Protein(s) from the Gram-Positive Bacterium *Clavibacter michiganensis* subsp. *michiganensis* Induces a Hypersensitive Response in Plants

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ABSTRACT

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The gram-positive tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis* induced a local necrotic response on four-o'clock (*Mirabilis jalapa*) and tobacco (*Nicotiana tabacum*) plants. This necrosis response was characteristic of the hypersensitive response (HR). The cell-free cul-

ture supernatant from strain CMM623 also induced a necrosis that was phenotypically similar to that induced by the bacteria. Inhibitors of plant metabolism suppressed the necrotic reaction of both *M. jalapa* and tobacco. The HR-inducing activity present in the supernatant was heat stable, sensitive to proteases, and had an apparent molecular mass in the range of 35 to 50 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The properties observed for the necrosis-inducing activity resembled harpin and PopA described from gram-negative phytopathogenic bacteria.

Gene-for-gene interactions are a widely occurring phenomenon in which single dominant alleles in plants and their pathogens determine whether the plant will be resistant or susceptible to attack (17). The major hypothesis to explain resistance proposes that a receptor present in the plant (encoded by *R* genes) specifically recognizes a molecule called an elicitor, which is produced by an *avr* gene in the pathogen (29,36,41). The resistant response is characterized by a hypersensitive response (HR) that is characterized by necrosis resulting from localized plant cell death at the site of infection (13,21,29).

The elicitors of gram-negative bacteria are produced directly or indirectly by the *avr* gene product. An elicitor produced indirectly by an *avr* gene product is represented by the low molecular weight gamma-lactones called syringolides from *Pseudomonas syringae* pv. *tomato* (35). A race-specific elicitor produced directly by an *avr* gene product is represented by AvrBs3 from *Xanthomonas campestris* pv. *vesicatora* (22,38) and AvrPto from *P. syringae* pv. *tomato* (34,37). Avr proteins seem to be recognized by the *R* gene product only inside the plant cell (8,32).

In addition to avirulence genes, bacteria require *hrp* (hypersensitivity response and pathogenicity) genes to induce a HR in nonhost or resistant cultivars (7,23). *hrp* gene products are also necessary to cause disease in compatible hosts (7). Most Hrp proteins appear to be involved in the assembly of a type III protein secretion pathway (6,15,39) through which bacterial virulence factors, including *avr* gene products and harpins (described below), traverse to the extracellular milieu or are injected into host cells (26,34,37,38,40). One of the *hrp* genes encodes a proteinaceous HR elicitor named harpin, which has been found in *P. syringae* (26), *Erwinia amylovora* (40), *E. chrysanthemi* (3), and recently in *E. carotovora* (11). Purified harpins are biologically active and, by themselves, can trigger a HR on a variety of different nonhost plants (3,40). This

plant defense response does not follow the gene-for-gene interaction (24,41). Another bacterial protein elicitor of the HR with some structural characteristics of harpins, named PopA, has been isolated from cell-free culture supernatants (CFCS) of *Ralstonia* (formerly *Pseudomonas*) *solanacearum* (1).

Although HR induction is widespread in phytopathogenic gram-negative bacteria, it has been reported only in two cases for gram-positive bacteria. In 1990, Gitaitis (20) described an apparent HR on four-o'clock (*Mirabilis jalapa*) and tobacco when the intercellular spaces of leaf panels were infiltrated with the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis*. Later, it was reported that the exopolysaccharide produced by this bacterium is not responsible for the necrosis induced on *M. jalapa* (4), suggesting that other molecules, which might include proteins, are involved in this effect. In the second case, Nissinen et al. (31) recently described a proteinaceous elicitor from CFCS of the potato pathogen *C. michiganensis* subsp. *sepedonicus*. This elicitor shares some harpin characteristics and induces HR only on tobacco.

In the current work, we described a proteinaceous HR-eliciting activity from the gram-positive phytopathogen *C. michiganensis* subsp. *michiganensis*, which elicits a HR on *M. jalapa* and tobacco plants.

