# Ultrastructure of Interactions Between *Xanthomonas campestris* pv. *vesicatoria* and Pepper, Including Immunocytochemical Localization of Extracellular Polysaccharides and the AvrBs3 Protein

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**The ultrastructure of interactions between the bacterial spot pathogen *Xanthomonas campestris* pv. *vesicatoria* and its host, pepper, was investigated. Development of colonies of the race 2 strain 85-10 and transconjugants expressing the cloned avirulence gene *avrBs3* on plasmids pL3XV1-6 and pD36 was examined in pepper cultivars Early Cal Wonder (ECW) and ECW-30R; these cultivars are isogenic apart from the absence or presence, respectively, of the *Bs3* allele for resistance to *X. c.* pv. *vesicatoria*. Resistance is expressed by the hypersensitive reaction (HR). Immunocytochemical staining allowed examination of the contribution of extracellular polysaccharide (EPS) to colony development and differentiation between material of bacterial and plant origin in the intercellular space. Early growth of colonies was identical in resistant and susceptible leaves. Bacteria were not specifically attached to mesophyll cells in resistant leaves but during compatible and incompatible interactions rapidly became coated by a thick layer of EPS within 4 hr after inoculation. Labeling with monoclonal antibodies (MAbs) A6 and D1 with specificity for the xanthan side chain was most dense around the bacterial cell wall; more extracellular label was found with A6. By contrast, with MAb B3, which has specificity for the pyruvylated terminal mannose of the xanthan side chain, label was primarily intracellular at the poles of bacterial cells. Labeling with B3 was greatly reduced in bacteria within tissue which had undergone the HR. The AvrBs3 protein was located by immunocytochemistry within the cytoplasm of cells of *X. c.* pv. *vesicatoria* overexpressing the *avrBs3* gene *in vitro* and in the plant. Exchange of signals between bacteria and plant during both the compatible and incompatible interactions was indicated by the localized deposition of paramural papillae in mesophyll cells. The collapse of pepper cells during the HR followed plasma membrane damage and vesiculation of the cytoplasm initially close to the bacterial colony. The histological studies have defined the structural and temporal framework within which recognition and response occur in pepper and indicate the changing conditions to which cells of *X. c.* pv. *vesicatoria* are exposed during colonization of the intercellular space.**

*Additional keywords:* bacterial pathogenicity, disease resistance, electron microscopy.

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Electron microscopy of plant-pathogen interactions allows definition of the structural and temporal framework within which recognition and response occur in the plant. Microscopical studies with fungal pathogens have provided valuable insights into the processes associated with successful parasitism and also the restriction of growth in resistant plants (Heath 1989; Mansfield 1990; Mendgen and Lesemann 1991; O'Connell *et al.* 1985, 1990). The successes achieved with fungi have, to some extent, been due to their production of characteristic infection structures and features of mycelial growth. In contrast to the fungi, most leaf-infecting bacterial pathogens are limited to the production of colonies within intercellular spaces or xylem vessels. The use of histochemical and immunocytochemical analyses of bacterial infections has, however, helped to define the microsites within which bacteria multiply and respond to the complex and largely undefined environment within the plant. The more detailed analyses have been on the infection of leaves by pseudomonads (Al-Issa and Sigee 1982; Brown and Mansfield 1988, 1991; Hildebrand *et al.* 1980, O'Connell *et al.* 1990), *Clavibacter michiganense* subsp. *michiganense* (Benhamou 1991) and *Xanthomonas campestris* pv. *malvacearum* (Al-Mousawi *et al.* 1982; Cason *et al.* 1978). In these studies the attachment and encapsulation of bacterial cells in plant cell wall-derived materials has been recognized as one mechanism of resistance. In the analysis of resistance of a cultivar of cotton classified as immune to *X. c.* pv. *malvacearum*, attachment and encapsulation of bacteria during the incompatible interaction were also considered to be required for the subsequent development of the hypersensitive resistance reaction (Cason *et al.* 1978). Here we have used electron microscopy to examine the genetically well-defined interaction between *Xanthomonas campestris* pv. *vesicatoria* and its host pepper (*Capsicum annuum* L.).

