Knot Formation Caused by *Pseudomonas syringae* subsp. *savastanoi* on Olive Plants Is *hrp*-Dependent

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ABSTRACT

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The virulence of *Pseudomonas syringae* subsp. *savastanoi*, which causes hyperplastic symptoms (knots) on olive plants, is associated with secreted phytohormones. We identified a Tn5-induced mutant of *P. syringae* subsp. *savastanoi* that did not cause disease symptoms on olive plants although it was still able to produce phytohormones. In addition, the mutant failed to elicit a hypersensitive response in a nonhost plant. Molecular characterization of the mutant revealed that a single Tn5

insertion occurred within an open reading frame encoding a protein 92% identical to the HrcC protein of *P. syringae* pv. *syringae*. Moreover, sequence analysis revealed that the gene encoding the HrcC protein in *P. syringae* subsp. *savastanoi* was part of an operon that included five genes arranged as in other phytopathogenic bacteria. These results imply that *hrp/hrc* genes are functional in *P. syringae* subsp. *savastanoi* and that they play a key role in the pathogenicity of this plant pathogen.

Additional keywords: *hrcC* gene, *hrpC* operon, *Olea europaea*, olive knot disease.

Pseudomonas syringae subsp. *savastanoi* (ex Smith, 1908) Janse (21) (= *P. savastanoi* pv. *savastanoi*) (10,45) causes hyperplastic symptoms on olive (*Olea europaea* L.), oleander (*Nerium oleander* L.), and ash (*Fraxinus excelsior* L.) as well as on other minor host plants. Symptom development on olive and oleander plants is associated with the ability of the bacterium to produce phytohormones (indole-3-acetic acid [IAA] and cytokinins) (19, 36,37) that alter the physiological hormone balance in infected tissues and cause the proliferation of plant cells surrounding the infection site. Although several studies have provided significant information on the genetic determinants and metabolic regulation of phytohormone production by *P. syringae* subsp. *savastanoi* (8,12,31,44), the involvement of other pathogenicity determinants in the interaction of this plant pathogen with its host plants still remains to be established.

The ability of many plant-pathogenic bacteria to cause disease in host plants and to induce a hypersensitive response (HR) in resistant and nonhost plants depends on *hrp* genes (24). At first, *hrp* genes were so named because the characteristic phenotype associated with *hrp* mutants is their inability to cause disease in susceptible host plants and the inability to elicit HR in nonhost plants as well as in resistant cultivars of susceptible plants (40). These genes are thought to be universal among necrosis-causing, gram-negative, plant-pathogenic bacteria (1) and have been extensively characterized in the gall-forming plant pathogen *Erwinia herbicola* (*Pantoea agglomerans*) pv. *gypsophilae* (28–30), although their role in the pathogenicity of a hyperplasia-causing *Pseudomonas* sp. such as *P. syringae* subsp. *savastanoi* has not yet been ascertained.

hrp genes are typically clustered; *hrp* gene clusters encode components of a type III protein secretion system that is believed to be used by phytopathogenic bacteria to transport, directly into the host cells, virulence proteins which are ultimately responsible

for (i) leakage of plant nutrients to the extracellular space of infected tissues and (ii) suppression of host defenses (7,14,17). *hrp* gene clusters have been identified and studied in plant-pathogenic bacteria of the genera *Pseudomonas*, *Erwinia*, *Ralstonia*, *Xanthomonas*, and *Pantoea* (24); they have been divided into two groups on the basis of regulatory systems, possession of similar genes, operon structures, and gene arrangements (1,2). Furthermore, nine *hrp* genes have been renamed *hrc* (for HR and conserved) to denote their conservation among the type III secretion systems of both plant and animal pathogens (5), and one of these genes, *hrcC*, has been particularly well studied (2,6,9,18).

In order to identify virulence determinants other than phytohormones in *P. syringae* subsp. *savastanoi*, a collection of Tn5-induced mutants was generated from a strain isolated from an olive plant (35). Some of these mutants have been described previously; however, in those cases, Tn5 insertions occurred in DNA regions different from the *hrp* gene cluster of *P. syringae* pv. *syringae* strain 61, although the presence of *hrp/hrc* genes was previously indirectly revealed in *P. syringae* subsp. *savastanoi* by Southern hybridization (35). Additional Tn5 mutagenesis of *P. syringae* subsp. *savastanoi* have led to identification of another mutant (ITM317-1421) that was unable to elicit a hypersensitive reaction in a nonhost plant (tobacco) and also failed to cause disease symptoms on olive plants. In the present study, we demonstrate that inactivation of the *hrp* gene cluster completely abolished knot formation by *P. syringae* subsp. *savastanoi* in spite of a normal production of IAA and cytokinins. We provide evidence that the transposon insertion occurred in a functional homologue of the *hrcC* gene that plays a key role in the interaction between *P. syringae* subsp. *savastanoi* and olive plants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *P. syringae* subsp. *savastanoi* strain ITM317 (37) and its mutant ITM317-1421 were grown at 26°C on King's medium B (KB) (23), or Woolley's medium (41), as indicated. Miller's minimal A medium (27) was used to evaluate the prototrophy of the Tn5-

