Specificity of the Envelopment of Bacteria and Other Particles in Cotton Cotyledons

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ABSTRACT

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During the hypersensitive response of cotyledons of incompatible cotton line Im 216 following infiltration with a suspension of *Xanthomonas campestris* pv. *malvacearum*, bacterial cells were observed by transmission electron microscopy to be enveloped by fibrillar materials and often covered by more dense cuticlelike materials on the host cell wall surfaces. Cells of the same pathovar were never enveloped in compatible cotton line Ac 44, whereas all cells of incompatible pathovar *X. campestris* pv. *campestris* were enveloped. In Ac 44, envelopes formed (without an accompanying hypersensitive response) around cells of compatible *X. campestris* pv. *malvacearum* that had been killed by heat, rifamycin, or ultraviolet light. Polystyrene latex particles 0.5 μm in diameter were not enveloped in cotyledons of either cotton line. However, the Gram-positive bacterium *Micrococcus lysodeikticus* and corn starch grains were both enveloped in Ac 44. These observations suggest that bacteria and other hydrophilic particles are generally enveloped at the surface of cotton cell walls, but that during compatible interactions envelopment is actively prevented.

Additional key words: bacterial blight of cotton, hypersensitive response.

When aqueous suspensions of *Xanthomonas campestris* pv. *malvacearum* (Smith 1901) Dye 1978b (= *X. malvacearum*) are infiltrated into cotyledons of cotton line Im 216, which is immune to bacterial blight, the bacteria are enveloped by fibrillar material with an outer border of more dense cuticlelike material during the first 4 hr following inoculation (5). Evidence of a hypersensitive response (HR) is apparent within a few hours in severely damaged membrane systems of host cells closest to the bacteria. With the exception that bacteria are enveloped only in incompatible host plants, the sequences of ultrastructural changes during host cell degradation and tissue collapse appear similar during compatible and incompatible interactions in cotton (*Gossypium hirsutum* L.) (1,5). However, the incompatible response occurs more quickly.

Envelopment of bacteria at host cell wall surfaces has also been observed in other plants (9,13,15,17). In tobacco, cells of incompatible and saprophytic bacteria are enveloped, but compatible bacteria are not (12,15,17). Tobacco rattle virus particles infiltrated into tobacco leaves attach end-on to cell walls, but apparently are not enveloped (10) and neither are polystyrene particles or asbestos fibers enveloped in tobacco (G. A. de Zoeten and L. Sequeira, *unpublished* [described in (22)]). This specificity, as well as the development of membrane vesicles and thick cell wall appositions at the bacterial attachment site, led some authors to infer that attachment is specific for incompatible or saprophytic bacteria and that envelopment is an essential part of the HR (12,22). They suggested that specificity of attachment is due to recognition and binding of a bacterial cell surface component by an agglutinin on the host cell wall.

In bean and soybean, however, envelopment appears to be nonspecific; it sometimes occurs with compatible, incompatible, and saprophytic bacteria (6,9,13,19). Less electron-dense material accumulates beneath the enveloping films than in tobacco, and there is little ultrastructural evidence that these hosts participate actively in envelopment. Since similar films occur in uninfiltrated and water-infiltrated bean and soybean leaves (9,13), the authors

concluded that the enveloping films consist of materials dissolved during water infiltration and deposited during subsequent transpiration. At 12 hr after inoculation of bean leaves, however, differences between compatible and incompatible interactions were evident (13). Nearly all colonies were still bound by films, but cells of compatible *Pseudomonas syringae* pv. *syringae* van Hall 1902 (Dye 1978b) had multiplied more, were less tightly packed, and were surrounded by a more electron-lucent matrix than were those of the four incompatible pathovars that were studied.

In cotton, as in tobacco, bacteria have been observed consistently to be enveloped in incompatible host lines (2,3,5), and never in compatible lines (1,3). Electron micrographs of the enveloping structures, however, reveal these to be more similar in electron-lucency and lack of host cell wall appositions to the structures in bean and soybean than to those in tobacco.

The present study was undertaken to explore the specificity with which cotton cotyledons enveloped bacteria and other particles with the objective of determining whether a recognition event is prerequisite to envelopment.