In leaves of susceptible cultivars of pepper, cells of *X.*

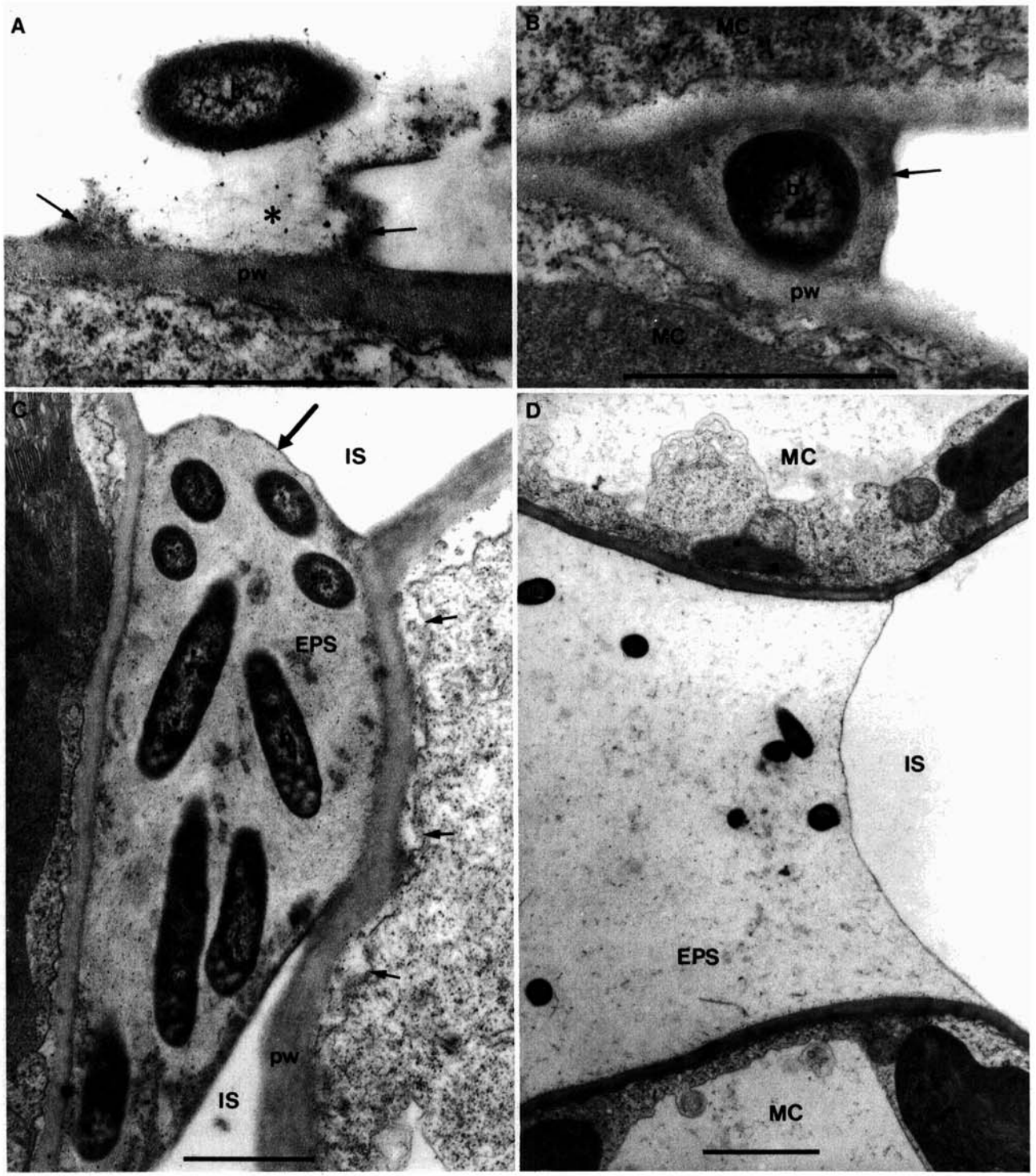


**Fig. 1.** Colony development examined by conventional staining. A and B, show bacterial cells (b) in close association with the plant cell wall (pw), 2 hr after inoculation. Bar = 1 μm. In **A,** the bacterium *X. c.* pv. *vesicatoria* 85-10 (pL3XV1-6) is apparently being deposited onto the plant cell wall (pepper cv. ECW-30R, incompatible interaction) and is surrounded by fine fibrils of EPS (asterisk) and amorphous material (arrows) derived from the inoculum droplet. In **B,** a bacterial cell (*X. c.* pv. *vesicatoria* 85-10 in ECW, a compatible interaction) has become lodged at the junction between two spongy mesophyll cells (MC). The bacterium is encapsulated within fibrillar EPS and a layer of amorphous electron-dense deposits (arrow). **C,** An expanding colony of *X. c.* pv. *vesicatoria* 85-10 (pL3XV1-6) in cv. ECW-30R within the intercellular space (IS) between mesophyll cells 8 hr after inoculation. Note the darker staining pellicle (large arrows) at the surface of dense EPS surrounding bacteria. The colony appears to have distorted (but not degraded) the wall of one mesophyll cell which has some plasma membrane convolution (small arrows). Bar = 1 μm. **D,** Bacterial cells of *X. c.* pv. *vesicatoria* 85-10 (pL3XV1-6) are widely dispersed within EPS, which has filled large volumes of the intercellular space in cv. ECW, 30 hr after inoculation. During colonization of this susceptible cultivar no major changes are observed in the structure of adjacent mesophyll cells. Bar = 2 μm.

*c.* pv. *vesicatoria* multiply rapidly, giving rise to water-soaked lesions and, later, the brown necroses characteristic of bacterial spot disease (Stall and Cook 1966). Mechanisms of pathogenicity in *X. c.* pv. *vesicatoria* are poorly understood. No low molecular weight plant toxins have been reported in *X. c.* pv. *vesicatoria* and, in contrast to *X. c.* pv. *campestris*, plant cell wall-degrading enzymes do not have a major role in pathogenicity (Beaulieu *et al.* 1991; Daniels *et al.* 1991; Tang *et al.* 1991). The production of extracellular polysaccharide (EPS), which contains xanthan, is recognized to have a role in symptom development in diseases caused by *X. c.* pv. *campestris* (Barrère *et al.* 1986; Coplin and Cook 1990; Daniels *et al.* 1991; Hötte *et al.* 1990; Koplin *et al.* 1992; Ramirez *et al.* 1988) but the importance of EPS in *X. c.* pv. *vesicatoria* has not been examined. A cluster of genes necessary for basic pathogenicity in susceptible plants and for the induction of the hypersensitive reaction in resistant plants (*hrp* genes) has been cloned from *X. c.* pv. *vesicatoria* (Bonas *et al.* 1991). Six *hrp* loci have been identified in *X. c.* pv. *vesicatoria* and found to be regulated by medium composition and plant signals (Schulte and Bonas 1992). Recent DNA sequence analysis indicates that the products of certain *hrp* genes share homology with proteins in *Shigella* and *Yersinia* which are involved in the export of virulence factors (Fenselau *et al.* 1992).

Several genes for resistance to bacterial spot have been identified in pepper. The *Bs1, Bs2,* and *Bs3* genes determine resistance to particular races of *X. c.* pv. *vesicatoria* carrying matching avirulence genes (Minsavage *et al.* 1990). Race-specific resistance to *X. c.* pv. *vesicatoria* is expressed by the hypersensitive reaction (HR) leading to the collapse and desiccation of infection sites (Bonas *et al.* 1989; Minsavage *et al.* 1990). Growth of bacteria is restricted following the collapse of tissue during the HR (Stall and Cook 1966; Herbers *et al.* 1992). The avirulence gene, *avrBs3* in *X. c.* pv. *vesicatoria* which corresponds in a gene-for-gene manner to the plant *Bs3* gene has been subjected to detailed molecular characterization (Bonas *et al.* 1989; Knoop *et al.* 1991; Herbers *et al.* 1992). Recent studies have shown that the *avrBs3* gene is constitutively expressed in *X. c.* pv. *vesicatoria* but for induction of the HR all of the *hrp* loci need to be intact (Knoop *et al.* 1991). The function of *avrBs3* and the nature of the signal molecules that trigger the transduction pathway leading to the HR in peppers expressing the *Bs3* gene is not known.

In this microscopical study of bacterial growth and the plant's response during the *avrBs3*/*Bs3* interaction, the contribution of EPS to colony development has been examined by immunocytochemistry using polyclonal antiserum to whole bacterial cells and monoclonal antibodies raised to xanthan. In addition, for the first time, the product of an avirulence gene, the AvrBs3 protein, has been localized by immunogold labeling. The interactions examined are summarized in Table 1.

## RESULTS

### Colony development in the plant.

A striking feature of colony development within intercellular spaces was the speed with which cells of *X. c.* pv. *vesicatoria* became dispersed within a fibrillar matrix clearly stained with uranyl acetate and lead citrate (Fig. 1A–D). At all times after inoculation the fibrillar material was labeled by the polyclonal XV1 antiserum raised to whole bacteria (Fig. 2A, B) and by the monoclonal antibody A6 which has specificity for the xanthan side chain (Fig. 3B, D, F). Neither XV1 nor A6 antibodies reacted with plant cell wall material; the matrix surrounding *X. c.* pv. *vesicatoria* cells was therefore identified as bacterial EPS, containing xanthan.

Bacterial numbers have been shown to increase at similar rates for the first 2 days after inoculation during compatible and incompatible *X. c.* pv. *vesicatoria*/pepper interactions governed by *avrBs3* (Herbers *et al.* 1992 and unpublished results). Time-course studies using electron microscopy showed that the initial patterns of colony development were also identical in the different interactions examined. Stages of colony development are illustrated in Figure 1 in which examples have been selected from both compatible and incompatible interactions. Following injection, as the water within the intercellular space evaporated, bacteria and other suspended material appeared to dry down onto the plant cell wall as illustrated in Figure 1A. At this early stage, 2 hr after inoculation, direct wall-to-wall contact between bacteria and plant cells was sometimes observed, but cells of *X. c.* pv. *vesicatoria* were often already surrounded by a layer of fibrillar EPS (Figs. 1A, 2A, 3B). Amorphous electron-dense material was occasionally observed around individual bacteria 2 hr after inoculation (Figs. 1A, 1B, 2A). This material was not labeled by the antibodies XV1 or A6 or the other monoclonals to xanthan, B3 or D1. It appeared identical to deposits found at cell junctions in conventionally stained specimens and was therefore probably of plant rather than bacterial origin. Figure 1C shows the typical appearance of a growing colony as observed during the compatible and incompatible interactions. During the early stages of the interaction between *X. c.* pv. *vesicatoria* and pepper there was, therefore, no indication of the specific attachment or encapsulation of bacterial cells in resistant leaves.