MATERIALS AND METHODS

Bacterial strains and culture media. *C. michiganensis* subsp. *michiganensis* strain CMM623 was obtained from X. Besoain, Universidad Católica de Valparaíso (this strain was isolated from Quillota, Chile) and CMM100, which is a plasmid-free derivative of NCPPB382 (30), was a gift from R. Eichenlaub, Universität Bielefeld, Germany. Strains were routinely grown at 26°C in M523 medium, which contains per liter 10 g of sucrose, 8 g of casein hydrolysate, 4 g of yeast extract, 2 g of K₂HPO₄, and 0.3 g of MgSO₄·7H₂O. The minimal medium used was a modification of the semiselective medium for *C. michiganensis* (SCM) (14) and contains per liter 0.25 g of MgSO₄·7H₂O, 0.5 g of KH₂PO₄, 2 g of K₂HPO₄, 10 g of sucrose, and 0.1 g of yeast extract.

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Preparation of CFCS. Bacteria were grown for 48 h in yeast extract glucose MgSO_4 (YGM) (12) or M523 medium. After centrifugation at $8,000 \times g$ for 15 min at 4°C , the culture supernatant was filtered through a $0.45\text{-}\mu\text{m}$ ME25 membrane filter (Schleicher & Schuell Inc., Keene, NH). Sterility of CFCS preparations was confirmed by culturing. After the addition of phenylmethylsulfonyl fluoride (PMSF) (0.5 mM final concentration), this preparation was immediately used either for infiltration assays on *M. jalapa* and tobacco plant leaves or for purification and characterization of the elicitor.

For preparation of the bacteria to be used in infiltration assays, overnight M523 cultures were washed, suspended in sterile water, and adjusted to appropriate cell concentrations by measuring optical density at 600 nm .

Plant growth and inoculation procedures. *M. jalapa* plants were grown in soil from seed under greenhouse conditions with temperatures between 15 to 35°C for 2 to 3 weeks (two-leaf stage). Twenty-four hours before inoculation of bacteria or supernatants, plants were transferred to a growth chamber with a 16-h light/8-h dark photoperiod at 25°C and a light intensity of $100\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$. Tobacco plants (*Nicotiana tabacum* L. var. Xanthi) were grown under similar conditions, and prior to inoculation, they were moved to a chamber at 20°C .

Plants were infiltrated with *C. michiganensis* subsp. *michiganensis* or CFCS with a 1-ml needleless syringe through a small hole made in the plant leaf with a surgical blade. Inoculated *M. jalapa* or tobacco were moved back to the growth chamber under the same conditions prior to inoculation and visually monitored for reactions.

Active plant metabolic inhibitors were infiltrated as described in He et al. (25). Briefly, cycloheximide, α -amanitin, sodium vanadate, and lanthanum chloride were used at final concentrations of $70\text{ }\mu\text{M}$, $200\text{ }\mu\text{M}$, $50\text{ }\mu\text{M}$, and 1 mM , respectively.

Heat and protease treatment of CFCS. CFCS contained in Eppendorf tubes were heated to 100°C in a boiling water bath for 10 min.

The chemical nature of the necrosis-inducing activity was investigated by treatment of CFCS with different proteases. Pronase (Sigma Chemical Co., St. Louis) and proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, IN) were used at concentrations of 1 mg/ml and $350\text{ }\mu\text{g/ml}$, respectively. Both reactions were incubated at 37°C for 30 min, and the protease was then inactivated either by boiling the sample in a water bath for 5 min or by adding PMSF (1 mM final concentration).

To determine whether *C. michiganensis* subsp. *michiganensis* secretes pectinases into the medium, we followed the method described by He et al. (27). Briefly, $5\text{ }\mu\text{l}$ of culture was placed on the surface of A1 medium agar (2) that was supplemented with sodium polygalacturonate (Sigma Chemical Co.) at 0.4% final concentration. After 24 h at 28°C , plates were flooded with a 1% cetyltrimethylammonium bromide solution. The formation of a clear area around the colony indicated the secretion of pectinases.

Partial purification of the HR-inducing activity. CFCS were prepared from CMM623 grown in YGM, and they were fractionated on a DEAE cellulose column (Whatman International, Ltd., Maidstone, England). This column was previously equilibrated with BT buffer (20 mM Bis-Tris, $\text{pH } 6.0$) (31). After the column had been washed with the same buffer, proteins were eluted with a NaCl step gradient (100 , 200 , 300 , 500 , and $1,000\text{ mM}$ in BT buffer). Fractions were dialyzed against 50 mM phosphate buffer, $\text{pH } 7.0$, and used in plant reaction assays. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard. CFCS proteins or the eluted fractions from the DEAE cellulose column were concentrated by trichloroacetic acid (TCA) precipitation (28) and separated by discontinuous 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was silver-stained (according to the Millipore In-

vestigator TM 2-D electrophoresis system manual; Millipore Corp., Bedford, MA). A preparative gel containing CFCS proteins was cut into 0.5-cm slices and the polypeptides were eluted, renatured (10), and used in HR assays.