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induced mutant of *P. syringae* subsp. *savastanoi*. *Escherichia coli* strains TG1 and HB101 (34) were grown overnight on Luria-Bertani (LB) agar or in LB broth (34) at 37°C. Plasmid pBluescript SK II (Stratagene, La Jolla, CA) and its recombinant derivatives were propagated in *E. coli* TG1. When appropriate, antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 20 µg/ml; and chloramphenicol, 50 µg/ml.

Hypersensitivity assays and pathogenicity tests. The Tn5-induced mutant ITM317-1421 of *P. syringae* subsp. *savastanoi* and its parental strain ITM317 were tested for hypersensitive reaction on tobacco plants, as described previously (35), by using bacterial suspensions containing about 3×10^8 CFU/ml, as determined by a viable cell count on KB agar. To evaluate the pathogenicity of the mutant that failed to induce an HR, bacterial suspensions containing about 1×10^6 and 2×10^7 CFU/ml were inoculated into three wounds made in the bark of five 1-year-old olive stems; the parental strain was used for comparison. The experiments were repeated three times. The population dynamics within olive plants of the parental strain and its mutant derivative were determined after inoculation with about 10^5 CFU per inoculation site, as reported previously (35).

Production of phytohormones. The ability of *P. syringae* subsp. *savastanoi* strain ITM317 and its Hrp mutant ITM317-1421 to produce IAA and cytokinins in culture was assessed in Woolley's medium supplemented with 0.5 mM L-tryptophan. The bacteria were grown with shaking in the above-mentioned medium, and after 6 days incubation, the cells were removed by centrifugation. The resulting supernatants were extracted four times with an equal volume of ethyl acetate at pH 2.8 and subsequently at pH 8.5. The organic extracts were evaporated under reduced pressure at 30°C and the residues dissolved in methanol. The IAA content in the acidic extracts and the cytokinin activity of the basic extracts were evaluated as described previously (35).

Genetic techniques, DNA manipulations, and sequencing. Tn5-induced mutants of strain ITM317 were obtained as described previously (35). Genomic DNA of *P. syringae* subsp. *savastanoi* was extracted with the AquaPure Genomic DNA Isolation Kit (Bio-Rad Laboratories, Hercules, CA). Plasmid DNA of *P. syringae* subsp. *savastanoi* and *E. coli* was isolated with the Quantum Prep Plasmid Miniprep Kit (Bio-Rad). Plasmid DNA of *P. syringae* subsp. *savastanoi* was isolated according to the method of Hansen and Olsen (16) with some modifications. Standard procedures were followed for restriction endonuclease digestions, ligations, and agarose gel electrophoresis (34). DNA labeling, hybridization, and detection were carried out according to the supplier's instructions, using a nonradioactive digoxigenin-labeling and detection kit (Roche Molecular Biochemicals, Monza, Italy). To determine if a single Tn5 insertion had occurred in the genome of mutant ITM317-1421, total DNA preparations

were digested to completion with *Eco*RI or *Eco*RI/*Hind*III restriction endonucleases. The DNA fragments were separated by agarose gel electrophoresis and hybridized with plasmid pGS9 (carrying Tn5). Polymerase chain reaction (PCR) primers hrcC-2F (5'-GACCGGCTTGGTCAGGAAT-3') and hrcC-2R (5'-CGGCTTTTCCCGGATTTCT-3') were used to amplify a 593-bp DNA fragment from strain ITM317. The final concentrations of the components in the PCR reaction mixture were 1 ng of genomic DNA per µl, 1 mM MgCl₂, 10 mM Tris-HCl, 50 mM KCl, 0.5 µM (each) primer, 0.2 mM (each) deoxynucleoside triphosphate (dNTP), and 0.03 units of *Taq* DNA polymerase per µl. PCR amplifications were performed in a thermocycler (Model PE 9700; Applied Biosystems, Foster City, CA) under the following conditions: an initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 45 s, 55°C for 1 min, and 72°C for 2 min; followed by a single final extension at 72°C for 10 min. The *Eco*RI Tn5-containing fragment from the mutant was cloned into the plasmid vector pBluescript SK II (Stratagene) and the recombinant plasmid was introduced into *E. coli* TG1 by electroporation as described previously (35). After gel purification, the two *Eco*RI/*Hind*III fragments from the above recombinant plasmid were individually subcloned into the same plasmid vector. Regions flanking the Tn5 element were sequenced by using, as a primer, a single oligonucleotide complementary to and extending outward from the ends of the inverted repeat of transposon (33) and by primer walking. DNA sequencing was performed with BigDye Terminator Chemistry from Applied Biosystems on an ABI 310 Sequencer. Sequenced DNA fragments were examined for the presence of open reading frames (ORF) or for similarity with deposited sequences using the ORF Finder Program or the BLASTN and the BLASTX programs (3). All of the above-mentioned programs are available through the National Center for Biotechnology Information (available online at the NCBI website).

