Clavibacter michiganensis subsp. *sepedonicus* Elicits a Hypersensitive Response in Tobacco and Secretes Hypersensitive Response-Inducing Protein(s)

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ABSTRACT

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Strains of *Clavibacter michiganensis* subsp. *sepedonicus*, causal agent of bacterial ring rot of potato, showed marked differences in virulence on host plants. When infiltrated into tobacco leaves, virulent strains caused a rapid localized necrotic response (within 24 to 48 h) characteristic of the hypersensitive response (HR), whereas nonpathogenic strains did not. Concentrated cell-free culture supernatants (CCS) from virulent strains caused a necrotic reaction on tobacco, whereas CCS from nonpathogenic strains did not. The necrosis-inducing activity was heat stable and pro-

tease sensitive. Inhibitors of eukaryotic metabolism suppressed the necrotic reaction of tobacco to CCS. No necrotic response was observed when host plants were infiltrated with either cells or CCS from virulent strains. HR-inducing protein(s) from a virulent strain separated from the majority of other proteins on DEAE cellulose at 250 to 300 mM NaCl. Ammonium sulfate-precipitated proteins from a virulent strain produced a necrotic reaction at a total protein concentration of 18 µg/ml, whereas those from a nonpathogenic strain did not, even at a concentration of 180 µg/ml. We conclude that virulent strains of *C. michiganensis* subsp. *sepedonicus* elicit a typical HR in tobacco and secrete proteinaceous elicitor(s) of the nonhost HR.

Additional keywords: *Corynebacterium sepedonicum*.

The gram-positive bacterium *Clavibacter michiganensis* subsp. *sepedonicus* causes bacterial ring rot of potato (16). In recent years, the number of crop failures due to this disease has been reduced by widespread adoption of a limited generation certified seed program in which there is zero tolerance for the disease (27, 42). However, eradication of the disease has not yet been achieved (19–21, 42). Certification of seed lots based on pathogen detection rather than symptom detection is being implemented to limit the spread of latent infections, which are especially common in resistant cultivars and climates uncondusive to symptom expression. The pathogen is spread during harvesting, storage, and cutting of tubers to produce seed pieces. Disease severity is affected by the environment (54), cultivar (7, 18, 28, 41), and pathogen strain (7, 11). Despite interest in elucidating the molecular biology of pathogenesis in *C. michiganensis* subsp. *sepedonicus*, progress has lagged behind that of gram-negative phytopathogens due to a lack of methods for genetic analysis of this and other coryneform bacteria. Recently, methods have been developed for transformation of *C. michiganensis* subsp. *sepedonicus* and related subspecies (38, 43); however, other basic tools, such as transposons and marker exchange methods, remain to be developed.

Biological interactions useful for studying pathogenicity of bacteria on plants need not be limited to those involving disease. On the contrary, studies of incompatibility, which results from the in-

teraction of a pathogen with a nonhost or a resistant host, have proven very productive. For example, several genera of gram-negative phytopathogenic bacteria elicit a hypersensitive response (HR), visible macro- and microscopically as rapid, localized plant cell death, when infiltrated into leaves of a nonhost plant, such as tobacco (25, 36). From numerous studies, it is known that the *hrp* gene clusters in *Erwinia amylovora* (9), *Xanthomonas campestris* (3, 13), *Pseudomonas syringae* (39, 46), and *Ralstonia solanacearum* (14) are required for HR induction on nonhosts and for pathogenicity on hosts (26). Avirulence genes also are required to elicit a HR on resistant host cultivars, and evidence is mounting that Avr proteins transit the *hrp*-encoded secretion system (1, 48).

