Evidence for a New Class of Peptide Elicitor of the Hypersensitive Reaction from the Tomato Pathogen *Pseudomonas corrugata*

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Contribution of the U.S. Regional Pasture Research Laboratory, University Park, PA.

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We thank R. G. Pedersen and S. Donahue for their technical assistance, K. Miller and F. Lukezic for their helpful suggestions in preparation of this manuscript, and J. Baker for the data reported in Table 1.

Accepted for publication 25 April 1995.

ABSTRACT

Gustine, D. L., Sherwood, R. T., and Moyer, B. G. 1995. Evidence for a new class of peptide elicitor of the hypersensitive reaction from the tomato pathogen *Pseudomonas corrugata*. Phytopathology 85:848-853.

Three elicitor-active fractions were isolated from culture fluids of the rough isolate of *Pseudomonas corrugata*, a tomato pathogen that elicits the hypersensitive reaction (HR) in tobacco. Lyophilized, filtered culture fluids were extracted with methanol-ethyl acetate (80:20, vol/vol) to produce a water-soluble crude extract (CE). CE was partitioned with ethyl acetate to produce water-soluble aqueous (AQ) and organic (EA) fractions containing purified elicitor. *P. corrugata* and the three fractions were tested for their ability to elicit HR in tobacco leaves, pith necrosis in tomato, and K^+/H^+ exchange in tobacco tissue culture. Only *P. corrugata* elicited pith necrosis in tomato; both bacteria and the fractions elicited HR in tobacco leaves; and bacteria and EA elicited K^+/H^+ exchange in tobacco cells. The primary component in EA, HR2, did not cause a spreading lesion in tobacco and was therefore not a toxin. HR2 was found to be 95% pure by analytical high-performance liquid chromatography, to contain a fluorescent chromophore, and to be a peptide. HR2 contains glutamic acid, tyrosine, aspartic acid, glycine, alanine and/or arginine, isoleucine and/or leucine, and methionine and/or valine (approximate molar ratio 10:3:1:1:1:1:1). Since *P. corrugata* is a useful biocontrol agent, these elicitors may have potential use in biocontrol.

Additional keywords: medicarpin, phytoalexin.

The hypersensitive reaction (HR) comprises a localized battery of plant defense responses that are manifested by development of chlorosis, rapid cell death, collapse of cells, and formation of dry tissue. While this process is initiated within 12 h, it is usually complete within 24 h. In contrast, pathogenesis and development of disease lesions is not localized and develops over several days. Underlying the visible defense responses of HR are many characterized physiological and biochemical responses: phytoalexin biosynthesis (16), K^+/H^+ exchange (2,3), active oxygen burst (10,11, 15), synthesis of lytic enzymes (12,24), cell wall strengthening by cross linking (7,8), increase in hydroxyproline-rich glycoproteins (6), and lipid peroxidation of sites within the plasma membrane (28,31,37). All or some of these responses may be observed during an incompatible reaction (15).

Initiation of these responses and subsequent HR apparently is dependent on elicitors that originate within the plant-microbe complex. A number of HR elicitors from bacteria and fungi have been described. Protein products of the *hrp* gene family may be bacterial HR elicitors. An *hrp* gene protein product is sufficient to cause HR in nonhosts and disease symptoms in the host (1,18, 19,39). The transcripts of *avr* genes, the determinants of host specificity, may be responsible for the formation of elicitor products (34). The *avr* gene in the microbe determines the specificity of the interaction, as defined by the gene-for-gene theory (21). Protein products of the *avr* gene interact with the corresponding plant resistance gene but do not cause disease symptoms (19,39). In addition to bacterial elicitors, fungal elicitins and hydrophobins also initiate the HR (27,35).

Pseudomonas corrugata is an economically important soilborne pathogen of tomato (33) and pepper (25). Two Australian strains of *P. corrugata* have been discovered that significantly diminish take-all disease of wheat and increase growth of healthy plants (32). This work suggests that *P. corrugata* is potentially useful in biocontrol applications as proposed by Kovacevich and Ryder (23). *P. corrugata* also causes HR in nonhost tobacco leaves (10,11,15) and elicits phytoalexin (medicarpin) biosynthesis in white clover callus (16), K^+/H^+ exchange in tobacco leaf disks (15), and active oxygen burst in white clover suspension cultures (11). Because *P. corrugata* is pathogenic on an economically important crop, its direct use in biological control is not advisable. Isolation and identification of *P. corrugata* metabolites that elicit nonhost defense responses may yield a potentially useful biological control agent and thus obviate field application of a plant pathogen.