MATERIALS AND METHODS

Cotton lines. A susceptible line, Ac 44, and a line that is immune to bacterial blight, Im 216, were used in these experiments (7). Plants were grown in a greenhouse with a daily maximum temperature of 32 ± 2 C and a minimum temperature of 21 ± 1 C at night. Relative humidity was near 100% at night and averaged 59% ± 11% during the day. Cotyledons of both lines were fully expanded 2 wk after planting and were used at this stage in all experiments except one, in which immature cotyledons were used 1 wk after planting.

Bacteria. An aggressive strain of *X. campestris* pv. *malvacearum* race 3 was used (1). *X. campestris* pv. *campestris* (Pammel 1895) Dye 1978b (= *X. campestris*) was obtained from A. Novacky of the University of Missouri. *Micrococcus lysodeikticus* dis-IIP$^+$ was obtained from E. A. Grula of Oklahoma State University. Maintenance of stock cultures and preparation of inocula were as previously described (7). All live bacterial inocula were suspensions of $1-5 \times 10^8$ colony-forming units (cfu) per milliliter in sterile, aqueous saturated solutions of calcium carbonate (7).

Inoculum suspensions of 10^9 cfu of *X. campestris* pv. *malvacearum* per milliliter were killed, where specified, immediately before inoculation. Killing was by exposure to boiling water for 10 min, by exposure to a 25-watt General Electric germicidal lamp at 29 cm (approximately 3 joules/m^2/sec) for 4 min, or by exposure to 200 μg/ml of rifamycin SV (sodium salt, Sigma Chemical Company, St. Louis, MO 63178) at 30 C for 60 min. Killed cells were centrifuged for 10 min at 1,700 g at room temperature, the supernatant fluid was decanted, and the pellet was resuspended in half the original volume of sterile saturated calcium carbonate solution. Aliquots plated on nutrient agar (Difco Laboratories, Detroit, MI 48233) indicated that survival rates from the three killing treatments were less than 10^{-7}, 10^{-4}, and 10^{-4}, respectively.

Other particles. An aqueous suspension of spherical polystyrene latex particles (0.50 μm in diameter; Pelco, Tustin, CA 92680) was centrifuged for 10 min at 1,700 g at room temperature. The supernatant fluid was decanted, and the pellet was resuspended to a concentration at 5×10^8 particles per milliliter in sterile, saturated calcium carbonate solution with and without 5×10^8 cfu of bacteria per milliliter.

Our source of starch grains was Argo brand wet milled cornstarch (Best Foods, Englewood Cliffs, NJ 07632), which contains only 0.35% protein and 0.08–0.1% carbon tetrachloride-extractable lipid (Chuck Mally, *personal communication*). A suspension was prepared by mixing 1 g of cornstarch with 9 ml of water, allowing the mixture to settle at room temperature for 15 min, and withdrawing an aliquot of the fluid.

Inoculation and sampling. The abaxial surfaces of two or more cotyledons were infiltrated with each aqueous suspension of inoculum by using a syringe without a needle (1). Infiltrated cotyledons lost their water-soaked appearance within 1 hr. Tissue samples were excised 5, 8, and 24 hr after infiltration and prepared for transmission electron microscopy as previously described (1). Samples were never subjected to vacuum infiltration, which disrupts host-bacterial associations (8). From 20 to 75 electron microscope fields containing bacteria and/or particles, with the exception of starch grains, were observed for each type of infiltrated material, and from 11 to 75 electron micrographs were made of each type of infiltrated material. Only 10 starch grains were found and photographed.

RESULTS

Twenty-four hours after infiltration of cotyledons with bacterial suspensions, envelopes were observed around *X. campestris* pv. *malvacearum* in mature cotyledons of the bacterial-blight-immune incompatible cotton line Im 216 (Fig. 1), but not in cotyledons of the susceptible cotton line Ac 44 (Figs. 2 and 3). Since Hildebrand et al (13) observed heavier enveloping films around bacteria in young bean leaves, we included immature Ac 44 cotyledons in this study, but observed no envelopment of *X. campestris* pv. *malvacearum* in them (Fig. 3). In Ac 44, however, envelopes were observed around cells of *X. campestris* pv. *campestris*, which is not a pathogen in cotton (Figs. 4 and 5). Heat-killed cells of *X. campestris* pv. *malvacearum* were enveloped within 5 hr in both incompatible (Im 216, Fig. 6) and compatible (Ac 44, Fig. 7) lines. Cells of *X. campestris* pv. *malvacearum* killed by rifamycin or ultraviolet light were also enveloped in Ac 44 within 5 hr (Figs. 8 and 9).