Induction of an HR-like reaction by a gram-positive plant pathogen was first described with the discovery of a hypersensitive-like reaction induced on four o'clock (*Mirabilis jalapa*) and tobacco by *C. michiganensis* subsp. *michiganensis* (24). All strains that were pathogenic on tomato also induced a hypersensitive-like reaction on four o'clock (24), agreeing with observations first made by Klement (37) that linked elicitation of the HR to pathogenicity in gram-negative bacteria. We have never successfully induced an HR on *M. jalapa* infiltrated with *C. michiganensis* subsp. *sepedonicus* (C. Ishimaru and M. Metzler, unpublished data); however, we find that this bacterial species does induce a necrotic response on tobacco. We demonstrate here that virulent strains produce a necrotic response, resembling the HR, on tobacco, whereas spontaneously attenuated nonpathogenic strains do not. We provide evidence that this necrotic response is a true HR, and we further show that an HR on tobacco can be elicited by preparations of extracellular proteins produced only by virulent strains. We also present a partial purification of HR-inducing protein(s).

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MATERIALS AND METHODS

Bacterial strains. The *C. michiganensis* subsp. *sepedonicus* strains used in this study were obtained from various sources. All originally were isolated from infected potatoes (Table 1). Strains 3M, 3NM, Cs4, R5, and R8 are spontaneously attenuated strains, and their identity as *C. michiganensis* subsp. *sepedonicus* was confirmed by immunofluorescence and polymerase chain reaction (34). Strains were stored at -80°C in DM medium (45) containing 20% glycerol. They were grown in either DM or YGM medium (17), either on agar or in liquid on a rotary shaker (280 rpm) at 26°C . *Escherichia coli* DH5 α (Clontech, Palo Alto, CA) was grown on 2 $\times$ YT agar (50) at 37°C . *Erwinia amylovora* strain 3305 and *R. solanacearum* 3238 biovar IV (National Collection of Plant Pathogenic Bacteria, Harpenden, England) were grown at 29°C on nutrient broth-yeast extract medium (51) and YGM, respectively.

Virulence tests. Virulence of *C. michiganensis* subsp. *sepedonicus* strains was evaluated in potato (*Solanum tuberosum* L.), the only natural host, and eggplant (*S. melongena* L.), an artificial host commonly used as an indicator plant. Eggplants (cv. Black Beauty) that were 28-days old were grown in a greenhouse and inoculated by injecting stems at a single site with $\sim 20\ \mu\text{l}$ of bacterial suspension (10^8 cells per ml) in quarter-strength Ringer's solution (55). Daytime conditions were 24°C , with a 15-h photoperiod supplemented with sodium vapor lights, and the nighttime temperature was 19°C . Foliar wilt severity was rated 18 days after inoculation, using the 1 to 12 double-logarithmic Horsfall-Barratt scale (33), in which 1 indicates no symptoms and 12 indicates visible symptoms on 100% of the plant. Results from a minimum of six plants per strain were averaged to obtain mean values of virulence.

S. tuberosum cv. Red Pontiac was used to evaluate virulence in potato. Plantlets were generated by micropropagating stem sections as previously described (23,35). After 21 to 28 days, plantlets were removed from tissue-culture medium. To promote bacterial entry, plantlet roots were trimmed aseptically to remove about one-third of the advancing root mass and immersed in a cell suspension of each strain. After soaking for 30 min, the plantlets were planted in soil. Six plantlets per strain were included to calculate the mean severity of foliar wilt symptoms 12 weeks after inoculation, as described above for eggplant.

Test for necrotic response in tobacco. Tobacco plants (*Nicotiana tabacum* L. 'Samsun' and 'Xanthi' ['Turk']) were grown in soil in $10.5 \times 10.5\text{-cm}$ pots, 13 cm deep, at 22 to 24°C , with a photoperiod of 16 h of light, 8 h of dark, and a light intensity of 200 to $300\ \mu\text{E m}^{-2}\text{ s}^{-1}$. For infiltrations, Samsun or Xanthi plants (6-leaf stage) were used. When flowering Xanthi plants were used, they were shifted to 19°C for at least 24 h prior to infiltration. Bacterial suspensions at OD_{650} (optical density at 650 nm) = 0.3 (in 5 mM potassium phosphate buffer, pH 7.0) or OD_{650} = 0.8 (in distilled water), giving cell densities of $\sim 5 \times 10^8$ and 1.3×10^9 CFU/ml, respectively, were infiltrated into intercellular spaces of fully expanded tobacco leaves with a hypodermic syringe, with or without a 26-gauge needle. Infiltrated areas were monitored for development of necrosis for 48 h postinoculation (hpi). Five to six inoculations were done for each strain. The resulting reactions were given numeral values from 0 to 3: 0 = no reaction; 1 = necrotic spots visible in infiltrated area; 2 = tissue collapse and necrosis at margins of infiltrated region; and 3 = complete collapse and necrosis of the entire infiltrated area.