RESULTS

Identification and phenotypic characterization of mutant ITM317-1421. Mutant ITM317-1421 of *P. syringae* subsp. *savastanoi* was obtained from strain ITM317 from olive by random Tn5 insertion using the suicide plasmid pGS9 as described previously (35). This mutant was unable to elicit an HR (Fig. 1) and did not induce disease symptoms (i.e., knot formation) on olive plants (Fig. 2). Moreover, the ability of the mutant to grow in olive plant tissues was markedly reduced (Fig. 3). The parental strain and the mutant reached population sizes of 1.7×10^6 and

Fig. 1. Hypersensitive reaction on tobacco 24 h after infiltration with bacterial suspensions of *Pseudomonas syringae* subsp. *savastanoi* strains. **A**, Treatment with sterile distilled water; **B**, parental strain ITM317; and **C**, Tn5-induced *hrcC* mutant ITM317-1421.

Fig. 2. Symptoms on olive stems 90 days after inoculation with *Pseudomonas syringae* subsp. *savastanoi* strains. **A**, Treatment with sterile distilled water; **B**, parental strain ITM317; and **C**, Tn5-induced *hrcC* mutant ITM317-1421.

3.6×10^5 CFU per inoculation site 5 days after inoculation, respectively. Thereafter, strain ITM317 continued to multiply, reaching a population size of 7.0×10^7 CFU per inoculation site 15 days after inoculation. On the contrary, the population size of the mutant decreased abruptly to 3.7×10^3 CFU per inoculation site at the end of the tests.

Both the parental and the mutant strains grew similarly in minimal broth medium or in KB medium. Moreover, both strains produced equal amounts of IAA and cytokinins in culture.

Physical characterization of Tn5 insertion and cloning of the Tn5-containing fragment from mutant ITM317-1421. Southern analysis of *EcoRI*- and *EcoRI/HindIII*-digested DNAs of the mutant strain indicated one and three DNA fragments, respectively, that hybridized with plasmid pGS9 (carrying Tn5). The results demonstrated that a single Tn5 insertion occurred in the genome. As expected, no hybridization signal was observed in the DNA of parental strain ITM317 (Fig. 4). Furthermore, the probe did not hybridize to plasmid DNA of the parental or mutant strains, indicating that transposon insertion occurred in the chromosome.

The *EcoRI* Tn5-containing fragment from the Hrp mutant ITM317-1421 was cloned into the plasmid vector pBluescript SK II and the resulting recombinant plasmid was named pITM-1421. *EcoRI* and *EcoRI/HindIII* digestions of this recombinant plasmid and Southern blot analysis confirmed that the clone contained the expected *EcoRI* fragment. The approximate size of this fragment was estimated to be 24.6 kb. The two *EcoRI/HindIII* fragments from plasmid pITM-1421, containing part of the Tn5 transposon (1.2 kb) and the flanking DNAs from mutant ITM317-1421, were individually subcloned; they were designated 1421-FrB and 1421-FrS and had approximate sizes of 18.1 and 3.1 kb, respectively (Fig. 5A). The DNA flanking Tn5 insertion within the 1421-FrS fragment was sequenced completely, whereas the DNA region adjacent to the transposon within the 1421-FrB fragment was only partially sequenced.

