scanning electron microscopy. J. Histochem. Cytochem. **25**:295–305.
15. **Horisberger, M., J. Rosset, and H. Bauer.** 1975. Colloidal gold granules as markers for cell surface receptors in scanning electron microscopy. Experientia **31**:1147–1149.
16. **Horisberger, M., and J. Rosset.** 1977. Gold granules, a useful marker for SEM, p. 75–82. *In* O. Jahari and R. P. Becker (ed.), Scanning electron microscopy, vol. II. ITT Research Institute, Chicago.
17. **Hossell, J. C., and J. H. Baker.** 1979. A note on the enumeration of epiphytic bacteria by microscopic methods with particular reference to freshwater plants. J. Appl. Bacteriol. **46**:87–92.
18. **Kennedy, B. W., and G. L. Ercolani.** 1978. Soybean primary leaves as a site for epiphytic multiplication of *Pseudomonas glycinea*. Phytopathology **68**:1196–1201.
19. **Koizumi, M.** 1971. A quantitative determination method for *Xanthomonas citri* by inoculation of detached citrus leaves. Bull. Hortic. Res. Stn. (Minist. Agric. For.) Ser. B (Okitsu) **11**:167–183.
20. **Kuan, T. L., G. V. Minsavage, and N. W. Schaad.** 1986. Aerial dispersal of *Xanthomonas campestris* pv. *campestris* from naturally infected *Brassica campestris*. Plant Dis. **70**:409–413.
21. **Leben, C.** 1969. Colonization of soybean buds by bacteria: observations with the scanning electron microscope. Can. J. Microbiol. **15**:319–320.
22. **Leben, C.** 1963. Multiplication of *Xanthomonas vesicatoria* on tomato seedlings. Phytopathology **53**:778–781.
23. **Lin, P. S. D., and R. Becker.** 1975. Detection of backscattered electrons with high resolution. Scanning Electron Microsc. **1975**:61–70.
24. **Miller, T. J., and H. O. Stone.** 1978. The rapid isolation of ribonuclease free immunoglobulin G by protein-A Sepharose affinity chromotography. J. Immunol. Methods **24**:111–125.
25. **Moon, N. J., and W. G. Henk.** 1980. Progression of epiphytic microflora in wheat and alfalfa silages as observed by scanning electron microscopy. Appl. Environ. Microbiol. **40**:1122–1129.
26. **Timmer, L. W., J. J. Marois, and D. Achor.** 1987. Growth and survival of xanthomonads under conditions nonconducive to disease development. Phytopathology **77**:1341–1345.
27. **Trejdosiewicz, L. K., M. A. Smolira, G. M. Hodges, S. L. Goodman, and D. C. Livingston.** 1981. Cell surface distribution of fibronectin in cultures of fibroblasts and bladder derived epithelium; SEM-immunogold localization compared to immunoperoxidase and immunofluorescence. J. Microsc. **123**:227–236.