Preparation and concentration of cell-free culture supernatants (CCS) with elicitor activity. *C. michiganensis* subsp. *sepedonicus* strains Cs4, R5, Cs7, R14, and CIC31 were grown in YGM medium for 2 to 3 days until cultures reached an $\text{OD}_{650} \approx 2.0$. Cells were removed by centrifugation ($10,000 \times g$ for 15 min), and supernatant was sterilized by passage through $0.2\text{-}\mu\text{m}$ cellulose acetate filters (Schleicher & Schuell, Dassel, Germany). The sterile culture supernatant was concentrated with Centricon 10 mini-concentrators (Amicon, Beverly, MA) or Vivaspin 15-ml concen-

trators (Vivasciences, Binbrook Lincolnshire, England), having a cut-off size of 10 kDa, and then was desalted by washing with 3 volumes of 50 mM potassium phosphate buffer, pH 7.0. The resulting concentrated CCS was diluted with phosphate buffer to give a final concentration 3- to 15-fold higher than the original supernatant. Protein concentration was determined by the Biorad protein assay (Biorad, Hercules, CA).

To test specific activities of elicitor preparations, *C. michiganensis* subsp. *sepedonicus* strains CIC31 (virulent) and Cs4 (non-pathogenic) were grown in 2 liters of YGM medium (300 rpm at 26°C) to an $\text{OD}_{650} \approx 2.0$. Cells were removed by centrifugation, and the supernatant was filter sterilized as above. Phenylmethylsulfonyl fluoride (PMSF) was added to give a final concentration of 0.5 mM. The supernatant was cooled to 4°C , and ammonium sulfate was added to give 40% saturation. After overnight precipitation at 4°C , the precipitated proteins were collected by centrifugation at 4°C at $10,000 \times g$ for 15 min. The pellet was suspended in 30 ml of 50 mM potassium phosphate buffer, pH 7.0, and concentrated and desalted with Vivaspin 15-ml filters as described above for concentrated CCS. These preparations were infiltrated into tobacco leaves after diluting to appropriate concentrations.

Characterization of elicitor activity in CCS. For tests of heat stability, 100- μl samples of CCS were heated at 100°C for 10 min in a boiling water bath and cooled on ice and clarified by centrifugation at 14,000 rpm for 10 min in a table-top microcentrifuge. To determine sensitivity to protease, 100- μl aliquots of CCS were treated with 30 to 40 μg of proteinase K per mg of total sample protein and incubated at 37°C for 30 min. The reaction was stopped by heating reaction mixtures for 5 min at 100°C . As a control for the protease treatment, the protease inhibitor PMSF was added to CCS to a final concentration of 0.5 mM in the presence and absence of proteinase K. PMSF-treated samples were incubated and heat treated as described for proteinase K-treated samples.

Partial purification of proteinaceous elicitor. Concentrated CCS ($50\times$) from CIC31 and CS3NM was prepared as described above, except that the supernatants were resuspended in 20 mM Bis-Tris (BT) buffer, pH 6.0. Samples (400 μl) were loaded onto a DEAE cellulose column that had been equilibrated with BT buffer (bed volume 1.5 ml), and the column was washed with 10 ml of BT buffer. Proteins were eluted with a sodium chloride step gradient in BT buffer, with 50 mM increments from 50 to 300 mM, a 500 mM step, and a final 950 mM step. The fractions collected were concentrated and washed with BT buffer with Vivaspin concentrators and resuspended to a final 400- μl volume. Extracellular polysaccharide concentrations were determined colorimetrically (22).

