Relation of Tobacco Hypersensitivity to Pathogenicity of *Erwinia rubrifaciens*

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ABSTRACT

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Thirty-five strains of *Erwinia rubrifaciens* derived from 16 counties in California induced the hypersensitive response (HR) in Havana tobacco leaves at high inoculum levels (3×10^9 cfu/ml) and were pathogenic in Hartley walnut trees. However, HR$^-$ mutants obtained by either transposon Tn10 insertion or nitrosoguanidine mutagenesis were equally pathogenic, indicating a lack of correlation between HR and pathogenicity. Endopolygalacturonic acid *trans*-eliminase (PATE) activity was present in osmotic shock extracts of both the wild-type strain and the mutant derivatives. These findings do not permit the use of the tobacco hypersensitivity reaction for a rapid means of screening for pathogenic strains of *E. rubrifaciens*. They also suggest that more than PATE is involved in the HR in tobacco.

Additional key words: *Juglans regia*, plasmid pSa.

Erwinia rubrifaciens Wilson et al, the causal agent of the deep bark canker disease of the Persian (English) walnut *Juglans regia* L., is endemic in California and probably originated from a single source (1).

This pathogen is transmitted by the mechanical harvester (8), and only a few cells of *E. rubrifaciens* are needed to establish infection, providing that infection sites (shaker wounds) are made almost simultaneously with initial deposition of the inoculum (21).

Following initial infection of the wound site, the bacteria systemically invade the tree (4) and eventually cause extensive necrosis of the nonfunctional secondary phloem (22). The nature of this necrosis is not well understood. However, we have shown previously that *E. rubrifaciens* induces the typical hypersensitive response (HR) in tobacco leaves infiltrated with cells of *E. rubrifaciens* or with a cell-free osmotic-shock extract of the bacterium (5). The HR inducer in the extract was identified (5) as endopolygalacturonic acid *trans*-eliminase (PATE) with a pH optimum of 9.1, which coincidentally is identical to the pH of the sap of *J. regia* 'Hartley' (H. Azad, *unpublished*). Less than 1 μg of purified PATE was sufficient to induce the HR reaction in a tobacco leaf panel within 60 min (5).

The HR in tobacco is routinely used to aid in the identification of bacterial pathogens (16), and considerable reliance is placed on it for virulence assessments since saprophytic bacteria do not induce an HR (12,13). Various pathogenic *Erwinia* species induce HR (6,14), so that HR induction by *E. rubrifaciens* is not unique for Erwiniae. Whether or not virulence of this pathogen is related directly to its ability to induce an HR has not been established. In the present communication, we have employed genetic approaches to answer this question.

MATERIALS AND METHODS

Bacterial strains. Strains of *E. rubrifaciens* were isolated from infected Hartley walnut trees located in various California counties (Table 1). The pathogen was isolated as described previously (4,7). *Escherichia coli* C600(pSa::Tn10) was obtained from Stephen Farrand, and the Tn10 insertion in plasmid pSa was mapped within the 10.5-kb site on the pSa map (23) by Robert C. Tait in our laboratory. *E. coli* J53-1(pSa) was provided by Esther Lederberg. *E. coli* HB101 F$^-$ *pro leu thi lac Y* Str *r- m-* EndoI-RecA$^-$ was obtained from Ray Rodriguez.

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Culture media. Strains of *E. rubrifaciens* were cultured on medium YDC (1) and medium 523 (9). Strains of *E. coli* were grown on medium 523 and LB agar (1.5%) plates and broth (18). Minimal agar medium 925 and PM buffer were formulated as described previously (15) except glucose, which was substituted for sucrose, was added separately after autoclaving. Antibiotic plates contained tetracycline (Tc) (30 μg/ml), kanamycin (Km) (50 μg/ml), chloramphenicol (Cm) (40 μg/ml), spectinomycin (Sp) (50 μg/ml), or gentamycin (Gm) (100 μg/ml) as specified in the text. All antibiotics were purchased from Sigma Chemical Company, St. Louis, MO 63178.

Nitrosoguanidine mutagenesis. *E. rubrifaciens* 6D380 cells were grown in medium 523 at 30 C for 7 hr with aeration. Nitrosoguanidine (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) (Aldrich Chemical Co., Milwaukee, WI 53233) was dissolved in 100 mM sodium citrate buffer, pH 5.5, and mutagenesis was performed as described previously (15). Auxotrophic mutants were eliminated by selection on minimal medium 925.