Sequence analysis of DNA regions flanking Tn5 insertion in mutant ITM317-1421. The DNA regions flanking the Tn5 insertion site in mutant ITM317-1421 of *P. syringae* subsp. *savastanoi* were sequenced and a sequence of 4,050 bp was obtained. In this sequence, the 9-bp target sequence 5'-CGTCGACC-3', corresponding to Tn5 insertion in the mutant, was located from

Fig. 3. Growth curves of *Pseudomonas syringae* subsp. *savastanoi* ITM317 and its Tn5-induced *hrcC* mutant ITM317-1421 in olive stem tissues. Data points represent means of three independent determinations and the error bars indicate standard error of the mean.

position 1868 to 1877. By using PCR primers *hrcC*-2F and *hrcC*-2R, located 316 bp upstream and 268 bp downstream from the Tn5 insertion site in the mutant, respectively, a DNA fragment of 593 bp was amplified from the wild-type strain ITM317. This fragment was sequenced and the results confirmed that it was identical to the DNA regions flanking the Tn5 element in the mutant ITM317-1421, except for the above-mentioned 9 bp directly repeated duplications of the target DNA caused by Tn5 insertion in the mutant (4). Sequence analysis and database searches revealed that Tn5 insertion occurred in mutant ITM317-1421, at 550 bases from the ATG translational start codon, within a homologue of the *hrcC* gene; in addition, they revealed that this gene of *P. syringae* subsp. *savastanoi* was part of the *hrpC* operon that includes five ORFs. These five *P. syringae* subsp. *savastanoi* genes were predicted to encompass nucleotides 675 to 896, 877 to 1314, 1319 to 3415, 3415 to 3615, 3621 to 3965, and on the basis of their homologies to previously characterized *hrp/hrc* genes of *P. syringae* pathovars, they were named *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV*, respectively. A homologue of *hrpE* that includes nucleotides 10 to 588 also was found upstream of the *hrpC* operon (Fig. 5B). The *hrpE* and the genes of the *hrpC* operon were co-

Fig. 4. Southern blot analysis of digested genomic DNAs from the wild-type strain ITM317 of *Pseudomonas syringae* subsp. *savastanoi* and its Tn5-induced mutant ITM317-1421 probed with pGS9 (carrying Tn5). Lane 1, digoxigenin-labeled molecular weight marker II (Roche Molecular Biochemicals, Monza, Italy); lane 2, *EcoRI*-digested DNA from strain ITM317; lane 3, *EcoRI*-digested DNA from mutant ITM317-1421; and lane 4, *EcoRI/HindIII*-digested DNA from mutant ITM317-1421. The numbers on the left refer to the weight marker in kilobases.

linear and had the same transcriptional orientation as in *hrp* clusters of other *P. syringae* pathovars (9); moreover, they began with a typical ATG translational start codon and were terminated by a TGA stop codon, except for *hrpT* and *hrpV*, which showed a TAG stop codon. A typical consensus promoter sequence (*hrp*-box), consisting of TGGAACC-16N-CCACNNA (43) as well as a putative ribosome binding site (GGAG), were found upstream of the *P. syringae* subsp. *savastanoi* *hrpC* operon. In addition, downstream of the *P. syringae* subsp. *savastanoi* *hrpV* gene, a typical rho-independent terminator, characterized by an inverted repeat and a poly(T) was identified (Fig. 5B). The predicted products, HrpE, HrpF, HrpG, HrcC, HrpT, and HrpV, encoded by the

above-mentioned *hrp/hrc* genes of *P. syringae* subsp. *savastanoi*, showed a high degree of identity/similarity, with the deduced products encoded by the corresponding genes of *P. syringae* pathovars (Table 1). The percentages of identity ranged from 76 to 100% in most cases. In particular, HrcC protein of *P. syringae* subsp. *savastanoi* was 92 and 79% identical to HrcC of *P. syringae* pvs. *syringae* and *tomato*, respectively. On the contrary, HrpF of *P. syringae* pv. *tomato* seems to be an exception as it was divergent and only 36% identical to the HrpF of *P. syringae* subsp. *savastanoi*.

Nucleotide sequence accession number. The nucleotide sequence, which includes the *hrpE*, *hrpF*, *hrpG*, *hrcC*, *hrpT*, and

Fig. 5. A, Map of the *EcoRI* Tn5-containing fragment cloned from mutant ITM317-1421 of *Pseudomonas syringae* subsp. *savastanoi*. 1421-FrS and 1421-FrB are 3.1- and 18.1-kb *EcoRI*/*HindIII* fragments, respectively, subcloned from pITM-1421; Tn5, transposon Tn5. Black boxes denote the parts of the fragments that have been sequenced; gray box denotes the Tn5 transposon. **B,** Map of the DNA sequence of the *hrpE* gene and *hrpC* operon from *P. syringae* subsp. *savastanoi*. A potential consensus promoter sequence (*hrp*-box) and an inverted repeat are shown in the enlargements. The position of Tn5 insertion has been determined on the basis of DNA sequencing and is marked with a downward black arrowhead.