TABLE 1. Disease symptom expression and necrotic reaction (NR) induced by strains of *Clavibacter michiganensis* subsp. *sepedonicus* on hosts

Strain	Potato ^a	Eggplant ^a	NR index ^b	Source of strains	Colony morphology
3M	1.00	1.17	0.0	S. H. De Boer	Mucoid
3NM	1.00	1.33	0.0	S. H. De Boer	Nonmucoid
Cs4	1.00	1.00	0.0	S. H. De Boer	Nonmucoid
R5	1.00	1.50	0.0	S. H. De Boer	Mucoid
R8	1.33	1.33	0.0	S. H. De Boer	Mucoid
Cs9	6.00	2.83	1.4	B. Rogers	Mucoid
Cs7	6.83	6.00	2.0	B. Rogers	Mucoid
Cs2	7.00	7.67	1.7	R. J. Copeman	Mucoid
Cs3-2	7.17	6.33	2.2	B. Rogers	Mucoid
R14	7.67	8.50	2.4	S. H. De Boer	Mucoid
Cs3-1	8.50	3.67	0.5	S. H. De Boer	Mucoid
3RC	10.83	10.50	2.8	S. H. De Boer	Mucoid
CIC31	6.5	8.75	2.7	C. Ishimaru	Nonmucoid

^a Symptoms were rated according to the Horsfall-Barratt scale (33), in which 1 = no symptoms and 12 = disease symptoms on 100% of the plant. Mean symptom ratings are based on six plants evaluated at 18 days postinoculation.

^b Tested on tobacco cv. Xanthi, mean necrotic reaction rating (from 0 to 3) for five to six plants of each strain.

Protein was loaded onto sodium dodecyl sulfate-10% polyacrylamide gels (SDS-PAGE) (6) and silver stained (silver staining kit, protein, Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

Characterization of the tobacco necrotic response, using metabolic inhibitors. Immediately prior to infiltration, cycloheximide, lanthanum chloride, and sodium vanadate were mixed with extracellular proteins from strain CIC31 prepared by 40% ammonium-sulfate precipitation, as described above, to give final inhibitor concentrations of 7.0×10^{-5} M, 1×10^{-3} M, and 5×10^{-5} M, respectively (30), and a final total protein concentration of 25 µg/ml. As a negative control, the inhibitors were mixed with distilled water to the same final concentration. α -Amanitin at 2×10^{-4} M was infiltrated into leaves 30 min before infiltration with the protein preparation (30). As a positive control, the protein preparation at the same final concentration was infiltrated into leaves without any added inhibitor.

RESULTS

***C. michiganensis* subsp. *sepedonicus* strains with different levels of virulence.** We assayed 13 strains of *C. michiganensis* subsp. *sepedonicus* for pathogenicity and virulence on both egg-

plant and potato. In both hosts, virulence of strains ranged from nonpathogenic to highly virulent (Table 1). All of the strains that were nonpathogenic on potato also were nonpathogenic on eggplant. Six of the strains that were highly virulent on potato (disease index > 6) also were highly virulent on eggplant. However, two strains showed higher virulence on potato than on eggplant: Cs3-1 caused severe symptoms on potato but showed only mild symptoms on eggplant, and Cs9 was moderately virulent on potato but weakly virulent on eggplant.

Correlation of virulence of different *C. michiganensis* subsp. *sepedonicus* strains with the ability to elicit a hypersensitive-like response on tobacco. Infiltration of a bacterial suspension of the highly virulent *C. michiganensis* subsp. *sepedonicus* strain R14 into tobacco leaves elicited a necrotic reaction resembling a HR. Plant tissue in the infiltrated area collapsed within 18 hpi and became clearly necrotic at 24 to 36 hpi. By 48 hpi, the maximum response was observed, with the collapsed area becoming dry and papery (Fig. 1). No necrosis or chlorosis was observed beyond the infiltrated region. The reaction was slower than that obtained when leaves were infiltrated with *E. amylovora*, which showed a reac-

Fig. 1. Necrotic reaction elicited in tobacco cv. Xanthi leaves by virulent and nonpathogenic *Clavibacter michiganensis* subsp. *sepedonicus* strains 72 h postinoculation. Strains shown are **A**, R5; **B**, Cs3-1; **C**, Cs2; **D**, Cs7; **E**, Cs9; and **F**, R14.