To investigate the native molecular mass of the HR-inducing activity, CFCS were concentrated with a Centricon filter (Amicon, Beverly, MA) having a cut-off size of 3 kDa , and both the concentrate and pass-through material were used in infiltration assays. Also, CFCS were loaded onto a Sephacryl S-300 (Sigma Chemical Co.) gel filtration column ($1.5 \times 50\text{ cm}$) previously equilibrated with 20 mM Tris-HCl, $\text{pH } 8.0$; 10 mM β -mercaptoethanol; and 1 mM EDTA. One-milliliter fractions were collected and used immediately in infiltration assays.

RESULTS

Bacterial culture supernatants induce a HR. *C. michiganensis* subsp. *michiganensis* produced an apparent HR on *M. jalapa* when suspensions containing 5×10^8 colony forming units (CFU) per ml were infiltrated into the intercellular spaces of the leaves (Fig. 1A). Lower concentrations of bacteria, such as 10^7 CFU/ml, did not induce a visible necrosis in the plant after 6 days post-inoculation. The distribution of the bacteria in planta remained limited to the infiltrated area, i.e., bacteria were not found after

Fig. 1. Leaves of *Mirabilis jalapa* showing responses to bacteria and cell-free culture supernatants (CFCS). Leaves were infiltrated as described in Materials and Methods and photographed after 48 h. **A**, 1 = strain CMM623 at 5×10^8 CFU/ml, 2 = CFCS from CMM623 cells grown in M523 medium, 3 = strain CMM100 at 5×10^8 CFU/ml, 4 = CFCS from CMM100 cells grown in M523 medium, and 5 = M523 medium. **B**, 1 = CFCS from strain CMM623, 2 = heat-treated CFCS from strain CMM623, and 3 = proteinase K-treated CFCS from strain CMM623.

culturing stem sections of inoculated leaves. To determine whether *C. michiganensis* subsp. *michiganensis* produces a HR elicitor that is released outside the bacteria, we inoculated CFCS into *M. jalapa*. Figure 1A shows that CFCS induced a collapse of the infiltrated area that was similar to the response induced by bacteria. The tissue collapse was detected within 18 h after infiltration followed by a papery appearance after 3 to 4 days. The plasmid-free strain CMM100, as well as its CFCS, were also able to induce necrosis on *M. jalapa* leaves (Fig. 1A), showing that the CFCS-inducing activity is determined by chromosomal genes. When inhibitors of active plant metabolism (cycloheximide, α -amanitin, lanthanum chloride, and sodium vanadate) were infiltrated together with CFCS or bacteria, necrosis induction was prevented (data not shown). Inhibition of the HR in *M. jalapa* induced by bacteria or the HR in tobacco induced either by CFCS or bacteria was possible only when we used 5 mM lanthanum chloride and 0.5 mM sodium vanadate. These concentrations are fivefold and 10-fold higher, respectively, than those used by He et al. (25). Culture medium (M523 or YGM) did not induce any reaction in the plant (Fig. 1A). Similar results to those described here for *M. jalapa* were observed when bacteria and CFCS were infiltrated into tobacco leaves (data not shown).

Supernatant from a bacterial culture grown in minimal medium was also able to induce a HR on *M. jalapa* (data not shown).

The responsiveness of different plants to CFCS correlates with their response to live bacteria. Strain CMM623 and culture supernatants from the same bacterium were tested for biological activity by infiltration into the intercellular spaces of leaves in several different plant species. Among nine species of plants, strain CMM623 and its culture supernatants induced a HR only on *M. jalapa* and tobacco (Table 1). Therefore, there is agreement between the ability of the CFCS to induce a HR and the HR induced by strain CMM623 on the plants assayed. This result suggests that the elicitor activity found in the culture supernatant could be similar to, or the same as, the one produced by the bacteria in planta. More importantly, it suggests that the elicitor activity is not phytotoxic, but rather it provokes a specific response in the plant. It should be noted that CMM623 did not induce a HR on its natural host tomato, but instead it caused bacterial canker symptoms (data not shown). CFCS from *Escherichia coli* HB101 did not cause a reaction.