TABLE 1. Comparison of the predicted amino acid sequences for HrpE, HrpF, HrpG, HrcC, HrpT, and HrpV from *Pseudomonas syringae* subsp. *savastanoi* with those of homologous proteins from *P. syringae* pvs. *glycinea*, *phaseolicola*, *syringae*, and *tomato*^a

	Percentage of identity/similarity					
	HrpE	HrpF	HrpG	HrcC	HrpT	HrpV
<i>P. syringae</i> pv. <i>glycinea</i>	96/97	100	97/98	n.a.	100	97/98
<i>P. syringae</i> pv. <i>phaseolicola</i>	94/95	n.a.	n.a.	n.a.	n.a.	n.a.
<i>P. syringae</i> pv. <i>syringae</i>	82/90	91/96	83/91	92/95	89/98	90/97
<i>P. syringae</i> pv. <i>tomato</i>	77/88	36/60	76/86	79/87	79/91	79/90

^a n.a. indicates the sequence is not available in the database.

DISCUSSION

Prototrophic mutant ITM317-1421 was obtained by a single transposon (Tn5) insertion in the genome of a *P. syringae* subsp. *savastanoi* strain isolated from an olive tree. This mutant did not induce HR, even when highly concentrated bacterial suspensions (3×10^8 CFU/ml) were used; moreover, it did not cause knots on olive plants and was unable to multiply in host plant tissue. Phenotypic characterization showed that mutant ITM317-1421 was able to produce phytohormones and to multiply in culture similar to the parental strain. These results could suggest that Tn5 insertion occurred within an *hrp* gene; in addition, when DNA regions flanking Tn5 insertion in mutant ITM317-1421 were sequenced, a database search revealed that the predicted protein encoded by the inactivated gene was 92% identical to the HrcC protein of *P. syringae* pv. *syringae*. The *hrcC* gene is considered an essential gene of the *hrp/hrc* gene cluster because *hrcC* mutants of *P. syringae* typically exhibit a marked Hrp⁻ phenotype (9). Moreover, a clear Hrp⁻ phenotype also was detected in *hrcC* mutants of *Erwinia amylovora* (22), *X. campestris* (39), and *P. solanacearum* (*R. solanacearum*) (13). The encoded HrcC protein is thought to multimerize and form a channel for the translocation of proteins across the bacterial outer membrane (2,18) and is required for both HrpZ secretion and delivery of Avr signals (2,6). Sequence analysis of the DNA flanking Tn5 insertion in mutant ITM317-1421 revealed the presence of the two genes *hrpF* and *hrpG* upstream and *hrpT* and *hrpV* downstream of *hrcC*, respectively. Therefore, the mutated phenotypes in mutant ITM317-1421 also could be the result of a polar effect of Tn5 insertion on *hrpT* and *hrpV*. On the other hand, the effects of non-polar *hrcC*, *hrpT*, or *hrpV* mutations in *P. syringae* pv. *syringae* suggest that the mutated phenotypes in mutant ITM317-1421 are essentially the result of the *hrcC* inactivation (9,32). The functions for *hrpT* and *hrpV* also have been proposed (32): *hrpT* encodes a putative lipoprotein and might function as an HrcC chaperone; and *hrpV* encodes a negative regulator of *hrp* gene expression.

Because of the very high level of identity between the *hrp* genes sequenced in *P. syringae* subsp. *savastanoi* with their homologues of *P. syringae* pathovars, it also could be hypothesized that gene composition, gene arrangement, and operon organization of the *P. syringae* subsp. *savastanoi* *hrp* gene cluster mainly resemble those of the *hrp* gene cluster in *P. syringae* pathovars. In this regard, the levels of identity between the predicted products HrpE, HrpF, HrpG, HrcC, HrpT, and HrpV of *P. syringae* subsp. *savastanoi* genes and the homologous proteins in *P. syringae* pathovars seem to be related to the genetic distance between the above-mentioned plant-pathogenic bacteria. In fact, the levels of identity were higher in the case of comparison with the related pathovars *glycinea* and *phaseolicola*, both included in the same genomospecies as *P. syringae* subsp. *savastanoi*, than in the case of comparison with pathovars *syringae* and *tomato*, which were considered more divergent pathovars (11).