Fig. 2. Necrosis caused by 3× concentrated cellfree culture supernatant (CCS) of *Clavibacter michiganensis* subsp. *sepedonicus* in tobacco cv. Xanthi leaves 72 h postinoculation. **A**, Proteinase K-treated CCS from strain Cs7; **B**, heat-treated CCS from strain Cs7; **C**, untreated CCS from strain R5 (negative control); **D**, untreated CCS from strain Cs7; and **E**, phenylmethylsulfonyl fluoride-treated CCS from strain Cs7.

tion in less than 12 h, but it was in all ways identical to that obtained with *R. solanacearum*. Similar infiltrations with *Escherichia coli* DH5 α did not cause a reaction.

We found the necrotic response induced by *C. michiganensis* subsp. *sepedonicus* on tobacco to be less reliable than the HR obtained with *E. amylovora*, in that it seemed to depend more on the growth conditions of the plant. The most reliable results were obtained with cv. Xanthi grown at a daytime temperature of 22°C and light intensity of >300 $\mu\text{E m}^{-2} \text{s}^{-1}$ or with cv. Samsun grown at a daytime temperature of 24°C and light intensity of 200 $\mu\text{E m}^{-2} \text{s}^{-1}$.

When suspensions of each of the 13 *C. michiganensis* subsp. *sepedonicus* strains were infiltrated into leaves of Xanthi tobacco plants, they elicited differing degrees of necrosis (Table 1; Fig. 1). Strains that were nonpathogenic in both potato and eggplant did not cause a reaction on tobacco. Infiltrated areas appeared unaffected 72 hpi, although there was occasionally some slight chlorosis visible at 1 week postinfiltration. Highly virulent strains, i.e., those with disease indices greater than 6 on both potato and eggplant, usually triggered a strong localized necrotic response in tobacco (necrosis index of 1.7 to 3), similar to that caused by strain R14. The two strains that showed significantly different levels of virulence on potatoes and eggplant (Cs9 and Cs3-1) typically gave lower necrotic response indices, with collapse and necrosis occurring in patches rather than confluent throughout the infiltrated area (Fig. 1).

Induced necrotic response in tobacco by concentrated CCS. CCS from five strains was filtered to remove cells, desalted, and concentrated to $1/10$ the original volume. When infiltrated into tobacco, CCS from the virulent strains R14 and Cs7 caused collapse of the infiltrated area within 18 hpi, and necrotic lesions were clearly visible at 24 hpi. The reaction was identical in appearance to the response induced by living cells. CCS from three nonpathogenic strains, 3NM, Cs4, and R5, as well as *Escherichia coli* DH5 α , did not cause a necrotic response.

Characterization of the necrotic response-inducing factor produced by *C. michiganensis* subsp. *sepedonicus*. CCS of strains Cs7 and R14 that had been treated with proteinase K no longer elicited a necrotic reaction on tobacco (Fig. 2). However, inactivation also occurred on some occasions in the absence of proteinase K. When PMSF, a protease inhibitor, was added to the nonproteinase K-treated CCS, the supernatants retained the ability to induce the necrotic reaction (Fig. 2). This suggests that *C. michiganensis* subsp. *sepedonicus* produces both proteinaceous factor(s) that induce the necrotic response in tobacco, as well as extracellular factor(s), possibly protease(s), that inactivate the elicitor. The inducing activity also was relatively heat stable: heat treating for 10 min at 100°C did not affect necrosis-inducing ability (Fig. 2). Therefore, necrotic response induction by CCS appeared to depend on heat-stable proteinaceous factor(s). Because the desalting process removed all molecules of less than 10 kDa, the necrosis-inducing factor(s) apparently were greater than 10 kDa.

Elicitor activities of CCS. Proteins from CCS of virulent strain CIC31 and nonpathogenic strain Cs4 were fractionated by precipitation with 40% ammonium sulfate and, subsequently, were desalted and concentrated 1,000-fold over that of the original culture supernatant. When infiltrated into tobacco, the preparation from

CIC31 gave a necrotic response at a protein concentration as low as 18 $\mu\text{g/ml}$. The preparation from Cs4 was tested at concentrations up to 180 $\mu\text{g/ml}$, and no response was seen on tobacco.