Our group has reported on an uncharacterized phytoalexin elicitor (16) and presented preliminary evidence for two HR elicitors from *P. corrugata* (15). We report here the isolation of two products, designated HR1 and HR2, and evidence that these are small peptides, each containing a fluorescent chromophore.

MATERIALS AND METHODS

Culture and plants. Rough and smooth isolates of *P. corrugata* provided by F. L. Lukezic (26) were stored at 4°C on water agar slants and grown out at 25°C on yeast-dextrose-phytopeptone agar (YDPA) plates for 24 h (11). The rough colony type

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of *P. corrugata* elicits HR in tobacco leaves, while the smooth colony type does not elicit HR and is less virulent on tomato (11). Suspensions of *P. corrugata* cells in water (approximately 8 × 10^8 CFU ml^{-1}) prepared from YDPA plates (11) were used for whole-cell elicitor experiments and as inoculum for shake cultures. Cultures of *P. syringae* pv. *syringae* 61 Nal^R provided by C. J. Baker (3) were maintained on King's B agar (22) supplemented with 50 μg of nalidixic acid per milliliter. Suspensions of live *P. syringae* cells were prepared as described above for *P. corrugata*.

Tobacco (*Nicotiana tabacum* 'Havana 44') and tomato (*Lycopersicon esculentum* 'Big Boy') plants were grown from seed in peat-vermiculite potting mixture in 12.5-cm plastic pots in a greenhouse. Tobacco plants with five to seven fully expanded leaves were used for HR bioassays, and 6-week-old tomato plants were used for whole-plant stem pith necrosis assays.

Preparation of elicitor fractions. Fifty milliliters of modified King's B medium (16) in each of 40, 250-ml Erlenmeyer flasks was inoculated with 0.2 ml of rough *P. corrugata* cell suspension. The cultures were grown at 25°C for 24 h on an orbital shaker and then combined and centrifuged at 10,000 × *g* for 10 min. The supernatant fraction was filtered through a 0.45-μm HA filter (Millipore, Bedford, MA), and the culture fluid (1,800 ml) was frozen in ice cube trays. The frozen cubes were lyophilized to yield a solid mixture of medium components and *P. corrugata* metabolites in four flasks. The dry, solid product in each flask was extracted with 200 ml of methanol-ethyl acetate (80:20, vol/vol) with gentle shaking for 2 h. The yellow liquid from each flask was decanted, filtered through a 0.5-μm HF Teflon filter (Millipore), and evaporated to a syrup by flash evaporation (bath temperature, 30 to 35°C). The syrup was lyophilized and stored at –70°C. The lyophilized syrup was dissolved in 90 ml of 18-megaohm (MΩ) water and designated the crude extract (CE). Thirty milliliters of CE was stored at –70°C for use in high-performance liquid chromatography (HPLC), spectral analyses, and biological testing. The remaining 60-ml portion was used to prepare elicitor by three cycles of manual countercurrent distribution. For each cycle, the water phase was extracted six times with an equal volume of ethyl acetate. Cycle 1 was initiated by adding 60 ml of reagent grade ethyl acetate to 60 ml of CE; cycles 2 and 3 were initiated by adding 60 ml of 18-MΩ water to the ethyl acetate remaining after partitioning. The pooled ethyl acetate phases for cycles 1 and 2 were reduced in volume to 60 ml by flash evaporation. Those from cycle 3 were evaporated to dryness to yield an organic fraction (EA). The aqueous phase (AQ) from cycle 1 was saved for analyses and biological testing. Fractions CE, AQ, and EA were stored at –70°C for up to 24 months.

A portion of an AQ fraction (8 ml) was deionized by passage through a column bed of 2 g of AG-1 X8 resin (20 to 50 mesh, Cl^- form) (Bio-Rad Laboratories, Hercules CA). The deionized material was applied to a 3.7- × 9-cm SilicAR CC-7 column (Mallinckrodt, Paris, KY) equilibrated with ethyl acetate. When the leading band of yellow color had migrated halfway, the eluting solvent was changed to ethyl acetate-ethanol (9:1, vol/vol), and 12-ml fractions were collected. Fractions containing color were combined, reduced in volume, and applied to a 2- × 45-cm CM Sephadex column (20 mM sodium acetate buffer, pH 5.0), and 12-ml fractions were collected. After the first peak (designated HR2) emerged, the buffer strength was increased to 50 mM, and the second peak (designated HR1) was collected.