TABLE 1. The sources and origin of strains of *Erwinia rubrifaciens* used to study the relation of tobacco hypersensitivity and pathogenicity to walnut trees

Strain	California county	Source (reference)
6D380 (Type strain)	Solano	This article
6D365	Tehama	H. Azad and C. I. Kado (1)
6D366 and 6D373	Glenn	H. Azad and C. I. Kado (1)
6D374, 6D375, 6D376, and 6D379	Butte	H. Azad and C. I. Kado (1)
6D345	Colusa	N. Schaad, UC Davis
6D369 and 6D381	Yuba	H. Azad and C. I. Kado (1)
6D372 and 6D368	Sutter	H. Azad and C. I. Kado (1)
6D360 and 6D356	Sutter	N. Schaad, UC Davis
6D3 and 6D34	Yolo	E. E. Wilson, UC Davis
6D330	Yolo	Lee Jackson, UC Davis
6D370	Yolo	H. Azad and C. I. Kado (1)
6D364	Solano	H. Azad and C. I. Kado (1)
6D377	Contra Costa	H. Azad and C. I. Kado (1)
6D361	San Joaquin	N. Schaad, UC Davis
6D327	Stanislaus	J. Gardner, UC Davis
6D331, 6D332, 6D337, 6D339, 6D348, and 6D349	Stanislaus	N. Schaad, UC Davis
6D343 and 6D344	Merced	N. Schaad, UC Davis
6D363	Madera	W. Moller, UC Davis
6D367	Fresno	H. Azad and C. I. Kado (1)
6D371	Tulare	H. Azad and C. I. Kado (1)
6D378	Kings	H. Azad and C. I. Kado (1)

Transposon mutagenesis. Exponentially growing cells of *E. coli* C600(pSa::Tn10) and *E. rubrifaciens* 6D380 were mixed in equal proportions and mated on sterile membrane filters (Millipore 0.45 μM pore size, Millipore Corporation, Bedford, MA 01730) as described previously (11). Selections were made for Tc^r and Km^r in antibiotic agar medium 925. Transconjugants were verified as *E. rubrifaciens* as described previously (1,4) and checked for the transfer of plasmid pSa::Tn10 by the rapid mini-screen method (10). All of the colonies examined were cells of *E. rubrifaciens* harboring pSa::Tn10. These transconjugants were freed of the plasmid by growing them repeatedly in 4 ml of medium 523 containing acridine orange (40 μg/ml), at 30 C with aeration for 24 hr. This was done by transferring 10 μl of inoculum to fresh medium seven consecutive times after each 24-hr incubation. At these concentrations of acridine orange, no increase in spontaneous Tc resistance was observed with parental controls.

Transformation. Transformations were performed as described by Morrison (19).

***Trans*-eliminase assay.** Endopolygalacturonic acid *trans*-eliminase activity was assayed by the method of Nagel and Anderson (20). The reaction conditions of the assay were the same as those described previously (5).

Plasmid isolation and purification. Plasmids were isolated and analyzed by the method of Kado and Liu (10) or by the Froman method (23).

Hypersensitivity assay. Tobacco hypersensitivity assays were performed on the leaves of *Nicotiana tabacum* 'Havana' by using the method of Klement et al (12,13). Necrosis and death of the infiltrated portions of the leaf panels within 24 hr was scored as positive HR. For the assessment of polygalacturonate *trans*-eliminase inducing HR, osmotic shock fluids, prepared as described previously (5), were used.

Sterile distilled water and culture broth were used in control infiltrations. Positive controls also included each of the strains of *E. rubrifaciens* that was tested.

Pathogenicity assay. Cells of *E. rubrifaciens* were grown for 24 hr at 30 C on medium YDC and were resuspended in sterile PM buffer to a final cell density of 2×10^9 cfu/ml as determined turbidimetrically and confirmed by standard dilution plate assays on medium 523. All inocula were prepared the same day the inoculations were performed. Twelve-year-old trees (*J. regia* L. 'Hartley') on rootstocks of black walnut (*J. hindsii* Jepson) located in the Armstrong field plot, UC Davis, were used. Five sites on the trunk and primary branches of each tree, at least 1 m above the graft union were selected. Deep vertical wounds were made by driving a flat-bladed wood chisel (13-mm blade width) through the inner phloem region to the xylem. Inoculum (0.1 ml) was placed into each of the four wounds in the region of the phloem with a 1.0-ml sterile disposable pipette. The fifth wound, serving as control, was inoculated with the same amount of sterile PM buffer in the same manner as the other four. Two trees, with a total of 10 inoculations, were used per strain. Each inoculation site was marked by white paint. The inoculations were made on 15 October 1977 and on 27 July 1978, the times of the year when the cultivar Hartley plants are highly susceptible to infection (21).