The necrotic response—an active plant defense reaction. The eukaryotic metabolic inhibitors cycloheximide, α -amanitin, sodium vanadate, and lanthanum chloride were evaluated for their ability to interfere with the necrosis-inducing activity of CCS from virulent strain Cs7. CCS infiltrated into tobacco leaves along with metabolic inhibitors did not trigger any response, whereas the usual necrotic response was elicited in tobacco by untreated CCS (data not shown).

To confirm that the necrotic response was due to a nonhost plant defense reaction rather than a nonspecific toxin, leaves of cvs. Sangre and Ute Russet potato, as well as eggplant, were infiltrated with bacterial suspensions of virulent strains CIC31, Cs7, and R14, as well as concentrated CCS from CIC31 and R14. Neither bacterial cells nor CCS gave a rapid necrotic reaction in any of the host plants, although both induced a rapid necrotic response in tobacco. In contrast, *E. amylovora* elicited a strong HR in tobacco, potato, and eggplant. Furthermore, the ammonium sulfate-precipitated protein fraction from CIC31 did not induce a necrotic reaction on eggplant at a concentration of 180 μg of total protein per ml, although it induced a necrotic response on tobacco at 18 $\mu\text{g/ml}$.

Partial protein purification. When CCS proteins from strains CIC31 (virulent) and CS3NM (nonpathogenic) were separated on DEAE cellulose, necrosis-inducing activity by CIC31 was eluted at 250 and 300 mM NaCl. None of the fractions from CS3NM elicited a reaction (Table 2). SDS-PAGE of CCS from CIC31 and CS3NM showed many extracellular proteins (Fig. 3), as well as numerous differences between the two strains. In comparison, protein profiles of 250 mM salt fractions from CIC31 and CS3NM contained fewer major bands. In addition to several minor differences, one major band at ~25 kDa appeared in the 250 mM NaCl DEAE cellulose fraction from CIC31 but not in the same fraction from CS3NM.

DISCUSSION

We report the induction of a rapid localized necrotic response in leaves of the nonhost tobacco when infiltrated with virulent strains of the gram-positive plant pathogen *C. michiganensis* subsp. *sepedonicus*. Within 18 h, the infiltrated area collapsed, and by 36 h, the entire infiltrated area was necrotic, with collapse and necrosis contained strictly within the infiltrated area. By 48 h, the collapsed area was dry and papery. In contrast, induction of localized rapid necrosis was not observed in leaves of potato, the only known natural host of *C. michiganensis* subsp. *sepedonicus*, nor in eggplant, an artificial host. The necrotic reaction on tobacco was seen only with strains of *C. michiganensis* subsp. *sepedonicus* that were pathogenic on host plants. Strains that showed different levels of virulence on the two hosts tended to produce weaker, less reliable necrotic reactions. However, the lack of response of plants to nonpathogenic strains was consistent, in that nonpathogenic strains never induced localized necrosis.

The timing and localization of the necrotic reaction obtained in tobacco with *C. michiganensis* subsp. *sepedonicus* is the same as

TABLE 2. Hypersensitive response (HR)^a induced by DEAE cellulose fractions from *Clavibacter michiganensis* subsp. *sepedonicus*

Strain	Total protein ^b	Concentration of NaCl (mM) for eluted DEAE fractions ^c								
		0	50	100	150	200	250	300	500	950
CIC31 (virulent) ^d	+++	—	—	—	—	—	+++	+	—	—
3NM (nonpathogenic) ^e	—	—	—	—	—	—	—	—	—	—

^a A 30- μl desalted sample was infiltrated into tobacco cv. Xanthi and scored at 24 h postinoculation. + = mild HR; +++ = strong HR; and — = no HR.

^b Cellfree culture supernatants were concentrated, desalted, and brought up to 400 μl with Bis-Tris (BT) buffer before loading on the column.

^c Each fraction was concentrated, desalted, and brought up to 400 μl with BT buffer before infiltration.