Characterization of the HR-inducing activity from CFCS. We have shown that CFCS prepared from strain CMM623 grown in minimal or M523 media induced a necrosis response on *M. jalapa* and tobacco. To investigate the chemical nature of the elicitor, different treatments of the CFCS were performed. Elicitor activity was active on *M. jalapa* after 10 min of heat treatment (100°C) (Fig. 1B), although necrosis was induced only 40 h after infiltration and occasionally after 3 to 4 days. However, CFCS lost all HR-eliciting activity on *M. jalapa* when, prior to infiltration, they were digested with protease K (Fig. 1B) or Pronase for 30 min at 37°C. Similar results were found when treated CFCS were assayed on tobacco (data not shown).

TABLE 1. Plant specificity of hypersensitive response induced by cell-free culture supernatants (CFCS) from strain CMM623

Inoculated plant	Necrosis reaction ^a	
	CMM623 cells (5×10^8 CFU/ml)	CFCS from CMM623
<i>Mirabilis jalapa</i> (four-o'clock)	+	+
<i>Nicotiana tabacum</i> (tobacco)	+	+
<i>Lycopersicon esculentum</i> (tomato)	—	—
<i>Raphanus sativus</i> (radish)	—	—
<i>Capsicum annuum</i> (pepper)	—	—
<i>Brassica oleracea</i> (cabbage)	—	—
<i>Cucurbita pepo</i> (squash)	—	—
<i>Phlox</i> sp. (phlox)	—	—
<i>Apium graveolens</i> (celery)	—	—

^a +, necrotic response limited to infiltrated tissue; —, no response.

The proteinaceous elicitor is probably not a polygalacturonase enzyme, since we were not able to detect this enzyme activity in strain CMM623 (data not shown).

Partial purification of the elicitor. When CFCS were filtered through an Amicon filter (3,000 molecular weight exclusion), the concentrated material retained full elicitor activity, eliminating the possibility of low molecular weight elicitors such as syringolides (35). To determine the native size of the elicitor, we performed gel filtration chromatography on CFCS, and the column fractions were assessed for HR-inducing activity. The activity was consistently found in the 105-kDa molecular mass region (Fig. 2). HR-eliciting activity was also observed (although occasionally) migrating at molecular masses between 15 to 29 kDa and 440 kDa (Fig. 2). Similar results were observed in eight separate experiments.

To investigate whether the proteins from the sizing column were aggregates (misrepresenting the molecular mass), we separated CFCS proteins by SDS-PAGE. After elution, proteins were renatured and tested in plant reaction assays. Only those proteins in the molecular mass range of 35 to 50 kDa and 12 to 16 kDa had HR-inducing activity (Fig. 3). The same results were obtained in four separate experiments.

When CFCS were applied to a DEAE cellulose column, most of the elicitor activity eluted at 300 mM and a residual activity was found in the 500 mM NaCl-eluted material (Fig. 3). This elution profile is similar to that found for the proteinaceous *C. michiganensis* subsp. *sepedonicus* elicitor (31). The eluted material induced a necrotic response on *M. jalapa* and tobacco at a protein concentration as low as 7.3 µg/ml.

DISCUSSION

In this study, we present evidence that *C. michiganensis* subsp. *michiganensis* produces at least one protein involved in the induction of a HR on *M. jalapa* and tobacco. This protein activity was found in the CFCS, suggesting it is secreted outside the bacterium.

Fig. 2. Gel filtration chromatography of cell-free culture supernatants (CFCS) from strain CMM623. CFCS were loaded onto an S-300 Sephacryl column as described in Materials and Methods. Fractions were assayed for hypersensitive response, and those displaying necrosis-inducing activity on *Mirabilis jalapa* are indicated by shaded areas A, B, and C on the chromatogram. Open triangle indicates the elution of potassium ferricyanide, molecular mass 329.2 Da. Protein molecular weight markers (black circles; 443, 150, 66, and 29 kDa) were chromatographed under similar conditions as the sample, and protein absorbance was measured at 280 nm.

Bacterial pathogenicity factors (including elicitors) from gram-negative phytopathogenic bacteria are secreted by the specialized type III secretion encoded by the *hrp* genes that traverse both the cytoplasmic and outer membranes (15,23,39). Although there is homology between flagellar Fla/Fli proteins of *Bacillus subtilis* and some Hrp proteins of *X. campestris* pv. *campestris* (9,16), the absence of the outer membrane in gram-positive bacteria argues against a *hrp* secretion system. It is possible that the protein described here uses a secretion system similar to the one that secretes the 80-kDa pathogenicity factor internalin from *Listeria monocytogenes* (18,19).