Because of the striking secretion of EPS, the density of cells within colonies appeared to decrease rapidly (compare Fig. 1C and D) despite the rapid increase in bacterial numbers observed within the infected tissue and recorded in multiplication studies (Herbers *et al.* 1992). During the second day after inoculation, individual *X. c.* pv. *vesicatoria* cells were widely separated within the fibrillar matrix that filled the intercellular spaces in some regions of mesophyll tissue (Fig. 1D). No invasion of the xylem or degradation of plant cell walls was observed.

### Immunogold labeling of EPS.

The monoclonal antibodies allowed localization of different epitopes within the xanthan polymer. Representative results obtained with monoclonals A6 and D1 using leaf sections prepared 2, 20, and 48 hr after inoculation are illustrated in Figure 3. An identical distribution of label was found in cells of *X. c.* pv. *vesicatoria* 85-10 and 85-10 (pL3XV1-6) grown *in vitro* or in leaves of pepper cvs. ECW or ECW-30R. Transconjugant 85-10 (pD36) was not used in these experiments. The antibodies A6 and D1,

both directed against the xanthan side chain (Haaheim *et al.* 1989) produced a similar pattern of labeling around the bacterial cell wall (Fig. 3A–F) and no differences were observed between bacteria examined during compatible or incompatible interactions. A6 recognized more epitopes in the surrounding matrix including fibrils which were often decorated by linear arrays of gold particles (Fig. 3F). The labeling observed with A6 away from bacterial cells was generally similar to but less dense than that observed with the polyclonal XV1 serum (compare Figs. 2B and 3F). During the early stages of colony development, 2 hr after inoculation, both A6 and XV1 labeled the fibrillar material surrounding bacterial cells adjacent to the plant cell wall (Figs. 2A and 3B).

Monoclonal antibody B3 produced a distinctly different pattern of labeling, as illustrated in Figure 4. This antibody recognizes the pyruvylated terminal mannose of the fully acetylated xanthan side chain and is much less reactive than A6 and D1 to native xanthan (Haaheim *et al.* 1989). Gold label was almost entirely intracellular and was localized at the poles of actively multiplying bacteria in tissue sampled between 2 and 30 hr after inoculation. By 48 hr, bacteria in tissue undergoing the HR labeled very weakly with B3, whereas in susceptible leaves labeling of bacterial cells remained strong and highly localized (compare Figs. 4C and D).

**Immunogold labeling of the AvrBs3 protein.**

The incompatible interaction between *X. c.* pv. *vesicatoria* and pepper cv. ECW-30R is dependent on the expression of a functional *avrBs3* gene (Bonas *et al.* 1989; Knoop *et al.* 1991). The corresponding protein is constitutively expressed; its mode of action is unknown. One important question in understanding the biochemical function of the gene is the localization of the avirulence protein, AvrBs3. Attempts to localize the protein by immunocytochemistry in cells of *X. c.* pv. *vesicatoria* strain 71-21, from which the gene was isolated, or in cells of the transconjugant 85-10 (pL3XV1-6) grown *in vitro* or in the plant proved unsuccessful. To increase the expression of *avrBs3*, the 3.5-kb coding region was cloned into pDSK602. Western blot analyses showed that this construct allowed about a 20-fold increase in levels of AvrBs3 protein (J. C-S. and U.B., unpublished). Immunogold labeling localized AvrBs3 within cells of *X. c.* pv. *vesicatoria* 85-10 (pD36) grown *in vitro* and in the plant (Fig. 5). No label was found over plant cells. The same distribution of label was observed in cells of *X. c.* pv. *vesicatoria* 85-10 (pD36) grown *in vitro* and in ECW or ECW-30R plants, 4 and 24 hr after inoculation. One day after inoculation, the mean numbers ± SEM of gold particles per medial section of 50 separate cells of *X. c.* pv. *vesicatoria* 85-10 (pD36) grown *in vitro*, in cv. ECW, and in ECW-30R were 8.80 ± 1.53,