^d Two hundred, twenty micrograms of total protein was loaded on the column.

^e Three hundred, eighty micrograms of total protein was loaded on the column.

that for the HR described for gram-negative plant pathogens, which elicit an HR on tobacco at 8- to 24-h postinfiltration, depending on the bacterial species (32). Given the resemblance between the necrotic reaction we observe in tobacco and the HR described for gram-negative pathogens in terms of timing, localization, and association with pathogenicity, we conclude that *C. michiganensis* subsp. *sepedonicus* elicits a typical HR in tobacco.

The relatively high concentrations of cells used to obtain an HR with *C. michiganensis* subsp. *sepedonicus* are similar to concentrations needed for some other bacterial species. We used either 3×10^8 CFU/ml for bacteria resuspended in phosphate buffer or 1.3×10^9 CFU/ml for bacteria resuspended in water. In an extensive study of variables influencing the HR induced by gram-negative bacteria (32), two of the strains tested required 10^9 CFU/ml to cause an HR on cv. Glurk tobacco, whereas under certain conditions other strains caused an HR with as low as 10^6 CFU/ml. One possible concern with the high levels of bacteria we used is that, in some cases, compatible bacteria (*P. syringae* pv. *tabaci*) at high concentrations may produce HR-like necroses (37). In addition, with proper manipulation it is possible to induce necrosis in tobacco with nonpathogenic *P. fluorescens* (40). However, even at very high bacterial concentrations (8×10^9 cells per ml) in water or phosphate buffer, we found that nonpathogenic strains never caused an HR. Therefore, the necrotic reaction induced by virulent strains of *C. michiganensis* subsp. *sepedonicus* on tobacco is probably not an artifact of the high levels of bacterial inoculum used.

Only CCS prepared from virulent strains of *C. michiganensis* subsp. *sepedonicus* elicited the HR, which was the same in appearance and timing as that obtained with whole bacterial cells. The HR-inducing activity was protease sensitive and heat stable. Additionally, ammonium sulfate-precipitated proteins from nonpathogenic strain Cs4 produced no reaction on tobacco, even at concentrations 10× higher than that needed to obtain an HR with virulent strain CIC31, confirming the correlation between virulence and HR induction.

To demonstrate that the reaction induced by this elicitor preparation is an active plant defense response rather than a toxic response, we confirmed the necessity for active plant metabolism

for eliciting the HR. Coinfiltration of bacteria with various eukaryotic metabolic inhibitors has suggested that the HR is initiated by the plant and requires active plant metabolism. The availability of purified harpin_{PS} and harpin_{EA} established with certainty that the HR requires de novo gene expression and protein synthesis, calcium flux across membranes, and ATPase activity (29,30) and that the effect of the inhibitors is not due to the action of the inhibitors on the bacteria themselves. Similarly, we used four inhibitors of eukaryotic metabolism— α -amanitin (eukaryotic RNA polymerase II inhibitor), cycloheximide (80S ribosome inhibitor), vanadate (ATPase and phosphatase inhibitor), and lanthanum (calcium channel blocker)—to show that the HR induced by the crude protein preparations from *C. michiganensis* subsp. *sepedonicus* is dependent on active plant metabolism. The possibility still remains that we could be observing the action of a toxin that also is dependent on plant metabolism. However, it is unlikely that a toxin would depend, as the HR does, on all four of the aspects of plant cell metabolism that are inhibited by the various chemicals used.

The most well-studied classes of bacterial HR-inducing proteins are the harpins and PopA. Harpins have been isolated from *P. syringae* pv. *syringae* (30), *P. syringae* pv. *glycinea* and *P. syringae* pv. *tomato* (49), *E. amylovora* (52), and *E. chrysanthemi* (10), and PopA has been isolated from *R. solanacearum* (5). These heat-stable proteins elicit an HR that is similar to that induced by whole cells; the proteinaceous elicitor fraction from *C. michiganensis* subsp. *sepedonicus* resembles harpins in this aspect. However, it is now clear that HR-inducing activity in bacteria is not strictly linked to harpin expression (2,5,8), and the role of harpins in pathogenicity is still unclear (15). Thus, we cannot assume that the elicitor(s) described here have a clear role in either pathogenicity or induction of HR by whole cells.