Hypersensitive reaction. To assay the HR-eliciting activity of *P. corrugata* cells, CE, AQ, and EA, interveinal panels of tobacco leaves were pressure infiltrated (17) with 0.1 to 0.2 ml of each preparation until the entire panel was saturated with water. Bacteria were adjusted to concentrations of approximately 8 × 10^8 CFU ml^{-1}, and each elicitor fraction was prepared with A_{400} values of up to 4. Each treatment was replicated in two panels on each of two plants. A maximum of six panels per leaf was infiltrated. No adjacent panels were infiltrated, and no more than two leaves per plant were treated. Control panels were infiltrated with water. The extent of HR was assessed at 6, 24, and 48 h after infiltration. Rating values were 0.5 = any portion of a panel chlorotic; and 1, 2, 3, and 4 = 1/4, 2/4, 3/4, and 4/4 of panel tissue collapsed, respectively.

Pith necrosis assay. Approximately 0.1 ml of *P. corrugata* cell suspension or fraction was injected (1-ml syringe, 26-gauge needle) at the midpoint of a tomato plant internode. The plants were maintained for 7 days in the greenhouse and then analyzed. Each stem was sliced longitudinally with a razor blade, and the length of pith necrosis was measured. Each treatment was replicated three times.

K^+/H^+ exchange. Tobacco suspension cells were grown as previously described (2). EA stock solution (4 A_{400} units per ml) was added to 10 ml of MES (morpholineethanesulfonic acid) buffer (pH 6.0) (2) containing 50 mg of tobacco cells. Beginning at 0 time, the pH of triplicate treated suspensions and triplicate nontreated suspensions was measured at 30-min intervals. The pH decreased during the course of the experiment, but the change in pH relative to the buffer control was positive. The increase in the normalized pH represents an increase in potassium ions and a

TABLE 1. Elicitation of K^+/H^+ exchange in tobacco suspension cell culture as measured by change in pH[a]

Time (h)	Increase in pH units[b]		
		EA	
	MES buffer	0.1 ml	1 ml
0.0	0	0.000	0.000
0.5	0	0.050	0.100
1.0	0	0.060	0.130
1.5	0	0.045	0.150
2.0	0	0.050	0.175
2.5	0	0.050	0.185
3.0	0	0.060	0.205

[a] Data from C. J. Baker, Beltsville, MD.
[b] Organic fraction (EA) containing HR2 stock solution (4 A_{400} units per ml of EA) was added to 10 ml of MES buffer containing 50 mg of tobacco suspension cells per milliliter as previously described (2). Each pH value is the mean of three determinations; coefficient of variation was less than 5% for each value.

TABLE 2. Effects of *Pseudomonas corrugata* and fractions from *P. corrugata* culture fluids on tomato and tobacco

Preparation[a]	Extent of tomato pith necrosis[b] (mm)	Tobacco HR rating[c]
Pc rough	33.7 (4)	3.5 (10)
Pc smooth	16.9 (6)	1.5 (10)
CE rough	5.0 (4)	1.5 (4)
CE smooth	0.0 (6)	1.5 (8)
AQ rough	0.0 (2)	1.5 (4)
AQ smooth	0.0 (2)	0.5 (4)
EA rough	1.7 (4)	1.5 (4)
EA smooth	0.0 (6)	1.0 (8)

[a] Pc = *P. corrugata*; CE = crude elicitor; AQ = aqueous fraction after cycle 1 of ethyl acetate partitioning; and EA = organic fraction after cycle 3 of ethyl acetate partitioning.
[b] Each test solution or suspension (0.1 ml) was injected into the stem pith of 6-week-old tomato plants. The plants were maintained for 7 days in the greenhouse. The stems were sliced longitudinally with a razor blade, and the extent of necrosis was measured in millimeters above and below the injection site. Each value is the average of total length (normalized to 3.0 A_{400} units per ml) for stems of *n* (in parentheses) plants.
[c] Each test solution or suspension (0.2 ml) was infiltrated into a separate panel of the second, third, or fourth fully expanded leaf of a tobacco plant (cultivar Havana 44). The plants were placed under fluorescent lights in the laboratory and evaluated 24 h after infiltration. Hypersensitive reaction (HR) ratings: 0.5 = chlorotic; and 1, 2, 3, 4 = 1/4, 2/4, 3/4, and 4/4 of panel tissue collapsed, respectively. Each value is the average of ratings (normalized to 3.0 A_{400} units per ml) for leaf panels of *n* (in parentheses) plants.

corresponding loss of protons in the buffer medium (2). Gustine et al (15) established that *P. corrugata* and HR2 (EA) stimulate K^+ efflux in tobacco leaf disks.