Envelopes were not observed around 0.50-μm polystyrene particles 5 hr after their infiltration into Im 216 (Fig. 10) and Ac 44 (Fig. 11). When *X. campestris* pv. *malvacearum* and polystyrene particles were infiltrated together into cotyledons of the incompatible Im 216 line, envelopes formed around the bacteria, but not around isolated polystyrene particles (Fig. 12). Similar envelopment of bacteria, but not of isolated polystyrene particles, was observed following infiltration of Ac 44 with incompatible *X. campestris* pv. *campestris* together with polystyrene particles (Fig. 13). Where polystyrene particles occurred close to bacterial cells, however, the envelopes enclosed both (Fig. 14).

Cells of the Gram-positive saprophytic bacterium *M.*

lysodeikticus were enveloped within 5 hr of infiltration into Ac 44 (Fig. 15). Starch grains from maize were also enveloped in Ac 44 (Fig. 16).

There was considerable variation in the appearance of material inside the envelopes, which was usually fibrillar (eg, Figs. 1, 4, and 5), but sometimes granular (Fig. 6), and varied somewhat in electron density (low in Figs. 12 and 13, and high in Fig. 15). More
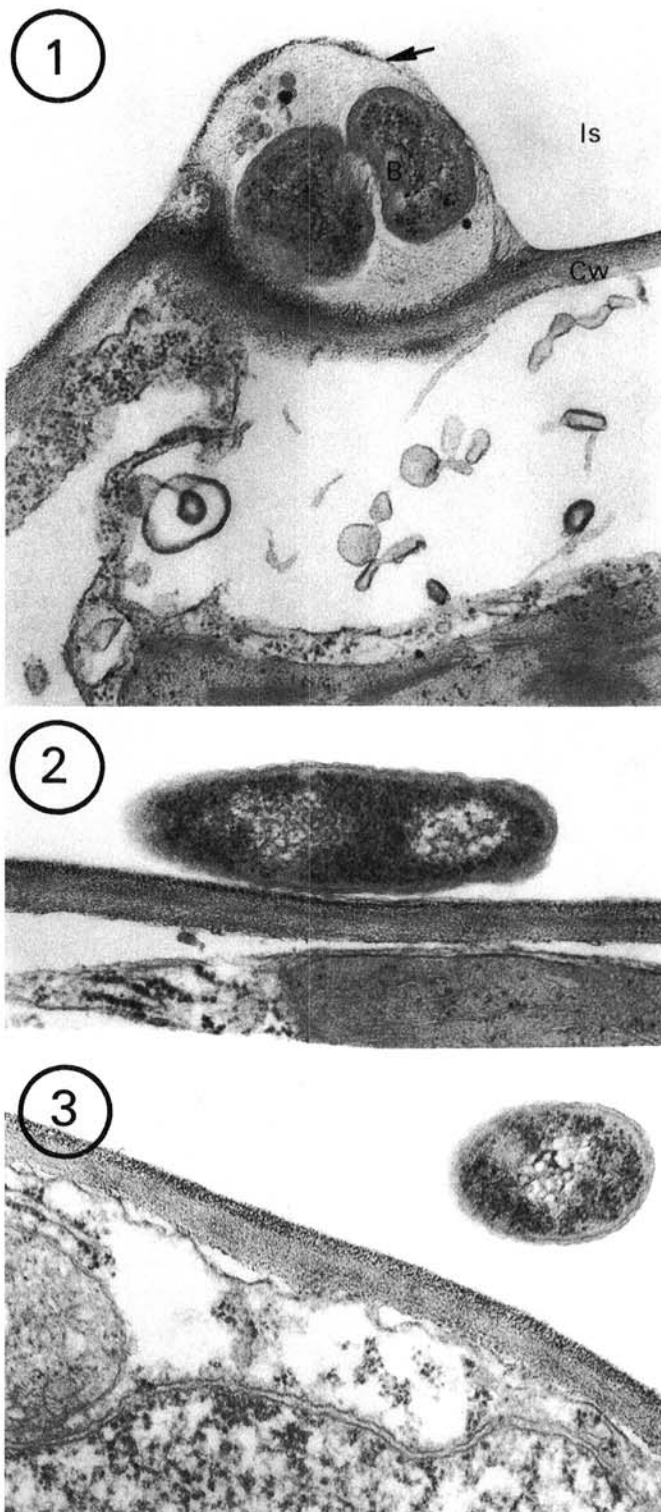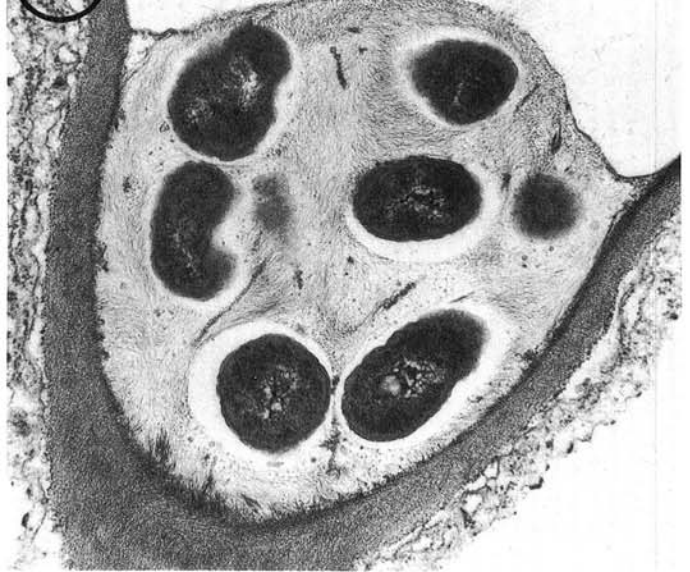