It is significant that a typical consensus promoter sequence, named *hrp*-box (43), was found upstream of the *P. syringae* subsp. *savastanoi* *hrpC* operon. In *P. syringae* pv. *syringae*, this conserved promoter sequence is recognized by the alternative sigma factor HrpL (42), encoded by the *hrpL* gene, which regulates the expression of *hrp* and *avr* genes. The identification in *P. syringae* subsp. *savastanoi* of an *hrp*-box upstream of the *hrpC* operon indirectly suggests that *hrpL* is also present in this plant pathogen and that the expression of its *hrp* gene cluster is regulated by a multicomponent regulatory cascade, as has been shown in *P. syringae* pv. *syringae* (42). An *hrp*-box motif is also located upstream of *virPphA* and *avrPphD* homologues that have been re-

cently identified in *P. syringae* subsp. *savastanoi*, indicating their possible regulation by HrpL (20). The *hrp/hrc* gene cluster encodes the type III secretion system responsible for the secretion of proteins, known as effector proteins, which play a key role in plant-microbe interaction (7,38). In this regard, VirPphA and AvrPphD, encoded by the above-mentioned genes, might be two of the effector proteins secreted by *P. syringae* subsp. *savastanoi* via the type III secretion system and may be involved in the interaction with its host plants. In particular, a homologue of *avrPphD* has recently been reported to play a key role in the virulence of the gall-forming plant pathogen *Erwinia herbicola* (*Pantoea agglomerans*) pv. *gypsophilae* (15) and this gene could have a similar importance in the virulence of *P. syringae* subsp. *savastanoi*. It was thought for many years that IAA and cytokinins produced by *P. syringae* subsp. *savastanoi* were the main factors responsible for the disease caused by this plant pathogen (36); however, *P. syringae* subsp. *savastanoi* mutants unable to produce IAA and/or cytokinins multiplied in the host tissues as well as the parental strain (19). In contrast, our results indicate that *hrcC* mutant ITM317-1421 was unable to multiply in the host tissues and its viability was also markedly reduced. Therefore, the *hrp* genes and the hypothetically secreted effector proteins seem to play a more fundamental role in *P. syringae* subsp. *savastanoi*-host interaction than phytohormone production. In fact, *hrp* genes and secreted effector proteins are believed to be responsible for leakage of plant nutrients and suppression of host defenses (7,14,17), and although no direct evidence is now available, the absence of both these activities could account for the marked decline of the viability of mutant ITM317-1421 in olive stems. Our results could also suggest that the pathogenetic mechanism leading to knot formation caused by *P. syringae* subsp. *savastanoi* is very similar to that of *Pantoea agglomerans* pv. *gypsophilae* (25); however, some relevant differences seem to exist. In fact, a *P. syringae* subsp. *savastanoi* mutant unable to produce IAA and cytokinins caused no overgrowth of the infected tissues (19); on the contrary, a similar mutant of *Erwinia herbicola* pv. *gypsophilae* unable to produce the above-mentioned phytohormones still caused galls of a reduced size (26).

In conclusion, our results indicate that some *hrp/hrc* genes are present in *P. syringae* subsp. *savastanoi* and that they are arranged and organized as in *P. syringae* pathovars. This, together with the identification and characterization of the *hrcC* mutant ITM317-1421 of *P. syringae* subsp. *savastanoi*, indicates that the hyperplastic symptoms caused by this plant-pathogenic bacterium are not only due to its ability to produce phytohormones but also depend on *hrp* genes whose function seems indispensable for pathogen multiplication within host tissues.

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LITERATURE CITED

1. Alfano, J. R., Charkowski, A. O., Deng, W. L., Badel, J. L., Petnicki-Ocwieja, T., van Dijk, K., and Collmer, A. 2000. The *Pseudomonas syringae* pathogenicity island has a tripartite mosaic structure composed of a cluster of type III secretion genes bounded by exchangeable effector and conserved effector loci that contribute to parasitic fitness and pathogenicity in plants. *Proc. Natl. Acad. Sci. USA* 97:4856-4861.
2. Alfano, J. R., and Collmer, A. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: Trafficking harpins, avr proteins, and death. *J. Bacteriol.* 179:5655-5662.
3. Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A