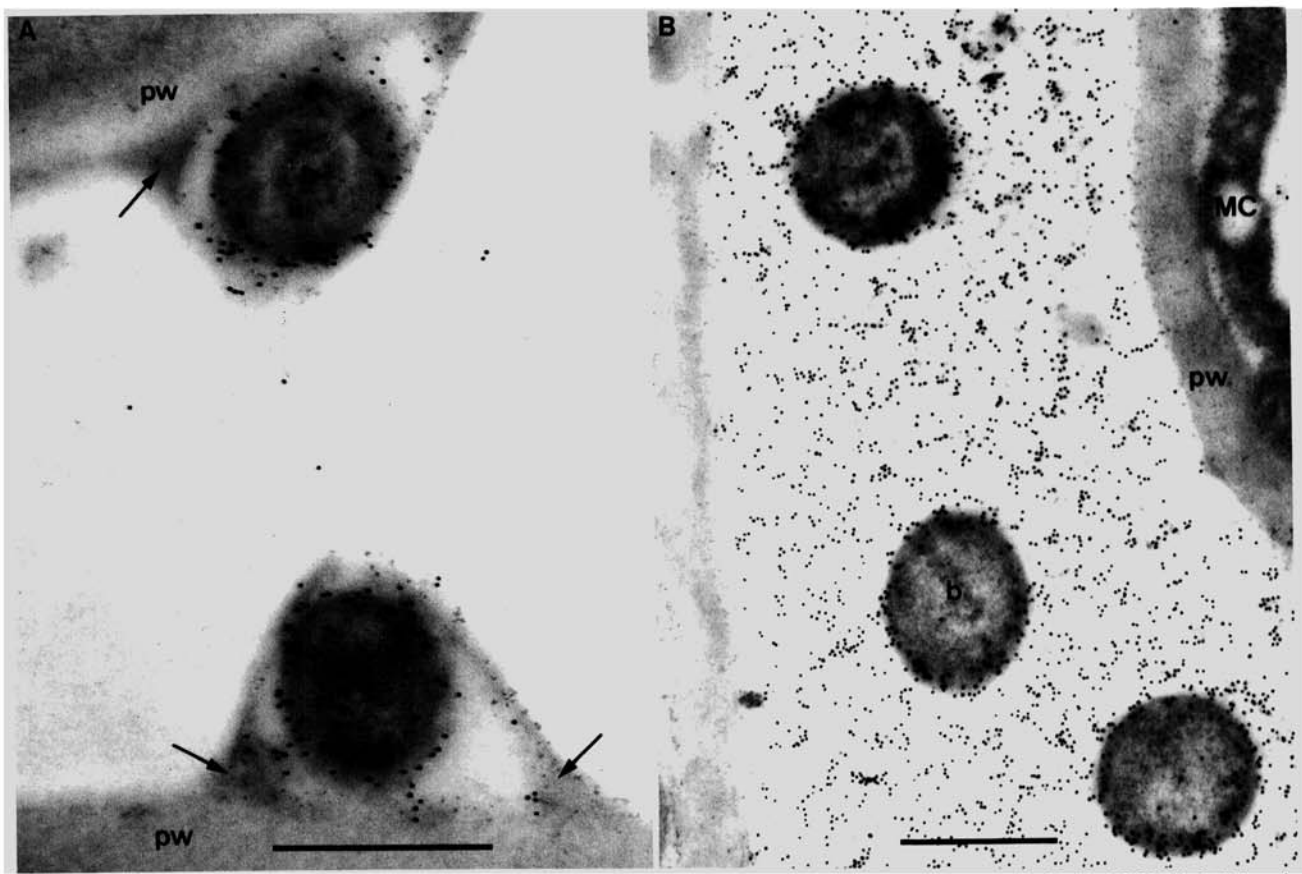


**Fig. 2.** Immunogold labeling with XV1 serum locates bacterial products. **A,** Two cells of *X. c.* pv. *vesicatoria* 85-10 (pL3XVI-6) 2 hr after inoculation of the resistant pepper cv. ECW-30R. The bacteria (b) have been deposited opposite each other, probably following evaporation of the same inoculum droplet. Note that the plant cell wall (pw) and amorphous encapsulating material (arrows) remain unlabeled. Bar = 0.5 μm. **B,** Part of a large colony of *X. c.* pv. *vesicatoria* 85-10 in ECW-30R, 30 hr after inoculation. Gold label decorates the bacterial cells and fibrils of EPS (note the linear array of particles). Bar = 0.5 μm.

$6.62 \pm 0.86$, and $6.9 \pm 0.87$, respectively. In a few transverse sections of bacteria, no label was observed. Quantitative analysis of the frequency of occurrence of gold label at different sites in and around bacterial cells confirmed that AvrBs3 was predominantly intracellular (Fig. 6). The numbers of gold particles found outside *X. c.* pv. *vesicatoria* cells expressing *avrBs3* were not significantly greater than the non-specific labeling observed around cells of *X. c.*

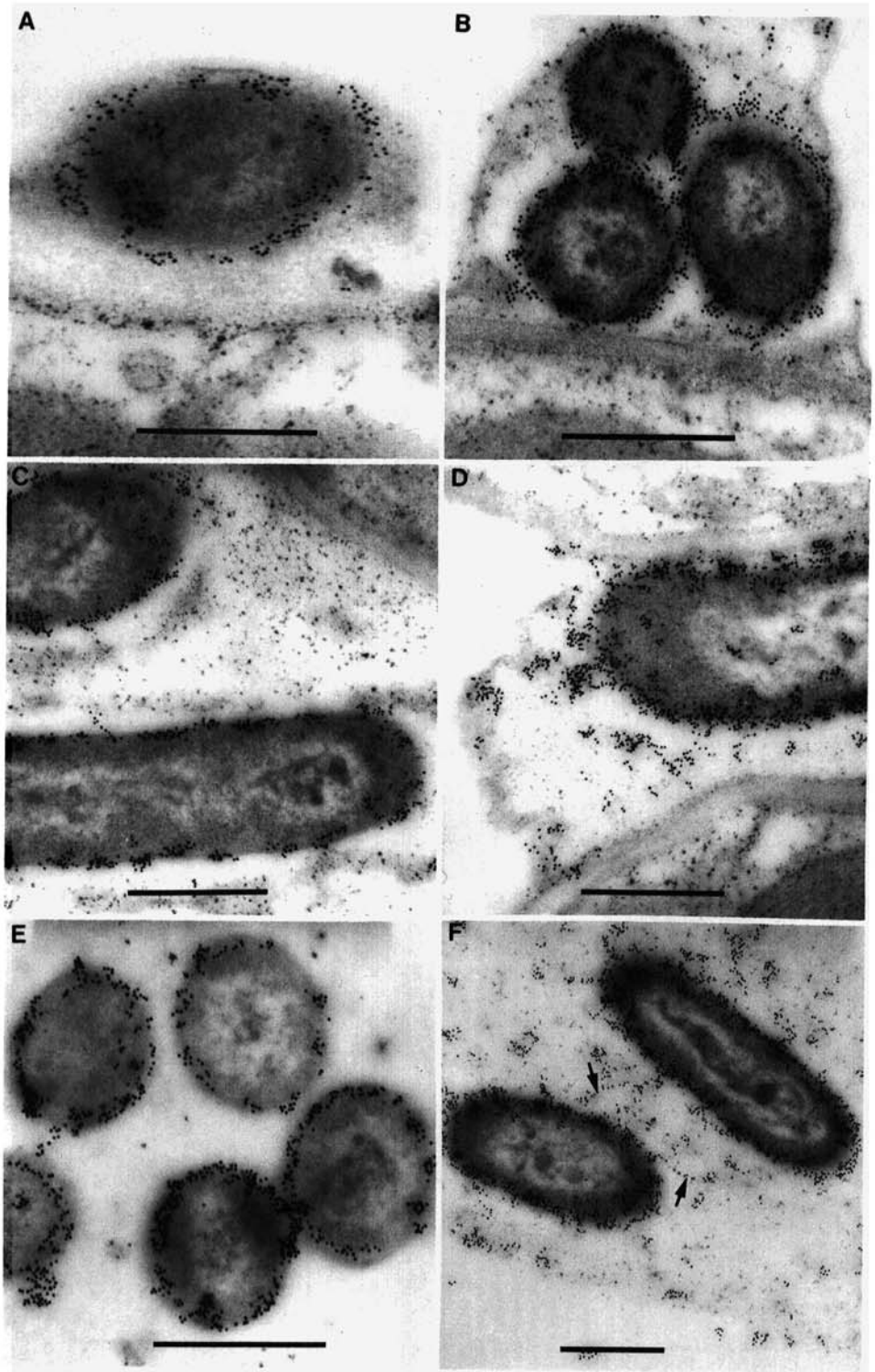


**Fig. 3.** Immunogold labeling with monoclonal antibodies to xanthan. **A, C, E,** *X. c.* pv. *vesicatoria* 85-10 in cv. ECW 2, 20, and 48 hr, respectively, after inoculation, labeled with monoclonal D1. **B, D, F,** *X. c.* pv. *vesicatoria* 85-10 (pL3XV1-6) in ECW-30R 2, 20 and 48 hr, respectively, after inoculation, labeled with monoclonal A6. Similar patterns of labeling were observed at each time after inoculation. Epitopes recognized by A6 include fibrils of EPS characterized by linear arrays of gold particles (arrowed) within the extracellular matrix. Bars = 0.5 $\mu$m.

pv. *vesicatoria* 85-10 or 85-10 with the vector pDSK602 alone. In some tangential and oblique sections (not used in the quantitative analysis), comparatively large areas of the bacterial cell surface and membrane were exposed but remained unlabeled with antiserum to AvrBs3 whether cells were grown in ECW or ECW-30R plants, or *in vitro*.