Figs. 1–3. Cotton cotyledons 24 hr postinfiltration with suspensions of *Xanthomonas campestris* pv. *malvacearum*. **1,** Mature cotyledon of Im 216, showing envelopment (arrow) of bacterial cells (×35,800). **2,** Mature cotyledon of Ac 44 (×43,800). **3,** Immature cotyledon (1 wk after planting) of Ac44 (×44,500). B = bacterium, Cw = cell wall, Is = intercellular space.

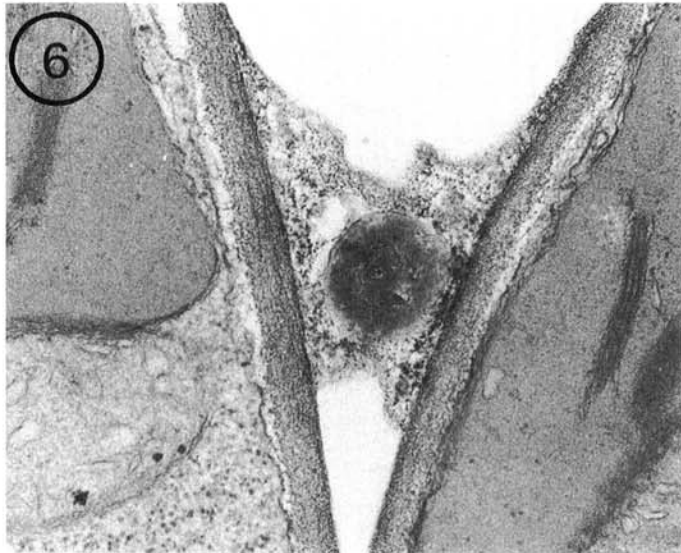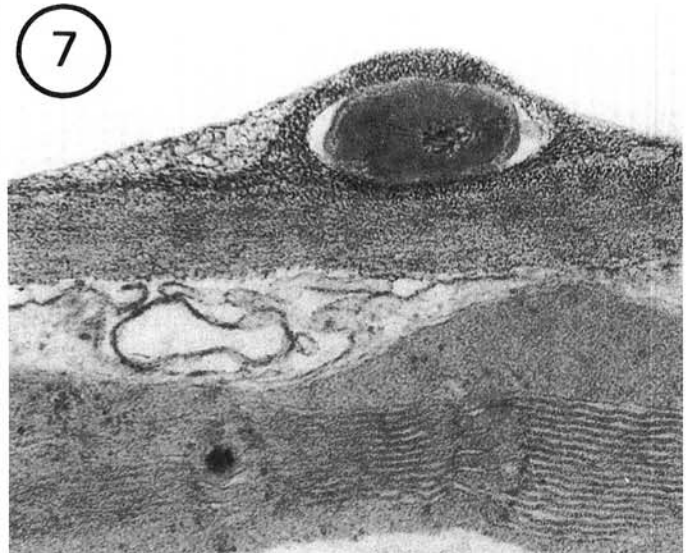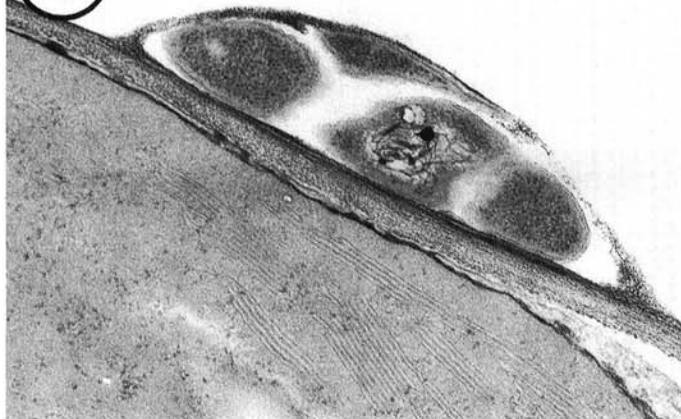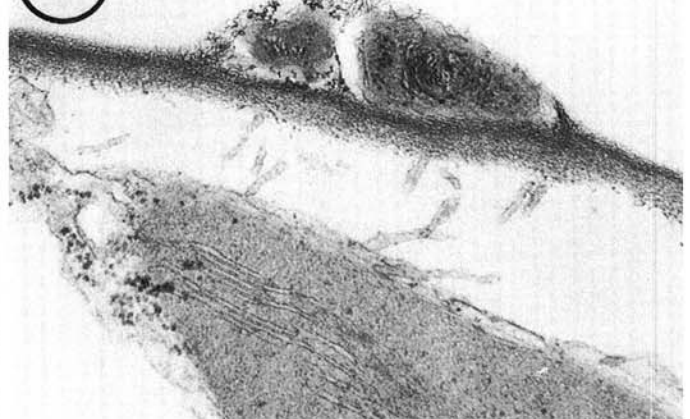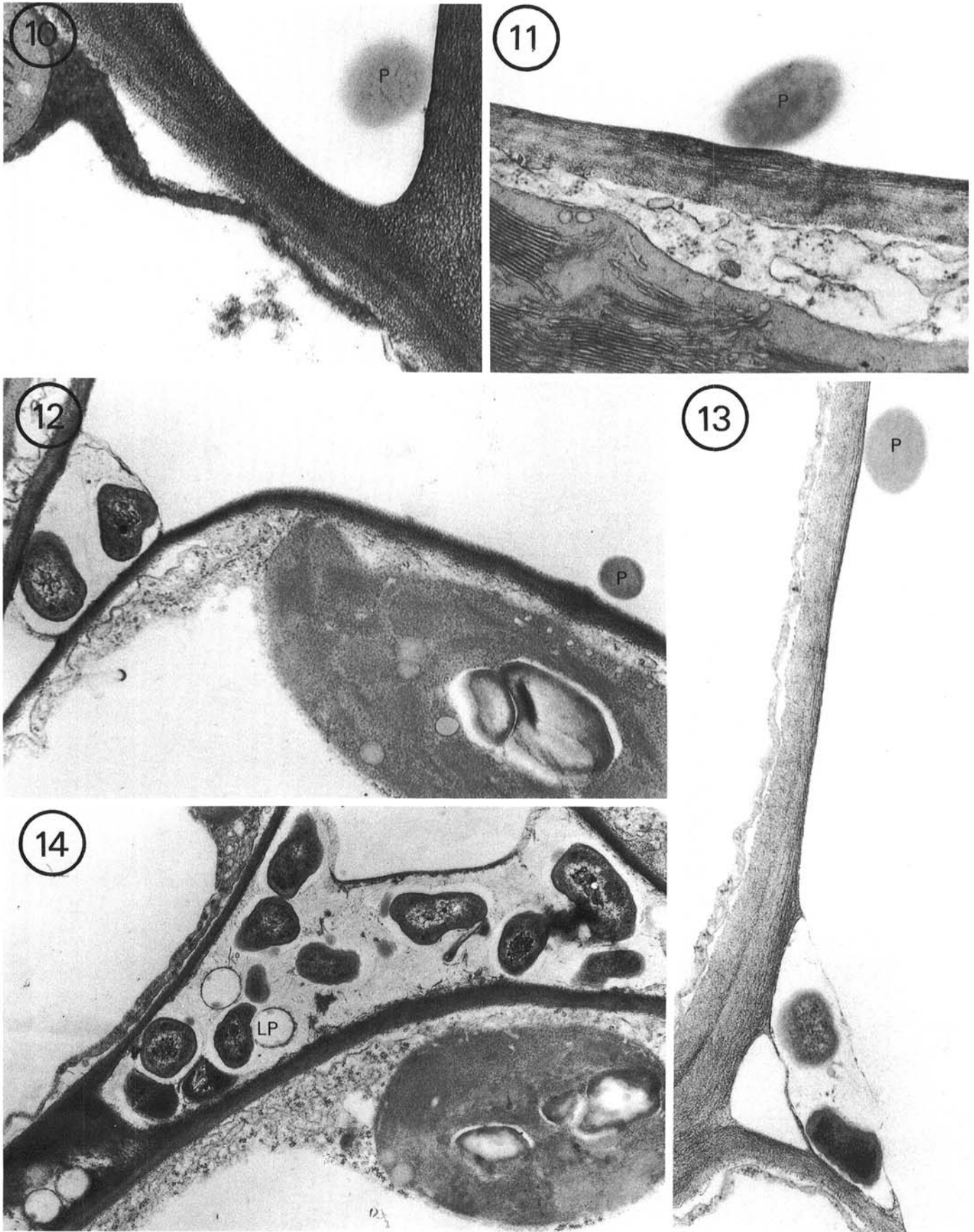**Figs. 4–9.** Cotton cotyledons postinfiltration with suspensions of live and killed bacteria. **4,** Ac 44 with *X. campestris* pv. *campestris* 8 hr postinfiltration (×40,250). **5,** Ac 44 with pv. *campestris* 24 hr postinfiltration (×29,750). **6,** Ac 44 with heat-killed pv. *malvacearum* 8 hr postinfiltration (×41,800). **7,** Im 216 with heat-killed pv. *malvacearum* 8 hr postinfiltration, showing vesiculation of the plasmalemma (×66,800). **8,** Ac 44 with rifamycin-killed pv. *malvacearum* 5 hr postinfiltration (×39,800). **9,** Ac 44 with ultraviolet light-killed pv. *malvacearum* 5 hr postinfiltration, showing plasmolysis and vesiculation of the plasmalemma (×39,800).