- new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
4. Berg, D. E. 1989. Transposon Tn5. Pages 185-210 in: *Mobile DNA*. D. E. Berg and M. M. Howe, eds. American Society for Microbiology, Washington, DC.
5. Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H. C., Hutcheson, S. W., Panopoulos, N. J., and van Gijsegem, F. 1996. Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Mol. Microbiol.* 20:681-683.
6. Charkowski, A. O., Huang, H. C., and Collmer, A. 1997. Altered localization of HrpZ in *Pseudomonas syringae* pv. *syringae* *hrp* mutants suggests that different components of the type III secretion pathway control protein translocation across the inner and outer membranes of gram-negative bacteria. *J. Bacteriol.* 179:3866-3874.
7. Collmer, A., Badel, J. L., Charkowski, A. O., Deng, W., Fouts, D. E., Ramos, A. R., Rehm, A. H., Anderson, D. M., Schneewind, O., van Dijk, K., and Alfano, J. R. 2000. *Pseudomonas syringae* Hrp type III secretion system and effector proteins. *Proc. Natl. Acad. Sci. USA* 97:8770-8777.
8. Comai, L., Surico, G., and Kosuge, T. 1982. Relation of plasmid DNA to indoleacetic acid production in different strains of *Pseudomonas syringae* pv. *savastanoi*. *J. Gen. Microbiol.* 128:2157-2163.
9. Deng, W. L., Preston, G., Collmer, A., Chang, C. J., and Huang, H. C. 1998. Characterization of the *hrpC* and *hrpRS* operons of *Pseudomonas syringae* pathovars *syringae*, *tomato*, and *glycinea* and analysis of the ability of *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* mutants to elicit the hypersensitive response and disease in plants. *J. Bacteriol.* 180:4523-4531.
10. Gardan, L., Bollet, C., Abu Ghorrah, M., Grimont, F., and Grimont, P. A. D. 1992. DNA relatedness among the pathovar strains of *Pseudomonas syringae* subsp. *savastanoi* Janse (1982) and proposal of *Pseudomonas savastanoi* sp. nov. *Int. J. Syst. Bacteriol.* 42:606-612.
11. Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F., and Grimont, P. A. D. 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremiae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). *Int. J. Syst. Bacteriol.* 49:469-478.
12. Glass, N. L., and Kosuge, T. 1988. Role of indoleacetic acid-lysine synthetase in regulation of indoleacetic acid pool size and virulence of *Pseudomonas syringae* subsp. *savastanoi*. *J. Bacteriol.* 170:2367-2373.
13. Gough, C. L., Genin, S., Zischek, C., and Boucher, C. A. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. *Mol. Plant-Microbe Interact.* 5:384-389.
14. Greenberg, J. T., and Vinatzer, B. A. 2003. Identifying type III effectors of plant pathogens and analyzing their interaction with plant cells. *Curr. Opin. Microbiol.* 6:20-28.
15. Guo, M., Manulis, S., Mor, H., and Barash, I. 2002. The presence of diverse IS elements and an *avrPphD* homologue that acts as a virulence factor on the pathogenicity plasmid of *Erwinia herbicola* pv. *gypsophillae*. *Mol. Plant-Microbe Interact.* 15:709-716.
16. Hansen, J. B., and Olsen, R. H. 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. *J. Bacteriol.* 135:227-238.
17. He, S. Y. 1998. Type III protein secretion systems in plant and animal pathogenic bacteria. *Annu. Rev. Phytopathol.* 36:363-392.
18. Huang, H. C., He, S. Y., Bauer, D. W., and Collmer, A. 1992. The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product, an envelope protein required for elicitation of the hypersensitive response in plants. *J. Bacteriol.* 174:6878-6885.
19. Iacobellis, N. S., Sisto, A., Surico, G., Evidente, A., and DiMaio, E. 1994. Pathogenicity of *Pseudomonas syringae* subsp. *savastanoi* mutants defective in phytohormone production. *J. Phytopathol.* 140:238-248.
20. Jackson, R. W., Mansfield, J. W., Ammoun, H., Dutton, L. C., Wharton, B., Ortiz-Barredo, A., Arnold, D. L., Tsiamis, G., Sesma, A., Butcher, D., Boch, J., Kim, Y. J., Martin, G. B., Tegli, S., Murillo, J., and Vivian, A. 2002. Location and activity of members of a family of *virPphA* homologues in pathovars of *Pseudomonas syringae* and *P. savastanoi*. *Mol. Plant Pathol.* 3:205-216.
21. Janse, J. D. 1982. *Pseudomonas syringae* subsp. *savastanoi* (ex Smith) subsp. nov., nom. rev., the bacterium causing excrescences on *Oleaceae* and *Nerium oleander* L. *Int. J. Syst. Bacteriol.* 32:166-169.