To assess susceptibility, all inoculation sites were examined on 1

TABLE 2. Hypersensitivity response on tobacco leaves in relation to mean canker extension on walnut trees inoculated with strains of *Erwinia rubrifaciens*

Strain	Hypersensitivity response[x]	Length of canker extension[y] above inoculation site (cm)	Length of canker extension[y] below inoculation site (cm)	Total length of canker extension[y] (cm)
6D374	+	6.9	14.1	21.0 a[z]
6D330	+	23.2	8.1	31.3 ab
6D339	+	31.9	13.6	35.3 abc
6D379	+	16.6	25.5	42.1 abcd
6D331	+	28.6	19.0	47.6 abcd
6D360	+	43.8	6.2	50.0 abcd
6D3	+	36.6	24.2	60.8 abcde
6D332	+	38.0	26.3	64.3 abcde
6D349	+	33.3	31.7	65.0 abcde
6D348	+	35.0	32.5	67.5 abcde
6D372	+	50.0	21.0	71.0 abcde
6D364	+	47.9	27.4	75.3 abcde
6D363	+	50.2	27.1	77.3 abcde
6D365	+	47.2	30.7	77.9 abcde
6D381	+	54.3	31.2	85.5 abcdef
6D343	+	55.6	30.0	85.6 abcdef
6D356	+	51.9	39.1	91.0 abcdef
6D380	+	54.6	38.5	93.1 abcdef
6D368	+	60.2	34.1	94.3 abcdef
6D344	+	50.5	47.5	98.0 abcdef
6D373	+	66.6	31.5	98.1 abcdef[z]
6D376	+	61.6	38.2	99.8 abcdef
6D337	+	85.1	33.4	118.5 bcdefg
6D361	+	88.5	32.9	121.4 bcdefg
6D367	+	82.5	39.4	121.9 bcdefg
6D375	+	92.0	31.4	123.4 bcdefg
6D327	+	92.8	34.1	126.9 cdefg
6D370	+	79.4	48.4	127.8 cdefg
6D371	+	91.3	36.6	127.9 cdefg
6D378	+	93.3	38.6	131.9 defg
6D369	+	109.2	36.7	145.9 efg
6D34	+	108.2	40.8	149.0 efg
6D345	+	123.1	49.4	172.5 fg
6D366	+	131.5	44.6	176.1 fg
6D377	+	153.8	42.5	196.3 g

[x] Inoculum (3.0×10^9 cfu/ml) used in infiltration of tobacco leaves.
[y] Figures represent an average of eight replicates. Data are rounded to the nearest millimeter.
[z] Entries with a common letter are not significantly different according to Duncan's multiple range test of mean differences, $P = 0.01$.

October 1978 and 15 August 1979, ~12–13 mo after each inoculation, and scored visually for the exudation of reddish-brown sap, which is a typical symptom of deep bark canker disease (4,7). The bark around each site and in both downward and upward directions from the site was then stripped away with a semicircular-bladed chisel. After being used on each tree, the chisel was flamed thoroughly to prevent cross contamination. The lengths of the lesions, detected by visual inspection of tissue in both vertical directions from the inoculation site were measured to the nearest millimeter. Sterilized forceps were used to obtain tissue samples (about 3 × 5 mm) from the diseased and apparently healthy internal regions that were exposed by the chisel. At least three samples from each inoculation site were plated immediately on YDC agar containing cycloheximide (250 μg/ml) to suppress possible fungal contaminants. These plates were taken back to the laboratory and incubated for 48 hr at 30 C. The bacteria from each plate were streaked on another series of YDC agar plates to verify that each colony produced the diffusible red pigment that is characteristic of *E. rubrifaciens* (1,7,21).