Figs. 10-14. Cotton cotyledons postinfiltration with suspensions of polystyrene particles alone (Figs. 10 and 11) and with bacteria (Figs. 12-14). **10,** Ac 44 with polystyrene particle 5 hr postinfiltration (×51,750). **11,** Im 216 with a polystyrene particle 5 hr postinfiltration (×41,800). **12,** Im 216 with polystyrene particle and pv. *malvacearum* 8 hr postinfiltration (×16,500). **13,** Ac 44 with polystyrene particles and pv. *campestris* 8 hr postinfiltration (×40,250). **14,** Ac 44 with polystyrene particles and pv. *campestris* enveloped together, 8 hr postinfiltration. The circular, electron-lucent areas (LP) are loci of polystyrene particles that were not retained in the fibrillar material during sectioning (×31,850). P = polystyrene particle, LP = location of polystyrene particle lost during sectioning.

fibrillar material was included within enveloping films with live bacteria (Figs. 1, 4, 5, 12–15) than with killed bacteria (Figs. 6–9). Despite their variability, clearly recognizable envelopes were found around all live incompatible bacteria, killed compatible and incompatible bacteria, live saprophytic bacteria, and starch grains that were observed. No envelopes or fragments of ruptured envelopes were observed near live, compatible pathogens or isolated polystyrene particles.