Analytical procedures. Spectra and absorbances of fractions were obtained with a scanning spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD) or a 990+ photodiode array detector (Millipore/Waters, Marlborough, MA). Analytical reversed-phase HPLC was conducted with a 0.46- × 12.5-cm 5-μm C-18 reversed-phase column (IBM Instruments, Danbury, CT) in 1% methanol and 2 mM acetic acid (pH 4.0) at 0.8 ml min^{-1}. The Waters photodiode array detector and software were used for detection.

Thin-layer chromatography (TLC) was conducted with Merck-SiGel 60 plates (0.25-mm-thick layer) (Merck, West Point, PA) developed in 1-butanol–glacial acetic acid–water (4:1:1, vol/vol/vol). Developed plates were observed under UV illumination and then sprayed with ninhydrin reagent (38).

Qualitative amino acid analysis. Forty-five microliters of AQ was incubated in microcentrifuge tubes with 0.38 units of proteinase K immobilized on agarose beads (Sigma, St. Louis, MO) and 50 mM sodium phosphate buffer (pH 7.5). After incubation for 0, 1, 2, 10, and 24 h at 30°C with shaking, the beads were removed by centrifugation, and the samples were analyzed by TLC as described above.

Duplicate samples of EA (approximately 10 A_{400} units each) were hydrolyzed in 1 ml of 47% hydriodic acid (HI) or 6 N HCl at 110°C for 18 h in sealed, evacuated tubes. Hydrolyzed samples were dried over KOH pellets and stored in a vacuum desiccator. Hydrolysates were dissolved in 3 ml of 18-MΩ water and filtered (0.45-μm Gelman Acrodisc, Gelman Sciences, Ann Arbor, MI). Free amino acids were derivatized with ortho-phthalaldehyde (OPA) (Pierce Chemical, Rockford, IL) and identified by reversed-phase HPLC (13,14).

RESULTS

The primary criteria for HR used for isolation of an HR elicitor were development of HR in tobacco, K^+/H^+ exchange, and phytoalexin induction. The visible tobacco HR caused by EA or HR2 from the rough isolate of *P. corrugata* was indistinguishable from that caused by the corresponding *P. corrugata* cells (data not shown). The visible tobacco HR caused by rough *P. corrugata*, EA, HR1, and HR2 was indistinguishable from that caused by *P. syringae* pv. *syringae* cells (data not shown). Infiltrated tissue, including the water control, was initially saturated with water and recovered within 2 to 4 h. Water control tissue had a healthy, turgid appearance thereafter. In bacteria- or elicitor-treated tissue, chlorosis was first evident 6 to 12 h after infiltration. This was followed by cell collapse and desiccation of the tissue within 8 to 24 h. All fractions from rough *P. corrugata* elicited accumulation of the phytoalexin medicarpin in white clover callus (data not shown; 15). However, the AQ and EA fractions appeared to have about one-tenth the phytoalexin elicitor activity of the CE fraction. EA elicited the K^+/H^+ exchange reaction (Table 1) in tobacco suspension cultures and in tobacco leaf disks (HR2 in Gustine et al [15]). The CE and AQ fractions were not tested. Increased pH in the medium on which the tobacco leaf disks were floated was accompanied by a corresponding increase in K^+ (15). The procedures for isolating the fractions were repeatable with regard to biological activity.

To distinguish between HR elicitor activity and toxin activity, the ability of *P. corrugata* and the isolated fractions to produce pith necrosis in tomato (host) was compared with their ability to induce HR in tobacco (nonhost). The data in Table 2 establish that *P. corrugata* cells, but not the three fractions, caused pith