RESULTS

Induction of HR. *E. rubrifaciens* induced the characteristic HR within 24 hr after infiltration of leaf panels of *N. tabacum* cultivar Havana with inoculum containing from 1.5×10^9 to 3×10^9 cfu/ml. All 35 strains listed in Table 2 induced the HR. When lower concentrations (10^6 to 10^7 cfu/ml) of *E. rubrifaciens* were tested, strains 6D3, 6D379, 6D365, 6D380, 6D344, 6D371, 6D378, 6D34, 6D345, and 6D366 continued to induce strong HR, but the remaining strains induced chlorotic responses.

Pathogenicity. Besides the capacity to induce the HR, all strains of *E. rubrifaciens* were tested for virulence on cultivar Hartley walnut trees. Individual trees were inoculated with each strain tested during the summer when the trees were most susceptible and were examined ~12 mo later. All of the inoculated sites showed the characteristic reddish-brown exudate trailing downward from the site of inoculation. Necrotic lesions occurred beneath the bark and extended vertically from the inoculation site in both directions. Cankers generally extended further in the upward than in the downward direction (Table 2). The range of total (upward plus downward) canker extension was from 0.2 to about 2.0 m. These observations corroborate a previous report that cankers extend further in the upward direction than downward (7). Canker formation by all strains of *E. rubrifaciens* tested clearly indicated that they were pathogenic. This was verified by the isolation of the organism from each canker region. In fact, the bacterium was also recovered from 45% of the tissue samples taken from regions about 20–30 cm beyond any visible symptoms above the canker, which confirms our earlier observations (4,7). No cells of *E. rubrifaciens* were recovered in control inoculation sites and in regions above the inoculation site.

Genetic analysis. To determine if the capacity to induce the HR and pathogenicity are directly correlated, HR⁻ mutants were constructed as follows: The transposon Tn10, which confers tetracycline resistance (2), was introduced into the chromosome of *E. rubrifaciens* 6D380 by matings with *E. coli* C600(pSa::Tn10). Transconjugants, obtained at an average frequency of 4.2×10^{-4} exconjugants per recipient cell, were then freed of the vector plasmid by curing with acridine orange. Selections were made on the basis of Tc^r and Km^s. Of 6,432 cfu tested, 57 colonies were of this phenotype. As shown in Table 3, five of the 57 mutants were HR⁻. All 57 mutants grew on minimal medium 925, indicating that the chromosomal insertion of Tn10 did not result in the formation of an auxotrophic mutant. The efficacy of acridine curing was verified by mini-screen analyses on each Tc^r Km^s exconjugant. That *E. rubrifaciens* 6D380 is free of any native plasmids was demonstrated previously (10). To verify that Tn10 had indeed translocated into the chromosome of *E. rubrifaciens*, the native plasmid pSa was transferred into *E. rubrifaciens* 6D380 (Tn10) by mating with *E. coli* J53-1(pSa). Randomly chosen Tc^r Km^s Cm^r transconjugant colonies were selected on the basis of pSa and Tn10 markers (see above). Five independent crosses were made and in each case the presence of pSa was verified by mini-screen analysis. Plasmid pSa DNA was purified from representative colonies of each of the crosses and subsequently transferred to *E. coli* HB101 by transformation. The resulting transformants were Tc^r and carried pSa antibiotic resistance markers, ie, Km, Cm, Gm, and mini-screen analysis verified that the transconjugants with these phenotypes carried plasmid pSa. The results of these analyses are summarized in Table 3.

Nitrosoguanidine mutants. Exponentially growing cells of *E. rubrifaciens* were mutagenized with nitrosoguanidine, and mutants were plated on minimal medium 925. All of the mutants recovered were screened for ability to induce the HR. One of the 186 mutants screened was HR⁻ and was prototrophic. This mutant, 6D380-N1, was fully virulent on Hartley walnut, producing the characteristic deep bark canker symptoms at and beyond the site of inoculation.

Relationship of HR to endopolygalacturonic acid *trans*-eliminase activity. Endopolygalacturonic acid *trans*-eliminase (PATE) is found in the periplasmic space of *E. rubrifaciens* and purified preparations of the enzyme induced the HR in tobacco (5). Analyses made on the Tn10- and nitrosoguanidine-induced HR⁻ mutants showed that these mutants retained PATE activity (Table 4). Analysis of the osmotic shock fluids of these mutants showed that when *E. rubrifaciens* harbored pSa, PATE activity was roughly 30% lower than the wild-type strain (Table 4).