**Plant responses.**

The first clear response during compatible or incompatible interactions was the localized minor convolution of the plasma membrane adjacent to *X. c.* pv. *vesicatoria* cells in contact with the mesophyll cell wall 2 hr after inoculation (Figs. 1A and B). Localized accumulation of amorphous, paramural papillae had occurred after 8 hr in cells next to a few colonies of all strains (Fig. 7A) but the response was uncommon. Differences between compatible and incompatible interactions were not clearly observed until 20 hr after inoculation. Regions of cytoplasm in mesophyll cells of pepper cv. ECW-30R next to the transconjugants expressing *avrBs3,* that is 85-10 (pL3XV1-6) and 85-10 (pD36), became vesiculated, indicative of localized dysfunction of the plasma membrane which was often highly convoluted (Fig. 7B). This response was more frequent next to bacteria overexpressing *avrBs3* in pD36. The localization of this early stage of the HR appeared to be followed by general decompartmentation and cytoplasmic collapse (compare Fig. 7B, C) which was observed in all cells in tissue undergoing resistant reactions 48 hr after inoculation. A macroscopic HR developed in cv. ECW-30R inoculated with 85-10 (pD36) about 6 hr before the response was apparent following inoculation with 85-10 (pL3XV1-6). No cell collapse was observed during compatible interactions (all strains of *X. c.* pv. *vesicatoria* in cv. ECW, and 85-10, and 85-10 [pDSK602] in ECW-30R) at the last sampling time (48 hr).

## DISCUSSION

The accumulation of a fibrillar matrix around cells of *X. c.* pv. *campestris* and *X. c.* pv. *malvacearum* growing in their hosts cabbage and cotton, respectively, was described in previous ultrastructural studies (Cason *et al.* 1978; Al-Mousawi *et al.* 1982; Bretschneider *et al.* 1989). However, in this earlier work the identity and origin of the fibrils were unconfirmed. Our immunocytochemical experiments have shown that the apparently similar fibrillar matrix accumulating around cells of *X. c.* pv. *vesicatoria* during compatible and incompatible interactions

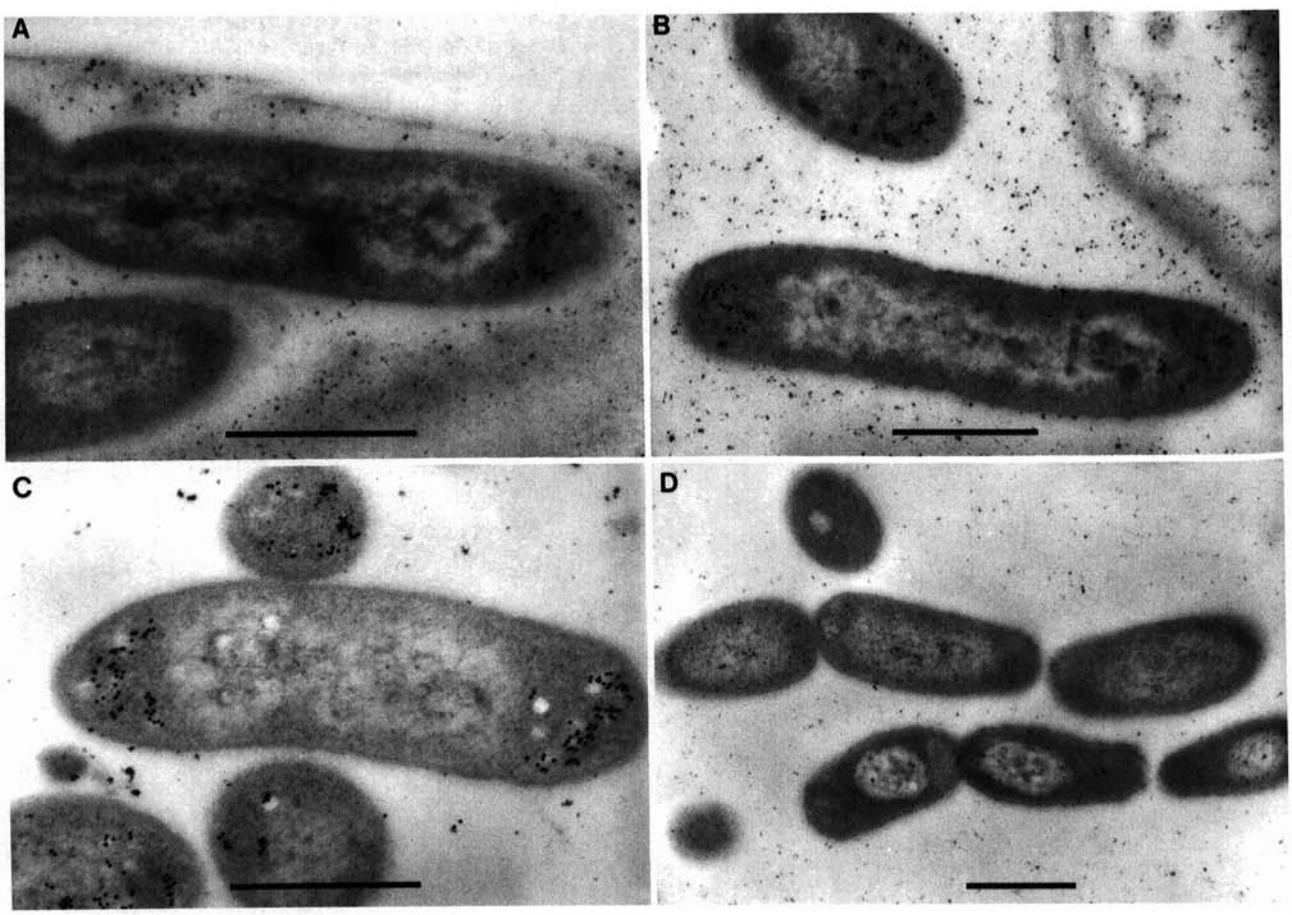


**Fig. 4.** Immunogold labeling with monoclonal antibody B3 which has high affinity for the pyruvylated terminal mannose of the fully acetylated xanthan side chain. Bars = 0.5 μm. **A, C,** Compatible interaction of *X. c.* pv. *vesicatoria* 85-10 in cv. ECW, 2 and 48 hr after inoculation and, **B, D,** incompatible interaction of *X. c.* pv. *vesicatoria* 85-10 (pL3XVI-6) in cv. ECW-30R, 2 and 48 hr after inoculation, respectively. Note the intracellular and polar location of label in bacterial cells (in A, B, and C) and the absence of label from bacteria in tissue which has undergone the HR (D).

in pepper is of bacterial origin and contains xanthan, the main component of EPS in xanthomonads (Coplin and Cook 1990).

The rapid synthesis of fibrillar EPS during the first hours after inoculation separated bacterial cells from each other and from the plant cell wall. The patterns of immunogold labeling to monoclonal antibodies A6 and D1, with the greatest density of reactive epitopes at the cell surface, were as expected from the known mechanism of biosynthesis of xanthan. In the proposed pathway, the pentameric repeating unit is assembled on an isoprenoid lipid carrier and acetylated and pyruvylated before polymerization. The displaced carrier lipid is recycled through a dephosphorylation step and the polymer extruded from the cell into the surrounding medium (Hassler and Doherty 1990; Ielpi *et al.* 1981; Jansson *et al.* 1975; Ramirez *et al.* 1988; Vanderslice *et al.* 1989). The intracellular location of epitopes reacting with monoclonal B3 was unexpected. Haaheim *et al.* (1989) found that B3 has specificity for the pyruvylated terminal mannose of the xanthan side chain. The polar location of B3 label may therefore represent sites of accumulation of the pyruvylated pentasaccharide repeating unit prior to polymerization which may lead to masking of the reactive epitope in *X. c.* pv. *vesicatoria*. The absence of labeling by monoclonal B3 during the later stages of the incompatible interaction suggests cessation of EPS biosynthesis. This may be part of the general inhibition of bacterial metabolism as a consequence of the HR or may be a more specific effect associated with regulation of EPS biosynthesis. Despite the absence of B3 labeling, a few cells of 85-10 (pL3XVI-6) appeared still to be dividing 48 hr after inoculation into the resistant pepper cv. ECW-30R (see Figs. 4D and 7C).

