A *Pseudomonas syringae* pv. *tomato* DC3000 Hrp (Type III Secretion) Deletion Mutant Expressing the Hrp System of Bean Pathogen *P. syringae* pv. *syringae* 61 Retains Normal Host Specificity for Tomato

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The plant pathogenic species *Pseudomonas syringae* is divided into numerous pathovars based on host specificity. For example, *P. syringae* pv. *tomato* DC3000 is pathogenic on tomato and Arabidopsis, whereas *P. syringae* pv. *syringae* 61 is pathogenic on bean. The ability of *P. syringae* strains to elicit the hypersensitive response (HR) in non-hosts or be pathogenic (or parasitic) in hosts is dependent on the Hrp (type III secretion) system and effector proteins this system is thought to inject into plant cells. To test the role of the Hrp system in determining host range, the *hrp/hrc* gene cluster (*hrpK* through *hrpR*) was deleted from DC3000 and complemented in trans with the orthologous cluster from strain 61. Mutant CUCPB5114 expressing the bean pathogen Hrp system on plasmid pCPP2071 retained the ability of wild-type DC3000 to elicit the HR in bean, to grow and cause bacterial speck in tomato, and to elicit a cultivar-specific (gene-for-gene) HR in tomato plants carrying the *Pto* resistance gene. However, the symptoms produced in compatible tomato plants involved markedly reduced chlorosis, and CUCPB5114(pCPP2071) did not grow or produce symptoms in Arabidopsis Col-0 although it was weakly virulent in NahG Arabidopsis. A hypersensitive-like collapse was produced by CUCPB5114(pCPP2071) in Arabidopsis Col-0 at 1×10^7 CFU/ml, but only if the bacteria also expressed AvrB, which is recognized by the *RPM1* resistance gene in Col-0 and confers incompatibility. These observations support the concept that the *P. syringae* effector proteins, rather than secretion system components, are the primary determinants of host range at both the species and cultivar levels of host specificity.

Pseudomonas syringae is a plant pathogen divided into more than 40 pathovars on the basis of its host specificity for different plant species (Hirano and Upper 2000). *P. syringae* pv. *tomato* causes bacterial speck of tomato and Arabidopsis but elicits the defense-associated hypersensitive response (HR) in bean, tobacco, and many other plants. In contrast, *P. syrin-*

gae pv. *syringae* 61 causes brown spot of snap bean but elicits the HR in tomato, tobacco, and other nonhost plants. We do not know the molecular basis for this host specificity, but there is substantial evidence that it involves the Hrp effector protein system.

P. syringae, like many other gram-negative phytopathogenic bacteria, is thought to inject effector (Avr or Hop) proteins into plant cells via an Hrp (type III secretion) system (Galán and Collmer 1999). Mutants defective in the Hrp secretion machinery are typically unable to elicit the HR in nonhosts or be pathogenic (or grow parasitically) in host plants. Many Hrp effector proteins have Avr designations because of the avirulence phenotype they confer on races of a given pathovar when tested in cultivars of the host species that carry a cognate resistance (*R*) gene. It is now established that host specificity at the race-cultivar level is determined in a gene-for-gene manner by such combinations of *avr* and *R* genes (Dangl and Jones 2001; Vivian and Arnold 2000). However, it is not known whether specificity at the higher level of pathovars and plant species is similarly controlled by the particular set of effector proteins deployed or by the Hrp secretion machinery. There also remains the formal possibility that host range is determined by other virulence factors unrelated to the Hrp effector protein system, although there is little support for this. For example, many *P. syringae* strains produce one or more phytotoxins, but these toxins are not host specific and their production has no apparent relationship with the host specificity of the pathovars producing them (Bender et al. 1999).