The necrosis-inducing protein described in this study and the elicitor protein recently described from *C. michiganensis* subsp. *sepedonicus* (31) share similar characteristics that include chromatographic behavior on DEAE cellulose, heat stability, and secretion to the culture supernatant. However, the elicitor from *C. michiganensis* subsp. *sepedonicus* induces a HR only on tobacco. Heat stability and secretion to the extracellular milieu are characteristics also shared by the gram-negative bacterial elicitor proteins harpin and PopA and some elicitors isolated from fungi (5,24,33). These results are suggestive of a new family of proteins from gram-positive bacteria with necrosis-inducing activity in plants.

The reaction induced in *M. jalapa* by culture supernatants from *C. michiganensis* subsp. *michiganensis* seems to be a true HR, since it depends on active plant metabolism. The involvement of plant metabolic processes was demonstrated in the HR elicited by *E. amylovora* harpin (25) and more recently by the *C. michiganensis* subsp. *sepedonicus* elicitor (31).

When proteins from CFCS were separated by a SDS-PAGE, isolated, and used in biological assays, HR-eliciting activity was detected within the molecular mass range of 35 to 50 kDa. This result suggests that more than one protein with HR-eliciting ac-

tivity is present. Alternatively, some activities and the weaker 15-kDa activity could be due to degradation of a larger elicitor protein. Interestingly, PopA from *R. solanacearum* and the various harpins are similar in size (e.g., 28 to 44 kDa) (1,11,26,40) to the elicitor described here. Our SDS-PAGE results also argue against high molecular weight elicitors as those observed after gel filtration chromatography. In this respect, it has been reported that harpin from *E. amylovora* also aggregates during gel filtration, eluting at a much higher molecular mass than the monomer (40).

Recently, Bermpohl et al. (4) reported that the HR-inducing factor from *C. michiganensis* subsp. *michiganensis* is strictly associated with viable cells. In agreement, inoculation of *C. michiganensis* subsp. *michiganensis* at concentrations of 5×10^8 CFU/ml in the presence of 50 µg of rifampicin or streptomycin per ml (bacterial protein synthesis inhibitor) did not induce a response in *M. jalapa* (data not shown). This suggests that, in planta, the elicitor needs to be produced by the bacteria and is not a preformed structural part of it. Surprisingly, Bermpohl (4) also reported that sterile filtrates from culture supernatants of strain NCPPB382 did not induce a HR on *M. jalapa*. In our hands, supernatants of the same strain did induce a response on *M. jalapa*, although this response was delayed and weaker compared with supernatants from strain CMM623. In addition, culture supernatants from the endophyte CMM100 (plasmid-free derivative of the pathogenic strain NCPPB382), also induced a necrotic response on *M. jalapa*, indicating the chromosomal location of the genes determining this activity. The conflicting results observed between Bermpohl (4) and us could be due to the age of the cultures. They used overnight cultures (there is no information about the growth phase of the bacteria), instead we used stationary phase or older cultures. HR-inducing activity was detected only in supernatants from cultures in or after the late log phase (data not shown). The different results could also be due to differences in host physiology, since we usually observed negative results with plants that had been grown at low temperatures (0 to 20°C). These cold-raised plants have succulent leaves and most of them have a red pigmentation in the stem.

The results of this study are significant, because they report a new class of proteins with HR-inducing activity in gram-positive bacteria and open important prospects for investigating secretion machinery in gram-positive bacteria.