One important difference between the *C. michiganensis* subsp. *sepedonicus* elicitor fraction and harpins is that some of the harpins are expressed only in specific minimal media (4,30,52). We were fortunate that the *C. michiganensis* subsp. *sepedonicus* elicitor activity is expressed in rich medium, because *C. michiganensis* subsp. *sepedonicus* grows very slowly on minimal agar medium and not at all in minimal liquid medium.

A purification step that has been useful with harpins from *P. syringae* pv. *syringae* (30) and *E. amylovora* (52) is to remove denatured proteins by centrifugation after heat treatment, leaving behind little other than the elicitor protein. This was not useful for *C. michiganensis* subsp. *sepedonicus*, because we never saw significant differences on SDS-PAGE between heat-treated, centrifuged samples and nontreated samples (data not shown). Using bovine serum albumin as a carrier to coprecipitate denatured proteins did not improve purification.

Even nonmucoid strains of *C. michiganensis* subsp. *sepedonicus* produce substantial quantities of extracellular polysaccharides (EPS) (31,53). Removal of EPS obviously is necessary to purify proteins from CCS, and in fact, we found that CCS proteins failed to fractionate when run on three different microscale high pressure liquid chromatography column types (strong anion exchange, gel filtration, and hydrophobic interactions columns). We found that the best way to fractionate proteins and remove EPS was to purify the proteins on DEAE cellulose with a sodium chloride gradient. Most EPS eluted with the void volume or at low salt (<150 mM), whereas proteins eluted at 200 mM NaCl and higher. The majority of the HR-eliciting activity was eluted in the 250 mM NaCl fraction, which contained very little EPS (data not shown). A comparison of proteins in the 250 mM NaCl fractions from virulent and nonpathogenic strains shows a few minor differences, plus one major band difference at ~25 kDa that is only in the virulent strain. This could be a candidate for an elicitor protein.

Harpins, as well as other virulence factors, are exported from gram-negative cells by the *hrp* (or type III) secretion apparatus. Although the elicitor(s) described here bear a superficial resemblance to harpins, any suggestion of the existence of an *hrp* locus

Fig. 3. Sodium dodecyl sulfate-10% polyacrylamide gel of cellfree culture supernatant proteins from virulent and nonpathogenic *Clavibacter michiganensis* subsp. *sepedonicus* strains (protein loaded in micrograms). Lane 1, strain CIC31 (11.4 µg); lane 2, strain CS3NM (11.9 µg). Lanes 3 and 4, proteins eluting from DEAE at 250 mM NaCl. Lane 3, from strain CIC31 (<0.35 µg); lane 4, from strain CS3NM (0.84 µg). Proteins were visualized by silver staining.

in *C. michiganensis* subsp. *sepedonicus* must consider the difference between the gram-negative and -positive cell wall. Although there is precedent for homologous transport systems in gram-positive and -negative bacteria (47), the gram-positive systems must have modifications to allow for the single cell membrane. A system in gram-positive pathogens homologous to the *hrp* system might be expected to conserve only the components involved in transport through the inner membrane (e.g., *hrcV*, *hrcR*, *hrcS*, *hrcT*, and *hrcU* [12,15]). Alternatively, due to the considerable genetic distance between gram-negative and -positive bacteria, it is reasonable to suggest that this elicitor protein could be secreted through an unrelated secretion system.

Determining the role of HR-eliciting factors in *C. michiganensis* subsp. *sepedonicus* infection will be difficult, because approaches for mutagenesis and complementation are limited for this species (44). Reverse genetics, in which a purified protein is sequenced at the amino acid level and used to identify the corresponding gene, have been instrumental in cloning genes from organisms that are recalcitrant to genetic manipulation. Thus, the finding that an HR can be elicited by a proteinaceous fraction from *C. michiganensis* subsp. *sepedonicus* provides an obvious route to isolating genes that may have a role in HR induction by *C. michiganensis* subsp. *sepedonicus*.

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