The hypersensitive response (HR) was apparent within 24 hr as macroscopically detectable necrosis in all cotyledons infiltrated with live, incompatible bacteria. The HR was not apparent in cotyledons infiltrated with saprophytic or compatible or killed-incompatible bacteria, although plasmolysis and vesiculation of the plasmalemma were often observed near saprophytic or killed bacteria (Figs. 7 and 9). No ultrastructural damage was observed within cells of cotyledons infiltrated with starch grains or with polystyrene particles alone.

DISCUSSION

Envelopment of bacteria accompanied the HR in both incompatible interactions that were studied (Im 216 with *X. campestris* pv. *malvacearum* and Ac 44 with pv. *campestris*). No traces of enveloping films were observed in the compatible interaction (Ac 44 with *X. campestris* pv. *malvacearum*). The compatible interaction was examined 24 hr after inoculation, an interval during which only about a two- to tenfold increase in bacterial numbers occurs (7). We cannot rule out the possibility that enveloping films formed earlier and then disappeared. However, bacterial multiplication alone does not account for the complete lack of envelopment observed at 24 hr, since we observed fibrillar materials and/or enveloping films around cells of pv. *malvacearum* following approximately 2,000-fold multiplication in

incompatible Im 216 leaves 6 days after a low level inoculation (2).

Although envelopment may be necessary for elicitation of the HR, envelopment alone is not sufficient to cause the HR, nor is it dependent on the HR, since the saprophyte *M. lysodeikticus*, starch grains, and killed *X. campestris* pv. *malvacearum* were enveloped in the absence of an HR. In tobacco also, saprophytic bacteria and dead phytopathogenic bacteria are enveloped and elicit local plasmolysis and vesiculation of the plasmalemma, without eliciting a macroscopically visible HR (12, 17).

Envelopment of infiltrated bacteria or particles does not appear to require specific surface features, since Ac 44 enveloped cells of Gram-negative *X. campestris* pv. *campestris*, cells of Gram-positive *M. lysodeikticus*, and starch grains. The surface components of *X. campestris* pv. *campestris* are lipopolysaccharide (LPS) (composition unknown) and exopolysaccharide (EPS), which consists of a β-(1,4)-glucan backbone with sidechains containing glucose, mannose, glucuronic acid, and pyruvic acid (14). The surface layer of Gram-positive *M. lysodeikticus* is peptidoglycan, an alternating copolymer of *N*-acetyl glucosamine and *N*-acetyl muramic acid crosslinked by an oligopeptide (16). The starch grains that are used contain so few minor constituents that their surface is principally glucan, and there is no histological evidence for a covering of other material (4). The observed envelopment of dead compatible bacteria in Ac 44 suggests that compatible bacteria also have the cell surface characteristics necessary for envelopment. While it is possible that the heat-killing treatment altered the bacterial cell surfaces, rifamycin inhibits bacterial transcription very specifically (11). Research in photobiology has indicated that chromosomal aberrations are the principal toxic effect of ultraviolet radiation on bacteria (20).