Our observations suggest that EPS has a major structural role in colony development and would be expected to modify the environment to which cells of *X. c.* pv. *vesicatoria* are exposed within the intercellular space. Genetic analyses have demonstrated a role for EPS in the pathogenicity of *X. c.* pv. *campestris* (Barrère *et al.* 1986; Ramirez *et al.* 1988) and other phytopathogenic bacteria for example, *Pseudomonas solanacearum* (Denny and Baek 1991), *Erwinia amylovora* (Steinberger and Beer 1988), and *Erwinia stewartii* (Coplin and Majerczak 1990). Further work is needed to determine the significance of EPS in the interaction between *X. c.* pv. *vesicatoria* and pepper.

Overexpression of the AvrBs3 protein allowed its detection by immunogold labeling. The AvrBs3-specific antiserum was raised to a fusion protein containing the N terminus and repeat region of AvrBs3 (Knoop *et al.* 1991). The localization found by immunocytochemistry confirms the results obtained by cell fractionation studies (Knoop *et al.* 1991). The intracellular location of AvrBs3 suggests that the molecule itself is not recognized by plant cells. It is, however, possible that the levels of extracellular AvrBs3 or peptides derived from the protein may have been too low for detection by the antiserum available. It is significant that similar localization was found *in vitro*

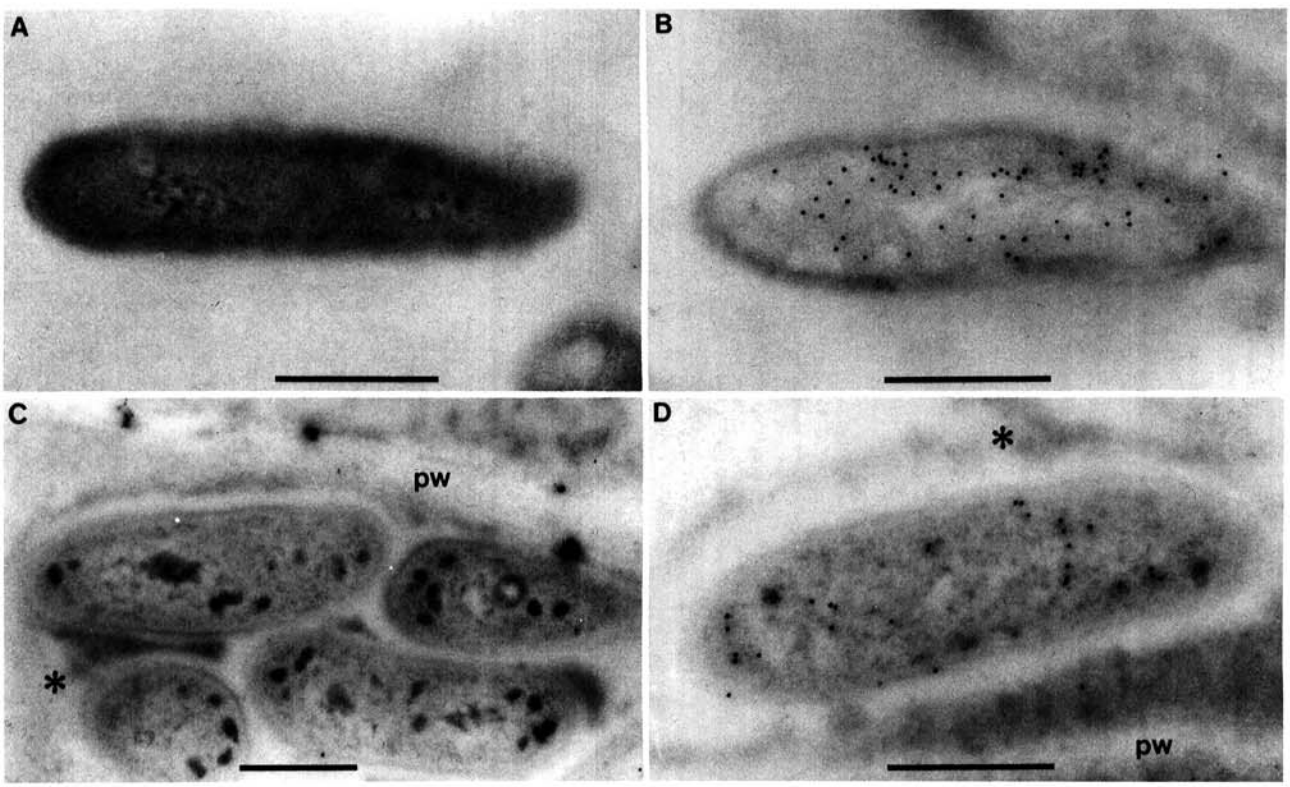


**Fig. 5.** Immunogold labeling locates the AvrBs3 protein. **A, B,** Cells of *X. c.* pv. *vesicatoria* 85-10 (pDSK602) and *X. c.* pv. *vesicatoria* 85-10 (pD36), respectively, grown *in vitro*. **C, D,** Cells of *X. c.* pv. *vesicatoria* 85-10 (pDSK602) and *X. c.* pv. *vesicatoria* 85-10 (pD36), respectively, in the intercellular spaces of leaves of cv. ECW 1 day after inoculation. Label has accumulated only in cells expressing *avrBs3*. The asterisk indicates EPS next to the plant cell wall (pw). Bar = 0.5 μm.

following growth in a complex medium and in the plant. Function of *avrBs3* has been found to be dependent on an intact *hrp* region in *X. c.* pv. *vesicatoria* (Knoop *et al.* 1991). The *hrp* genes are expressed in the plant but not in complex media (Schulte and Bonas 1992). Our results show, therefore, that activation of the *hrp* genes does not appear to allow major relocation of the AvrBs3 protein.

Our observations on *X. c.* pv. *vesicatoria* in pepper leaves show that during the first 4 hr after inoculation bacterial cells probably experience drastic changes in environment. Three distinctly different conditions are experienced: 1, within an aqueous droplet in the intercellular space; 2, in a concentrated suspension of cell wall debris as the water evaporates; and 3, in close contact with the plant cell wall, which is a source of freely available nutrients (Farrar and Lewis 1989; Hancock and Huisman 1981). Within the first 2 hr after inoculation EPS begins to accumulate around bacteria which have therefore begun to modify their own environment. It is intriguing to consider how these conditions might influence expression of *hrp* genes which are known to be regulated by many environmental factors (Arlat *et al.* 1992; Kamoun and Kado 1990; Rahme *et al.* 1991; Schulte and Bonas 1992). The application of immunocytochemical techniques to detect Hrp proteins in bacteria at different sites within the intercellular space and within the developing colony should provide further insight into the extent to which microenvironment might regulate the potential for *X. c.* pv. *vesicatoria* to interact with the plant.

The exchange of molecules between the plant and bacteria is soon buffered by a coat of EPS. Unless elicitors of the HR are able to diffuse through the matrix of EPS, the initial close contact may be critical for signal exchange. The importance of cell-to-cell contact was proposed by Stall and Cook (1979). Although contact may be important during the critical early stages of infection, we found no evidence for differential binding or attachment to plant cell walls during the incompatible interaction. Our findings contrast, therefore, with results obtained by Cason *et al.* (1978) who found that cells of *X. c.* pv. *malvacearum* were encapsulated and immobilized in fibrillar material thought to be of plant origin on the cell walls of a resistant cotton cultivar.