22. Kim, J. F., Wei, Z., and Beer, S. V. 1997. The *hrpA* and *hrpC* operons of *Erwinia amylovora* encode components of a type III pathway that secretes harpin. *J. Bacteriol.* 179:1690-1697.
23. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301-307.
24. Lindgren, P. B. 1997. The role of *hrp* genes during plant-bacterial interactions. *Annu. Rev. Phytopathol.* 35:129-152.
25. Manulis, S., and Barash, I. 2003. *Pantoea agglomerans* pvs. *gypsophillae* and *betulae*, recently evolved pathogens? *Mol. Plant Pathol.* 4:307-314.
26. Manulis, S., Haviv-Chesner, A., Brandl, M. T., Lindow, S. E., and Barash, I. 1998. Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and epiphytic fitness of *Erwinia herbicola* pv. *gypsophillae*. *Mol. Plant-Microbe Interact.* 11:634-642.
27. Miller, J. H. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
28. Mor, H., Manulis, S., Zuck, M., Nizan, R., Coplin, D. L., and Barash, I. 2001. Genetic organization of the *hrp* gene cluster and *dspAE/BF* operon in *Erwinia herbicola* pv. *gypsophillae*. *Mol. Plant-Microbe Interact.* 14:431-436.
29. Nizan, R., Barash, I., Valinsky, L., Lichter, A., and Manulis, S. 1997. The presence of *hrp* genes on the pathogenicity-associated plasmid of the tumorigenic bacterium *Erwinia herbicola* pv. *gypsophillae*. *Mol. Plant-Microbe Interact.* 10:677-682.
30. Nizan-Koren, R., Manulis, S., Mor, H., Iraki, N. M., and Barash, I. 2003. The regulatory cascade that activates the Hrp regulon in *Erwinia herbicola* pv. *gypsophillae*. *Mol. Plant-Microbe Interact.* 16:249-260.
31. Powell, G. K., and Morris, R. O. 1986. Nucleotide sequence and expression of a *Pseudomonas savastanoi* cytokinin biosynthetic gene: Homology with *Agrobacterium tumefaciens* *tmr* and *tzs* loci. *Nucleic Acids Res.* 14:255-265.
32. Preston, G., Deng, W. L., Huang, H. C., and Collmer, A. 1998. Negative regulation of *hrp* genes in *Pseudomonas syringae* by HrpV. *J. Bacteriol.* 180:4532-4537.
33. Rich, J. J., and Willis, D. K. 1990. A single oligonucleotide can be used to rapidly isolate DNA sequences flanking a transposon Tn5 insertion by the polymerase chain reaction. *Nucleic Acids Res.* 18:6673-6676.
34. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
35. Sisto, A., Morea, M., Zaccaro, F., Palumbo, G., and Iacobellis, N. S. 1999. Isolation and characterization of *Pseudomonas syringae* subsp. *savastanoi* mutants defective in hypersensitive response elicitation and pathogenicity. *J. Phytopathol.* 147:321-330.
36. Surico, G., and Iacobellis, N. S. 1992. Phytohormones and olive knot disease. Pages 209-229 in: *Molecular Signals in Plant-Microbe Communications*. D. P. S. Verma, ed. CRC Press, Boca Raton, FL.
37. Surico, G., Iacobellis, N. S., and Sisto, A. 1985. Studies on the role of indole-3-acetic acid and cytokinins in the formation of knots on olive and oleander plants by *Pseudomonas syringae* pv. *savastanoi*. *Physiol. Plant Pathol.* 26:309-320.
38. van Dijk, K., Fouts, D. E., Rehm, A. H., Hill, A. R., Collmer, A., and Alfano, J. R. 1999. The Avr (effector) proteins HrmA (HrpPsyA) and AvrPto are secreted in culture from *Pseudomonas syringae* pathovars via the Hrp (type III) protein secretion system in a temperature- and pH-sensitive manner. *J. Bacteriol.* 181:4790-4797.
39. Wengelnik, K., Marie, C., Russel, M., and Bonas, U. 1996. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. *vesicatoria* essential for pathogenicity and induction of the hypersensitive reaction. *J. Bacteriol.* 178:1061-1069.
40. Willis, D. K., Rich, J. J., and Hrabak, E. M. 1991. *hrp* genes of phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* 4:132-138.
41. Woolley, D. W., Schaffner, G., and Broun, A. C. 1955. Studies on the structure of the phytopathogenic toxin of *Pseudomonas tabaci*. *J. Biol. Chem.* 215:485-493.
42. Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. *J. Bacteriol.* 176:1025-1036.
43. Xiao, Y., and Hutcheson, S. W. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. *J. Bacteriol.* 176:3089-3091.
44. Yamada, T., Palm, C. J., Brooks, B., and Kosuge, T. 1985. Nucleotide sequences in the *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. *Proc. Natl. Acad. Sci. USA* 82:6522-6526.
45. Young, J. M., Saddler, G. S., Takikawa, Y., De Boer, S. H., Vauterin, L., Gardan, L., Gvozdyak, R. I., and Stead, D. E. 1996. Names of plant pathogenic bacteria 1864-1995. *Rev. Plant Pathol.* 75:721-763.