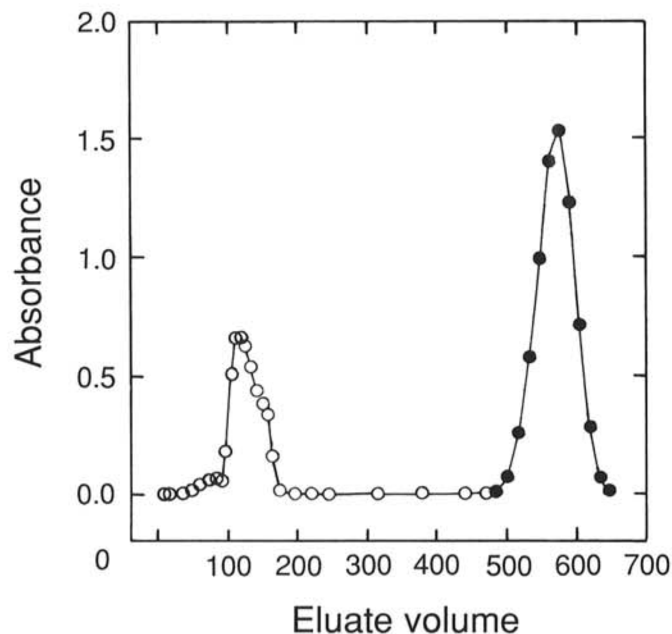

Fig. 1. Separation of rough *Pseudomonas corrugata* elicitors HR2 and HR1 by CM Sephadex chromatography. Concentrated *P. corrugata* culture fluid was partitioned with ethyl acetate, and the aqueous fraction was deionized and partially purified by silica gel column chromatography. This preparation was then applied to the CM Sephadex column. HR2 and HR1 were eluted with 20 and 50 mM sodium acetate buffers (pH 5.0), respectively. Absorbances were read at 405 (O——O, HR2) and 410 (●——●, HR1) nm.

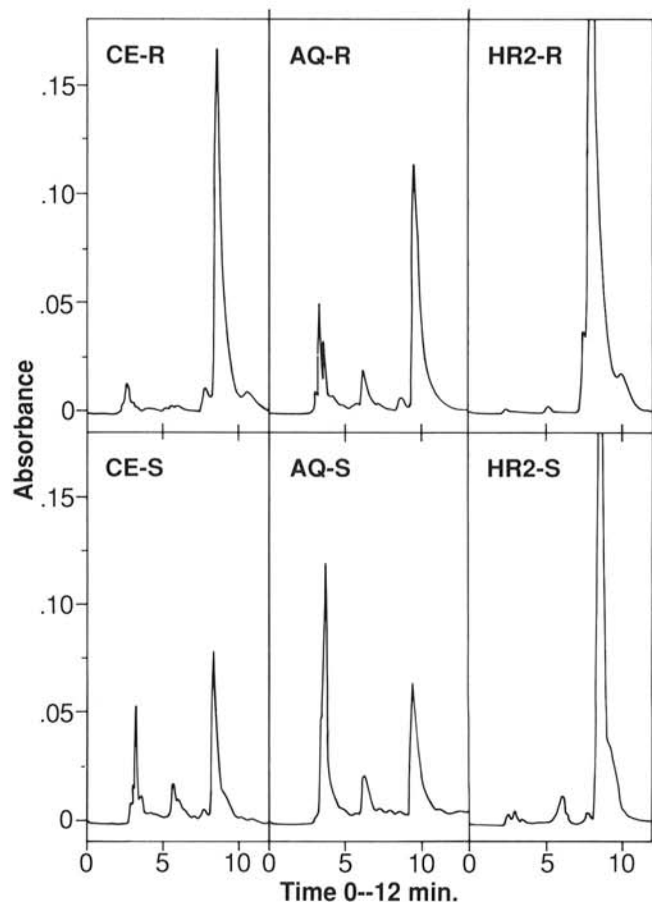

Fig. 2. Reversed-phase high-performance liquid chromatograms of three fractions from rough and smooth *Pseudomonas corrugata* culture fluids. Amounts of each solution injected varied from 5 to 40 μl, and the component concentrations were not normalized. CE-R = crude extract from rough colony type; AQ-R = aqueous phase; HR2-R = organic phase of CE-R partitioned against ethyl acetate; CE-S = crude extract from smooth colony type; AQ-S = aqueous phase; and HR2-S = organic phase of CE-S partitioned against ethyl acetate. Absorbance is reported at 254 nm.

necrosis in the 7-day tomato bioassay. While some toxin activity was present in CE from rough *P. corrugata*, none appeared to be present in AQ, nor was any present in CE and AQ from *P. corrugata* smooth cultures. The data also show that the cells from smooth *P. corrugata* cultures were less virulent in the tomato pith necrosis assay than were cells from rough *P. corrugata* cultures (Table 2). The CE, AQ, and EA fractions from both smooth and rough bacteria elicited visible HR in tobacco, but the level of HR was lower in smooth than in rough AQ and EA (Table 2). In contrast to a previous report (11), smooth *P. corrugata* elicited HR in tobacco, although much less than the rough isolate (1.5 for smooth versus 3.5 for rough). EA was not active as a toxin because it failed to produce pathogenic symptoms in tomato; it was active as an elicitor of HR because it produced the visible reaction on tobacco leaves.