Exchange of signals between *X. c.* pv. *vesicatoria* and plant cells during both the incompatible and compatible interactions was indicated by nonspecific, localized, papilla deposition. The papilla response in pepper was much less pronounced than in bean cells challenged by isolates of *Pseudomonas syringae* pv. *phaseolicola* (Brown and Mansfield 1988, 1991). The collapse of pepper cells during the HR followed localized vesiculation of the cytoplasm, which was associated with marked convolution of the plasma membrane next to the bacterial colony. This reaction indicates that early processes that lead to irreversible membrane damage may initially be localized to the site of first exposure to elicitors produced by adjacent bacteria. The cause of membrane damage may be an active response by the plant cell leading to localized generation of free radicals and subsequent lipid peroxidation (Ádám *et al.* 1989; Croft *et al.* 1990). It is possible that such a localized primary response involves activation of key enzymes rather than regulation at the level of transcription.

## MATERIALS AND METHODS

### Plants and bacteria.

Pepper cultivars Early Cal Wonder (ECW) and ECW-30R, which are near-isogenic apart from the absence or presence, respectively, of the *Bs3* allele, were grown to the six- to eight-leaf stage, and leaves were inoculated with bacterial suspension by injection as previously described (Bonas *et al.* 1989; Minsavage *et al.* 1990). Strains of *X. c.* pv. *vesicatoria* used were the race 2 strain 85-10 (which

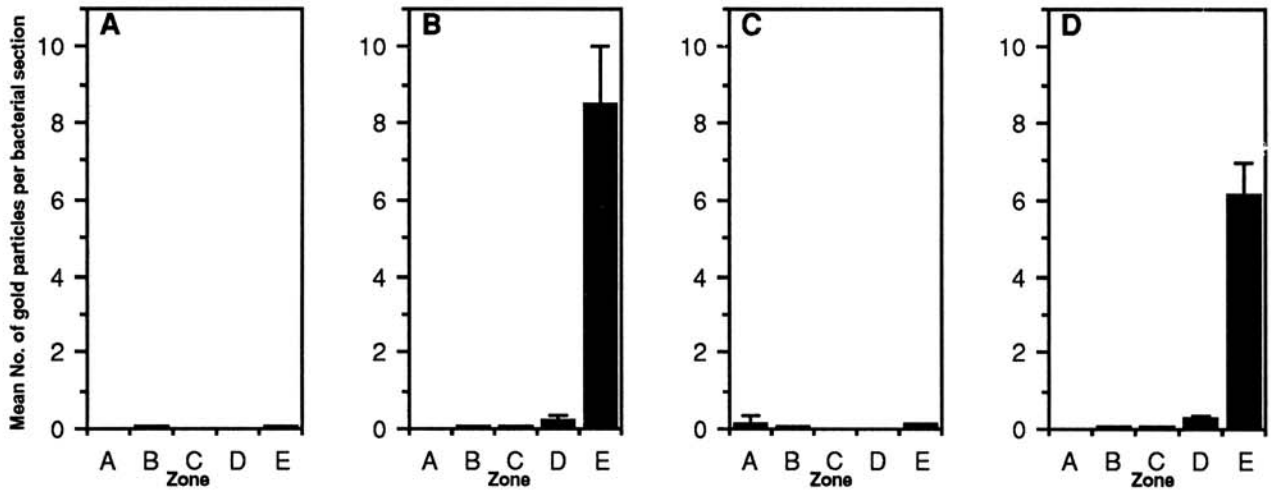


**Fig. 6.** Quantitative analysis of the distribution of immunogold label within cells of **A,** *X. c.* pv. *vesicatoria* 85-10 (pDSK602); **B,** *X. c.* pv. *vesicatoria* 85-10 (pD36) grown *in vitro* and **C,** *X. c.* pv. *vesicatoria* 85-10 (pDSK602) and **D,** *X. c.* pv. *vesicatoria* 85-10 (pD36) 1 day after inoculation into leaves of cv. ECW. Sections were labeled with antiserum to AvrBs3. Zones refer to locations; A, outside the bacterium; B, outside the bacterium associated with the outer membrane; C, associated with the periplasmic space; D, associated with the inner surface of the cell membrane and E, intracellular, away from the membrane. Mean numbers of gold particles recorded at each site in 50 medial sections of separate bacteria are given (bars = SEM).

lacks *avrBs3*) and transconjugants of 85-10 harboring pL3XV1-6, a pLAFR3-based clone containing *avrBs3* regulated by its own promoter (Bonas *et al.* 1989), and pD36. Plasmid pD36 contains the coding region of *avrBs3* cloned into pDSK602 resulting in overexpression of the AvrBs3 protein (J.C-S. and U.B., unpublished). The plasmid pDSK602 was kindly provided by Noel Keen, University of California, Riverside. Bacteria were prepared for inoculation as described by Bonas *et al.* (1989) and washed three times in 1 mM $MgCl_2$ to remove nutrients and EPS before resuspension at $5 \times 10^8$ cells per milliliter. Following injection, plants were maintained at 28° C day/22° C night under a 16-hrphotoperiod, with relative humidity not less than 80%. Tissues of each cultivar were examined 2, 4, 8, 20, 30, and 48 hr after inoculation with *X. c.* pv. *vesicatoria* 85-10 and 85-10(pL3XV1-6) and 4, 20, and 24 hr after inoculation with 85-10 (pD36) and 85-10 (pDSK602). The interactions examined are summarized in Table 1. Inoculation sites in leaves from two plants were examined at each time point with each interaction.

**Antibodies.**

Polyclonal antiserum XV1 was raised in rabbits injected with whole cells of *X. c.* pv. *vesicatoria* strain 82-8 recovered from intercellular fluids isolated from infected leaves of the susceptible cv. ECW 72 hr after inoculation according to the method of Klement (1965). The serum has affinity for commercial xanthan (J. C-S. and U.B., unpublished).

Monoclonal antibodies A6, B3, and D1 were obtained as mouse ascitic fluids from Lars R. Haaheim, University of Bergen (Haaheim *et al.* 1989). The antibodies were raised to purified xanthan from *X. c.* pv. *campestris*; A6 and