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LITERATURE CITED

1. Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitivity-like response on specific *Petunia* genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. EMBO (Eur. Mol. Biol. Organ.) J. 13:543-553.
2. Ausubel, F. M., Brent, R., Kingston, R., Moore, D., Seidman, J. G., Smith, J. A., and Struhl, K. 1992. Short Protocols in Molecular Biology. John Wiley & Sons, New York.
3. Bauer, D. W., Bogdanove, A. J., Beer, S. V., and Collmer, A. 1994. *Erwinia chrysanthemi* *hrp* genes and their involvement in soft rot pathogenesis and elicitation of the hypersensitive response. Mol. Plant-Microbe Interact. 7:573-581.
4. Bermpohl, A., Dreier, J., Bahro, R., and Eichenlaub, R. 1996. Exopolysaccharides in the pathogenic interaction of *Clavibacter michiganensis* subsp. *michiganensis* with tomato plants. Microb. Res. 151:391-399.
5. Billard, V., Bruneteau, M., Bonnet, P., Ricci, P., Pernollet, J. C., Huet, J. C., Vergne, A., Richard, G., and Michel, G. 1988. Chromatic purification and characterization of elicitors of necrosis on tobacco produced by incompatible *Phytophthora* species. J. Chromatogr. 44:87-94.
6. Bogdavone, A. J., Wei, Z.-M., Zhao, L., and Beer, S. V. 1996. *Erwinia amylovora* secretes harpin via a type III pathway and contains a homolog of *yopN* of *Yersinia* spp. J. Bacteriol. 178:1720-1730.
7. Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Pages 79-98 in: Current Topics in Microbiology and Immunology. Vol. 192. Bacterial Pathogenesis of Plants and Animals: Molecular and Cellular Mecha-

Fig. 3. Sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis of CMM623 proteins. Lane 1, cell-free culture supernatant proteins (26 µg). Lanes 2 (110 µg) and 3 (100 µg), proteins eluting from DEAE at 300 mM NaCl and 500 mM NaCl, respectively. Proteins were stained with silver. ++, eluted proteins caused complete necrosis of the entire infiltrated area. +, eluted proteins caused partial necrosis of the infiltrated area.