Purification of rough *P. corrugata* elicitor-active metabolites was followed by chromatographic analysis. Components in the AQ fraction were separated by CM Sephadex chromatography (Fig. 1). Analyses by TLC established a yellow green fluorescent spot as the major component in the first peak (HR2, R_f 0.6) and a similarly colored component in the second peak (HR1, R_f 0.35). HR1 and HR2 each elicited tobacco HR. TLC analysis of the AQ fraction confirmed the presence of the R_f 0.35 component and a lesser amount of the R_f 0.6 component. When the CE fraction was examined by TLC, both the R_f 0.35 and 0.6 components were found in preparations from rough and smooth cultures. Fractions from chromatography of rough CE on Sephadex G-25 containing either R_f 0.35 or R_f 0.6 also elicited HR in tobacco (D. L. Gustine and R. T. Sherwood, *unpublished*). When the EA fraction was analyzed by TLC, the R_f 0.6 component was primarily found. TLC plates were sprayed with many reagents, but none, including ninhydrin, gave an indication of specific classes of organic compounds. All attempts at purifying these compounds resulted in preparations with the second component, suggesting that each is unstable and spontaneously converts to the other. Because HR1 and HR2 are not stable and because of the ease in obtaining HR2 in the EA fraction, the results reported here are for analysis of HR2 in the EA fraction.

Figure 2 shows liquid chromatographic results for CE, AQ, and EA analyses. The peaks eluting at 3 to 4 min during HPLC separation were collected and compared by TLC with HR1 from CM Sephadex chromatography. In both cases, only the R_f 0.35 fluorescent compound was detected (data not shown), establishing that the component eluted at 3 to 4 min on HPLC and the R_f 0.35 component were the same. Similarly, when the peaks eluting at 8.5 to 9.5 min during HPLC (Fig. 2) were compared with the EA fraction and HR2 by TLC (data not shown), correspondence was established. HPLC analysis of fraction CE from rough and smooth cultures revealed that the major absorbing component at 254 nm was HR2 (Fig. 2, CE-R and CE-S). Peak integration analyses of EA (Fig. 2, HR2-R and HR2-S) indicated greater than 95% of the 254 nm-absorbing material was HR2.

To further characterize the elicitors, some of the EA and HR1 fractions were hydrolyzed in 0.1 N HCl, the products were separated by TLC, and the plate was evaluated. New fluorescing spots were seen along with unexpected ninhydrin-positive spots, indicating conversion of both HR1 and HR2 to products with primary amino groups and possibly indicating both compounds are peptides with blocked N terminal groups. HR2 in EA was subjected to total hydrolysis in 6 N HCl or 47% HI and the OPA-derivatized amino acids identified by HPLC. Table 3 shows that glutamic acid was the primary amino acid among several and indicates their molar ratios. Analysis by HPLC and TLC showed that 6 N acid hydrolysis destroyed the UV-absorbing chromophore of both HR1 and HR2 (15). Further proof that HR2 is a

TABLE 3. Proposed amino acid composition of HR2[a]

Amino acid	Approximate molar ratio HCl	HI
Glutamic acid	10.0	6.7
Tyrosine	2.5	2.1
Aspartic acid	0.8	0.9
Glycine	0.8	0.9
Alanine and/or arginine[b]	1.3	1.3
Isoleucine and/or leucine[b]	1.1	1.4
Methionine and/or valine[b]	1.0	1.0
Unidentified	1.0	1.0

[a] HR2 (3 mg of the organic fraction) was hydrolyzed in 1 ml of 6 N HCl or 47% hydriodic acid (HI) in sealed, evacuated glass tubes for 18 h at 110°C. Amino acids in hydrolysates were identified by high-performance liquid chromatography (HPLC) separation of ortho-phthalaldehyde derivatives.

[b] Derivatives not separated by HPLC.