DISCUSSION

E. rubrifaciens, the causal agent of deep bark canker disease of Persian walnuts, induces the HR response in Havana tobacco leaves. Although HR is considered diagnostic for pathogenicity of

TABLE 3. Comparative analysis of Tn10 insertional mutants of *Erwinia rubrifaciens*

Strain or mutant	Prototrophic	Resistance to: Sp[a]	Km	Cm	Tc	pSa present	HR
6D380	+	−	−	−	−	−	+
6D380 (pSa)	+	+	+	+	−	+	+
6D380(pSa::Tn10)	+	+	+	+	+	+	+
6D380::Tn10-(1 through 52)	+	−	−	−	+	−	+
6D380::Tn10-53	+	−	−	−	+	−	−
6D380::Tn10-54	+	−	−	−	+	−	−
6D380::Tn10-55	+	−	−	−	+	−	−
6D380::Tn10-56	+	−	−	−	+	−	−
6D380::Tn10-57	+	−	−	−	+	−	−
Escherichia coli J53-1(pSa)	−	+	+	+	−	+	−
E. coli C600-(pSA::TN10)	−	+	+	+	+	+	−

[a] Abbreviations: Sp = spectinomycin, Km = kanamycin, Cm = chloramphenicol, Tc = tetracycline, and HR = hypersensitive reaction in tobacco leaves.

TABLE 4. Endopolygalacturonic acid *trans*-eliminase (PATE) activities in relation to tobacco hypersensitivity

Strain/ mutant	Tobacco HR	PATE[a] induced HR	PATE (% sp. act.)
6D380	+	+	100
6D380 (pSA::Tn10)	+	+	73.9
6D380::Tn10-1	+	+	93.6
6D380::Tn10-53	−	+	88.9
6D380-N1	−	+	96.8
6D380::Tn10(pSa)	−	+	64.5

[a] PATE activity was released by osmotic shock of cells of *Erwinia rubrifaciens* as described previously (5). The osmotic shock fluid was concentrated by dialysis against glycerol at 4 C to 20% of its original volume and then dialyzed against distilled water at 4 C. The concentrated fluid was infiltrated into leaf panels of Havana tobacco. The response time for the HR was delayed at least 5 days.

certain *Pseudomonas* species, our present studies show the lack of correlative evidence with *E. rubrifaciens*. Thus, the potential of using the tobacco HR response as a determinative assay to screen for pathogenicity of *E. rubrifaciens* appears to be inapplicable. Although all of the isolates of *E. rubrifaciens*, which were derived from different regions in California, were pathogenic and induced the tobacco HR, no positive correlation between these two phenotypes was found. That all of the HR^- mutants remained pathogenic to cultivar Hartley walnut trees strongly indicates that mutations affecting HR do not affect the pathogenicity of *E. rubrifaciens*. We were unable to isolate naturally avirulent mutants, but those generated by mutagenesis were all auxotrophic and therefore their use in these experiments was inappropriate (H. Azad, *unpublished*). It was interesting that mutations resulting in the HR^- phenotype did not appreciably reduce the levels of PATE activity. The precise role of PATE in pathogenicity obviously cannot be explained by the present studies; however, the present data suggest that there may be additional components of *E. rubrifaciens* involved in HR induction. It is also feasible that although PATE activity is detectable, there is insufficient quantities of the enzyme elaborated by these HR^- mutants to elicit the HR. As suggested in Table 4, the presence of pSa may affect the synthesis of PATE. We have previously observed virulence suppression of *Agrobacterium tumefaciens* when pSa was introduced into this bacterium (3,17). Although the virulence of *E. rubrifaciens* to *J. regia* remains unaffected by pSa, further studies on the effect of this plasmid on PATE activity and perhaps other possible enzymatic activities may have merit. The enzyme clearly induces the HR in tobacco (5), but its role in disease production in walnut remains unclear. Both the wild-type strain and its HR^- mutant derivatives had comparable levels of PATE activity in osmotic shock fluid concentrates. The HR induced by infiltrating these preparations resulted in a delayed necrotic response in tobacco leaves. This may be explained by the fact that crude extracts were used, thus presumably insufficient amounts of the enzyme were present in the preparation either due to protease degradation or to inhibitors. Further studies on the effects of PATE on inducing necrosis of the inner phloem tissues of the walnut tree host should elucidate its role in pathogenicity.

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