The diversity of objects enveloped in cotton cotyledons is consistent with the conclusion of Hildebrand et al (13) that infiltration of leaves with water loosens from cell surfaces materials
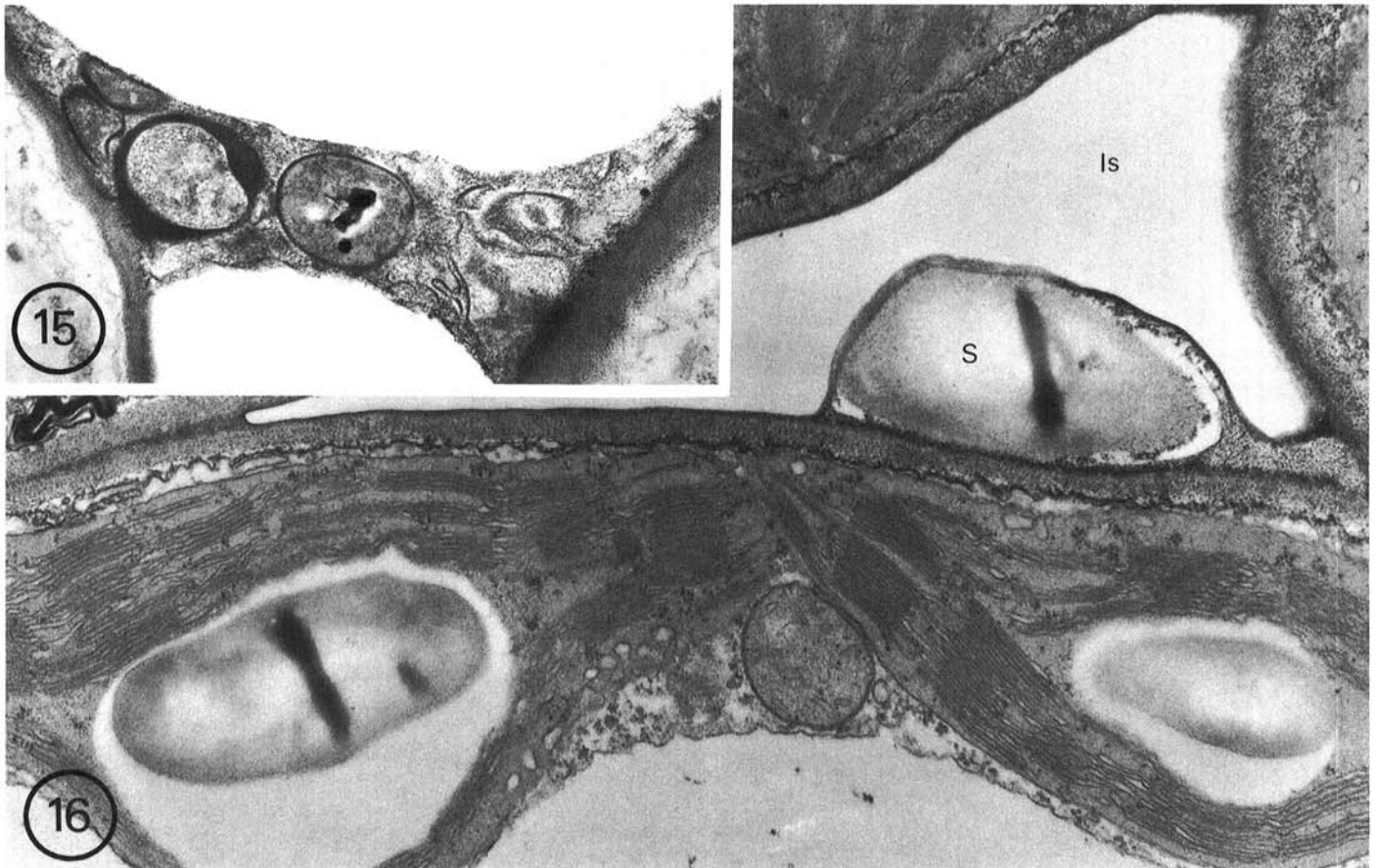
Figs. 15 and 16. Ac 44 cotton cotyledons 5 hr postinfiltration with *Micrococcus lysodeikticus*: **15,** showing two enveloped bacteria, and apparent fragments from at least one other (×50,880) or **16,** with starch grains (×38,500). Is = intercellular space, S = infiltrated starch grain.

that, when the water has evaporated, are deposited as films over intercellular objects by the retreating water meniscus. In our opinion, the failure of polystyrene particles to be enveloped is not inconsistent with this hypothesis. Polystyrene is hydrophobic, and the water meniscus probably retreated from it rather than coating it with a film. Although killed cells of *X. campestris* pv. *malvacearum* were consistently enveloped (Figs. 6–9), the greater amounts of fibrillar material within envelopes containing live bacteria (Figs. 1, 4, 5, and 12–15) suggest that bacterial activity also contributed to the structures either directly or by interaction with the host.

We suggest that in cotyledons and leaves of cotton, envelopment is not the result of a specific recognition event, but rather is the usual fate of bacteria and other hydrophilic particles, and that live compatible bacteria somehow prevent this from occurring. Envelopment may play a role in the HR, such as permitting contact between bacterial elicitor and host receptor molecules (21), and compatible bacteria have necessarily evolved a mechanism for preventing envelopment. Alternatively, the lack of envelopment of compatible bacteria may be only a side-effect of another activity characteristic of the compatible interaction, such as rapid synthesis of so much EPS (17,18) that the water meniscus does not recede to deposit a film close to the bacteria.

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