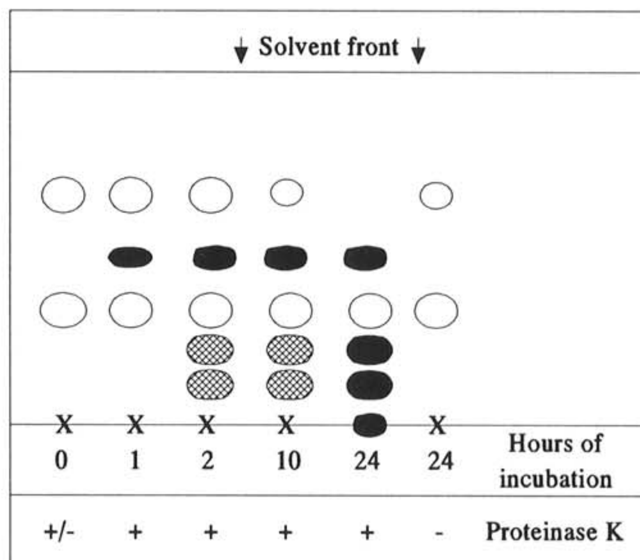

Fig. 3. Separation by thin-layer chromatography of fluorescent and ninhydrin-positive components after enzymatic hydrolysis of elicitor HR2 in the organic fraction from rough *Pseudomonas corrugata* with proteinase K. Incubations were in 50 mM sodium phosphate buffer (pH 7.5) for the indicated times; loaded silica gel plates were developed in 1-butanol–glacial acetic acid–water (4:1:1, vol/vol/vol). Unshaded ovals (R_f 0.35 and 0.6 compounds) = fluorescence under UV illumination; crosshatched ovals = fluorescence under UV and reaction with ninhydrin spray reagent; and dark ovals = reaction with ninhydrin only.

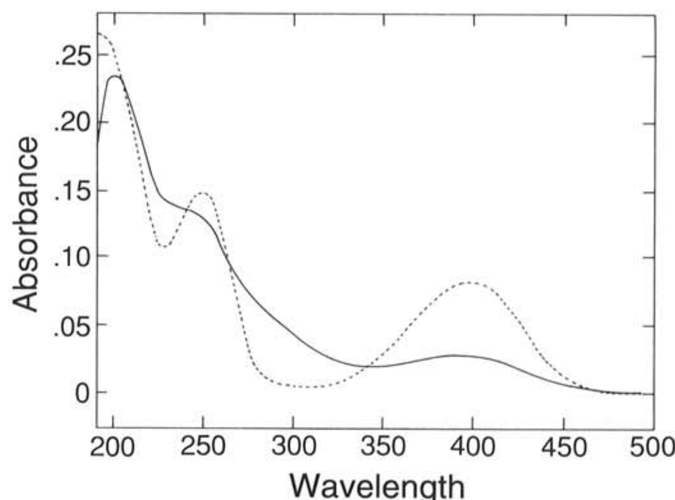

Fig. 4. Absorbance spectra of hypersensitive reaction elicitors HR1 (——) and HR2 (– – –) in fractions from rough *Pseudomonas corrugata*. Spectra were obtained in 1% methanol in 2 mM acetic acid (pH 4.0) from high-performance liquid chromatography photodiode array data.

peptide was obtained by incubating EA in proteinase K (Fig. 3). After 1 h of incubation, a ninhydrin-positive spot was detected. Longer incubation produced additional ninhydrin-positive spots; after 24 h, HR2 (R_f 0.6) had disappeared and four ninhydrin-positive spots were detected. HR1 was not cleaved by proteinase K. HR1 and HR2 were unaffected by 24 h of incubation in buffer.

Spectra were obtained from HPLC photodiode array analysis and revealed peak absorbances at 199, 242, and 392 nm for HR1 and at 192, 250, and 398 nm for HR2 (Fig. 4). Upon dialysis in Spectra/Por tubing (Spectrum Medical Industries, Houston, TX) (cutoff 3,500 Da) against water, HR2 readily passed through the membrane. Elution times of HR1 and HR2 from a Sephadex G-25 column in 0.1 M sodium phosphate buffer (pH 7.2) were consistent with a peptide of less than 3,500 Da. Analysis of HR2 by FAB-MS (fast atom bombardment-mass spectrometry) did not yield sufficient data to determine a molecular weight.