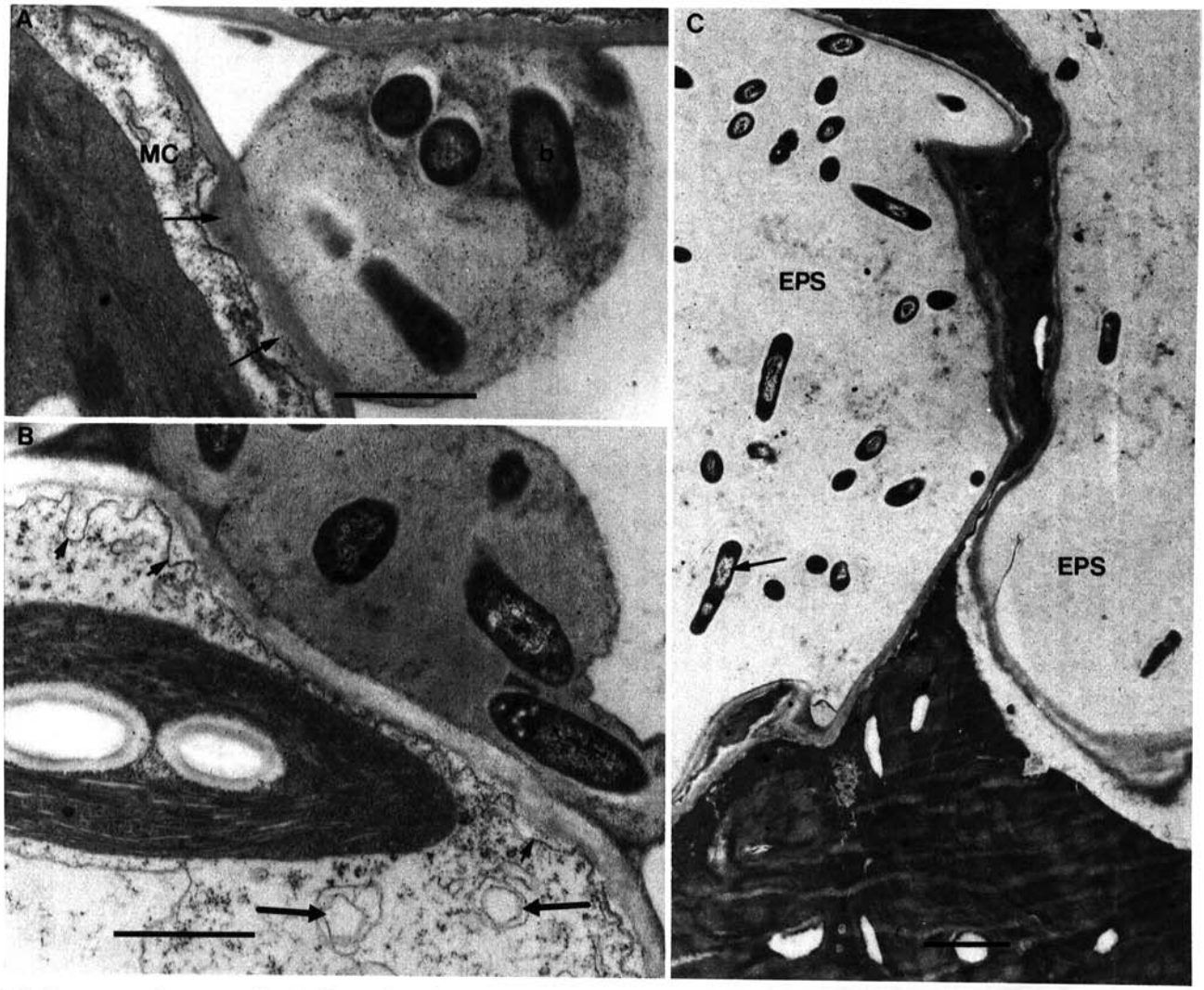


**Fig 7.** Responses of pepper cells. **A,** Formation of paramural papillae (arrowed) within a mesophyll cell (MC) in contact with a colony of *X. c.* pv. *vesicatoria* 85-10 in cv. ECW-30R. Bacterial cells (b) are embedded in EPS 8 hr after inoculation. Bar = 1 μm. **B,** Localized membrane convolution (small arrows) and vesiculation of the cytoplasm (large arrows) indicative of the onset of irreversible membrane damage during the HR in cv. ECW-30R, 20 hr after inoculation with *X. c.* pv. *vesicatoria* 85-10 (pL3XV1-6); bar = 1 μm. **C,** Final stage of the HR in ECW-30R, 48 hr after inoculation with *X. c.* pv. *vesicatoria* 85-10 (pL3XV1-6). The mesophyll cell has collapsed, its cytoplasm autolyzed and electron dense. Surrounding bacteria remain dispersed in EPS and are dividing (arrow) but show some vesiculation indicative of abnormal development; bar = 2 μm.

D1 were reported to be directed against the nonsubstituted trisaccharide side chain, whereas B3 required the fully acetylated side chain with the pyruvylated terminal mannose as the immunodominant part.

The AvrBs3-specific polyclonal antiserum is directed against a major part of the protein including the N terminal and repeat regions. The serum was affinity purified as described by Knoop *et al.* (1991).

**Electron microscopy and immunogold labeling.**

Pieces of inoculated leaf tissue (about 1.5 $mm^2$) were fixed in 2.5% glutaraldehyde in 45 mM sodium cacodylate buffer (CAB), pH 7.2, for 16 hr at 4° C. Vacuum infiltration of fixatives was not used because this displaced bacterial colonies from microsites in leaves. For conventional staining and immunogold localization of EPS, samples were then washed in CAB and postfixed for 1 hr in 1% osmium tetroxide in CAB. After washing in water, the samples were divided; half were dehydrated in ethanol and embedded in LR white resin (London Resin Co.) medium grade for immunocytochemistry and the remainder dehydrated in acetone and embedded in Epon-Araldite. For labeling of AvrBs3, within inoculated leaves or bacteria grown overnight in NYG broth (Daniels *et al.* 1984), postfixation with osmium tetroxide was omitted and samples were embedded in LR white resin. Polymerized blocks were sectioned on a Reichert UltraCut-E microtome using a diamond knife and sections (70–100 nm) were mounted on 300 mesh copper or gold grids. Epon-Araldite sections were stained in uranyl acetate and lead citrate (Brown and Mansfield 1988).

For immunogold localization all solutions were prepared in 20 mM Tris-buffered saline (pH 7.4) containing 0.1% bovine serum albumin (BSA) and 0.05% Tween 20 (TBST). After blocking in 2% BSA for 0.5 hr at room temperature, sections on uncoated gold grids were transferred to a 20-$\mu$l droplet of the primary antibody and were incubated for 16 hr at 4° C. The rabbit polyclonal antibody raised to whole bacteria (XV1) was diluted 1:500, the AvrBs3 antiserum diluted 1:50, and mouse monoclonal ascites (A6, B3, and D1) were diluted 1:2,000. After washing in a stream of TBST from a wash bottle, grids were incubated in the appropriate second antibody, either goat anti-rabbit IgG 10 nm gold (Amersham) or goat anti-mouse IgM 10 nm gold (Sigma) diluted 1:25 for 30 min at room temperature. After washing in TBST followed by distilled water, grids were dried and examined without further staining. All sections were viewed in an Hitachi H-7000 transmission electron microscope with an accelerating voltage of 75 kV.

**Table 1.** Interactions between cultivars of pepper and strains of *Xanthomonas campestris* pv. *vesicatoria* examined by electron microscopy

| | Cultivar of pepper | |
|---|---|---|
| Strain of *Xcv*[a] | ECW | ECW-30R |
| 85-10 | C[b] | C |
| 85-10(pL3XV1-6) | C | I[c] |
| 85-10(pDSK602) | C | C |
| 85-10(pD36) | C | I |

[a] For details of plasmids see Materials and Methods; pL3XVI-6 and pD36 contain the *avrBs3* gene, pDSK602 is the vector used in pD36.
[b] Compatible, water-soaked lesions produced.
[c] Incompatible interaction, HR in which tissue collapse starts to occur by 30 hr after inoculation with 85-10(pD36) and by 36 hr with 85-10(pL3XV1-6).


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