- nisms. J. L. Dangl, ed. Springer-Verlag, Berlin.
8. Bonas, U., and Van den Ackerveken, G. 1997. Recognition of bacterial avirulence proteins occurs inside the plant cell: A general phenomenon in resistance to bacterial diseases? *Plant J.* 12:1-7.
 9. Carpenter, P. B., Zuberi, A. R., and Ordal, G. W. 1993. *Bacillus subtilis* flagellar protein FliP, FliQ, FliR and FliH are related to *Shigella flexneri* virulence factors. *Gene* 137:243-245.
 10. Cortes, P., Buckbinder, L., Leza, M. A., Rak, N., Hearing, P., Merino, A., and Reinberg, D. 1988. EivF, a factor required for transcription of the adenovirus EIV promoter, binds to an element involved in Ela-dependent activation and cAMP induction. *Genes Dev.* 8:975-990.
 11. Cui, Y., Madi, L., Mukherjee, A., Dumenyo, C. K., and Chatterjee, A. K. 1996. The RsmA⁻ mutants of *Erwinia carotovora* subsp. *carotovora* strain Ecc71 overexpress *hrpN_{Ecc}* and elicit a hypersensitive reaction-like response in tobacco leaves. *Mol. Plant-Microbe Interact.* 9:565-573.
 12. De Boer, S. H., Van Vaerenbergh, J., Stead, D. E., Janse, J. D., and McKenzie, A. R. 1992. A comparative study in five laboratories on detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato stems and tubers. *Potato Res.* 35:217-226.
 13. Dixon, R. A., and Lamb, C. J. 1990. Molecular communication in interactions between plants and microbial pathogens. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:339-367.
 14. Fatmi, M., and Schaad, N. W. 1988. Semiselective agar medium for isolation of *Clavibacter michiganensis* subsp. *michiganensis* from tomato seed. *Phytopathology* 78:121-126.
 15. Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant-Microbe Interact.* 5:390-396.
 16. Fenselau, S., and Bonas, U. 1995. Sequence and expression analysis of the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. *Mol. Plant-Microbe Interact.* 8:845-854.
 17. Flor, H. H. 1942. Inheritance of pathogenicity in *Melampsora lini*. *Phytopathology* 32:653-669.
 18. Gaillard, J.-L., Berche, P., Frehel, C., Gouin, E., and Cossart, P. 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. *Cell* 65:1127-1141.
 19. Gallagher, R. B. 1996. Enter *Listeria*, unruffled. *Science* 271:1825.
 20. Gitaitis, R. D. 1990. Induction of a hypersensitivelike reaction in four-o'clock by *Clavibacter michiganensis* subsp. *michiganensis*. *Plant Dis.* 74:58-60.
 21. Goodman, R. N., and Novacky, A. 1994. The hypersensitive reaction in plants to pathogens. A resistance phenomenon. The American Phytopathological Society, St. Paul, MN.
 22. Gopalan, S., Bauer, D., Alfano, J., Loniello, A. O., He, S. Y., and Collmer, A. 1996. Expression of the *Pseudomonas syringae* avirulence protein AvrB in plant cells alleviates its dependence on the hypersensitive response and pathogenicity (Hrp) secretion system in eliciting genotype-specific hypersensitive cell death. *Plant Cell* 8:1095-1105.
 23. Gopalan, S., and He, S. Y. 1996. Bacterial genes involved in the elicitation of hypersensitive response and pathogenesis. *Plant Dis.* 80:604-610.
 24. Hahn, M. G. 1996. Microbial elicitors and their receptors in plants. *Annu. Rev. Phytopathol.* 34:387-412.
 25. He, S. Y., Bauer, D. W., Collmer, A., and Beer, S. V. 1994. Hypersensitive response elicited by *Erwinia amylovora* harpin requires active plant metabolism. *Mol. Plant-Microbe Interact.* 7:289-292.
 26. He, S. Y., Huang, H. C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* harpin_{ps}: A protein that is secreted via the *hrp* pathway and elicits the hypersensitive response in plants. *Cell* 73:1255-1266.
 27. He, S. Y., Lindeberg, M., Chatterjee, A. K., and Collmer, A. 1991. Cloned *Erwinia chrysanthemi* *out* genes enable *Escherichia coli* to selectively secrete a diverse family of heterologous proteins to its milieu. *Proc. Natl. Acad. Sci. U.S.A.* 88:1079-1083.
 28. Kaniga, K., Trollinger, D., and Galán, J. E. 1995. Identification of two targets of the type III protein secretion system encoded by the *inv* and *spa* loci of *Salmonella typhimurium* that have homology to the *Shigella* IpaD and IpaA proteins. *J. Bacteriol.* 177:7078-7085.
 29. Klement, Z. 1982. Hypersensitivity. Pages 149-177 in: *Phytopathogenic Prokaryotes*. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.
 30. Meletzus, D., and Eichenlaub, R. 1991. Transformation of the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* by electroporation and development of a cloning vector. *J. Bacteriol.* 173:184-190.
 31. Nissinen, R., Lai, F.-M., Laine, M. J., Bauer, P. J., Reilley, A. A., Li, X., De Boer, S. H., Ishimaru, C. A., and Metzler, M. C. 1997. *Clavibacter michiganensis* subsp. *sepedonicus* elicits a hypersensitive response in tobacco and secretes hypersensitive response-inducing protein(s). *Phytopathology* 87:678-684.
 32. Parker, J. E., and Coleman, M. J. 1997. Molecular intimacy between proteins specifying plant-pathogen recognition. *Trends Biochem. Sci.* 22:291-296.
 33. Parker, J. E., Schulte, W., Hahlbrock, K., and Scheel, D. 1991. An extracellular glycoprotein from *Phytophthora megasperma* f. sp. *glycinea* elicits phytoalexin synthesis in cultured parsley cells and protoplasts. *Mol. Plant-Microbe Interact.* 4:19-27.
 34. Scofield, S. R., Tobias, C. M., Rathjen, J. P., Chang, J. H., Lavalley, D. T., Michelmore, R. W., and Staskawicz, B. J. 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science* 274:2063-2065.
 35. Smith, M. J., Mazzola, E. P., Sims, J. J., Midland, S. L., Keen, N. T., Burton, V., and Stayton, M. M. 1993. The syringolides: Bacterial C-glycosyl lipids that trigger plant disease resistance. *Tetrahedron Lett.* 34:223-226.
 36. Staskawicz, B. J., Ausubel, F. M., Baker, B. J., Ellis, J. G., and Jones, J. D. 1995. Molecular genetics of plant disease resistance. *Science* 268:661-667.
 37. Tang, X., Frederick, R. D., Zhou, J., Halterman, D. A., Jia, Y. M., and Martin, G. B. 1996. Initiation of plant resistance by physical interaction of AvrPto and Pto kinase. *Science* 274:2060-2063.
 38. Van den Ackerveken, G., Marois, E., and Bonas, U. 1996. Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. *Cell* 87:1307-1316.
 39. Van Gijsegem, F., Genin, S., and Boucher, C. A. 1993. Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. *Trends Microbiol.* 1:175-180.
 40. Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257:85-88.
 41. Yang, Y., Shah, J., and Klessig, D. F. 1997. Signal perception and transduction in plant defense responses. *Genes Dev.* 11:1621-1639.