DISCUSSION

Taken together, our data establish that *P. corrugata* in culture produces at least two metabolites, HR1 and HR2, that apparently elicit HR. This is demonstrated by the findings that i) HR1 and HR2 elicit HR in tobacco leaves and ii) HR2 elicits increased pH in tobacco suspension cultures (HR1 was not tested). Additionally, we previously reported that HR2 and *P. corrugata* elicit K^+ efflux in tobacco leaf disks and HR2 elicits the biosynthesis of the phytoalexin medicarpin in white clover callus tissue (15). Furthermore, elicitation of HR by HR2 (in EA) cannot be ascribed to its functioning as a toxin, because unlike live *P. corrugata,* EA did not cause pith necrosis in tomato (Table 2). The difference in the extent of virulence and the similarity of visible tobacco HR caused by rough and smooth *P. corrugata* strains (Table 2) also point to HR2 functioning as an HR elicitor. Tobacco HR caused by HR2 in EA from rough or smooth *P. corrugata* cultures was confined to the leaf area infiltrated and did not spread, as would be expected with a toxin. Sometimes tissue infiltrated with CE became flaccid and black gray within minutes and did not recover. Its appearance was atypical of HR and was perhaps the result of inorganic salts of the medium remaining in the CE (5). *P. syringae* produced HR in the tobacco leaf infiltration assay indistinguishable from that caused by rough *P. corrugata*. Cells of saprophytic *P. fluorescens* did not elicit tobacco HR in this assay, and the response was similar to that for water infiltration controls (D. L. Gustine, *unpublished*). Volumes of methanol or ethyl acetate equivalent to those used in the isolation procedure were evaporated, and the residue was taken up in water. When these residues were infiltrated into tobacco leaf panels or injected into tobacco stems, no necrosis or HR symptoms were detected.

Our data also establish that HR2 is a small peptide: i) acid hydrolysis released amino acids from HR2; ii) proteinase K released ninhydrin-positive products from HR2; and iii) amino acids, primarily glutamine and tyrosine, were present in EA hydrolysates. HR1 was hydrolyzed to ninhydrin-positive products and may also be a peptide, as suggested by Gustine et al (15). Attempts to determine the structure of HR2 were unsuccessful, but further work is underway. Preliminary results show that HR2 is a peptide of less than 3,500 Da and either contains a chromophore or copurifies with a fluorescent compound. It is unlikely that a minor component of EA other than HR2 is responsible for the elicitor activity. For this to be true, each minor component would have to fortuitously cochromatograph with a fluorescent compound in three different separation systems (countercurrent distribution, CM Sephadex, and Sephadex G-25). Similarly, fractions from CM Sephadex or from Sephadex G-25 containing the R_f 0.35 component elicited tobacco HR. Cochromatography of elicitor-active minor components might be conceivable for one of the elicitor fractions but not for two elicitor fractions that have different polarities.

We do not know whether HR1 and HR2 are encoded by *hrp* genes. Their size and fluorescent properties are not consistent with those of harpin or other *hrp* expression products (19,39). Expression of HR1 and HR2 could also be determined by *avr* genes (4,34), but *avr* mutants of *P. corrugata* are not yet available to test this possibility.

HR1 and HR2 differ from the race-specific proteins produced by *Cladosporium fulvum* race 9, which elicit host defense responses. A fungal protein (AVR9) isolated from intercellular fluids of infected tomato leaves elicited HR on tomato cultivars carrying the *Cf9* resistance gene (9). The *avr* gene encodes for a 7.5-kDa protein containing the secretory leader sequence, which is processed to a mature, 28-amino acid peptide with six cysteine residues (36). This protein was shown to confer avirulence to pathogenic *C. fulvum* races.

HR1 and HR2 are also different from fungal elicitins that are responsible for the tobacco incompatible reaction (also called HR) and produce remote, systemic necrosis (20,27). These elicitins can also give rise to protection against subsequent inoculation with pathogenic *Phytophthora nicotianae* (30). Elicitors HR1 and HR2 in this report are much smaller than the 10-kDa elicitins (20,27,29,30) and contain a chromophore lacking in the elicitins. We found HR in tobacco only within the boundaries of the initial infiltrated area. HR1 and HR2 did not cause remote, systemic reactions.

HR1 and HR2 are different from a third group of fungal proteins, the hydrophobins, which are a relatively small, cysteine-rich, hydrophobic group of proteins (35).

Thus, *P. corrugata* elicitors HR1 and HR2 appear to be peptide elicitors unlike any previously reported. Elucidation of their structures and characterization of their genetic regulation will add to our understanding of why bacteria elicit the plant defense responses that comprise the hypersensitive reaction. Their usefulness in biocontrol remains to be addressed.

LITERATURE CITED

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