The Hrp secretion system must deliver effector proteins across the bacterial envelope, the plant cell wall and plasma membrane. Genes directing type III secretion through the bacterial envelope are conserved in plant and animal pathogens (Hueck 1998). These *hrc* (HR and conserved) genes are clustered with the *hrp* genes in a central region of the Hrp pathogenicity island of *P. syringae* (Alfano et al. 2000). The functions of the *hrp* genes include regulation of the Hrp system and translocation of effector proteins across the plant cell wall and plasma membrane. Because of adaptation to host environments, extracellular components of type III secretion systems are predicted to be more variable than components in the bacterial envelope, and such variability was used to successfully identify an extracellular component of the *Salmonella enterica* type III secretion system (Li et al. 1995). It is not known whether extracellular components of the *P. syringae* Hrp translocator are adapted for different hosts, and complete sequences of *hrp/hrc* gene clusters from different pathovars have not been available for such comparisons. In this context, it is noteworthy that a pioneering

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report that *hrp* genes “are conserved and interchangeable among pathovars of *Pseudomonas syringae*” was almost certainly based on highly conserved *hrc* genes (Lindgren et al. 1988), thus leaving unresolved the interchangeability of complete Hrp systems and their variable extracellular components. For example, the HrpA pilus subunit proteins of *P. syringae* pv. *syringae* 61 and *P. syringae* pv. *tomato* DC3000 are only 28% identical (Deng et al. 1998; Roine et al. 1997).

The *hrp/hrc* gene cluster and exchangeable effector locus from *P. syringae* pv. *syringae* 61, carried on cosmid pHIR11, is sufficient to enable nonphytopathogenic bacteria, such as *P. fluorescens* and *Escherichia coli*, to elicit the HR in tobacco (*Nicotiana tabacum*), but not *N. benthamiana* or soybean (Collmer et al. 2000; Huang et al. 1988). The complete sequence of the *P. syringae* DNA in pHIR11 has been determined (He et al. 1993; Heu and Hutcheson 1993; Huang et al. 1993, 1995; Lidell and Hutcheson 1994; Preston et al. 1995). It appears that pHIR11 enables bacteria carrying it to elicit the HR in tobacco because it encodes a functional Hrp system and HopPsyA (HrmA), an effector that triggers the HR in tobacco but not *N. benthamiana* or soybean (Alfano et al. 1997; Collmer et al. 2000). When various cloned *P. syringae* *avr* genes are expressed in nonpathogens carrying pHIR11, elicitation of the HR is observed if the inoculated test plants carry cognate *R* genes (Gopalan et al. 1996; Pirhonen et al. 1996). These and other observations indicate that the Hrp system of one *P. syringae* pathovar can deliver effector proteins from another pathovar. However, none of these nonpathogens expressing pHIR11 and various effector genes is able to grow in planta, and the question remains whether the Hrp secretion machinery of a given strain of *P. syringae* is adapted for pathogenesis and, thus, has a determinative role in host specificity.

We report here the completion of the sequence of the *P. syringae* pv. *tomato* DC3000 *hrp/hrc* cluster, comparison of the complete *hrp/hrc* clusters of *P. syringae* pathovars *tomato* and *syringae*, deletion of the entire *hrp/hrc* cluster from DC3000, and analysis of the interaction with different plants of the DC3000 Δ *hrp/hrc* mutant carrying the *hrp/hrc* cluster of strain 61.

RESULTS

Comparisons of the complete *hrp/hrc* clusters of *P. syringae* pv. *syringae* strain 61 and *P. syringae* pv. *tomato* strain DC3000.

To comprehensively compare the *hrp/hrc* gene clusters of these two *syringae* pathovars, we completed the DNA sequence analysis of the portions of the *P. syringae* pv. *tomato* DC3000 *hrpJ* and *hrpU* operons that were not reported previously (Alfano et al. 2000). The BLAST algorithm was used to identify orthologous open reading frames (Altschul et al. 1997). Each *hrp/hrc* gene then was compared between strains 61 and DC3000 using the Pairwise BLAST utility at NCBI. Results from amino acid comparisons are summarized in Figure 1B as percent similarity. The gene arrangement was as predicted (Alfano et al. 2000). The three proteins encoded within the *hrp/hrc* clusters of these pathovars with the least similarity were HrpA, the major subunit of the Hrp pilus (Wei et al. 2000); HrpF, a hydrophilic protein of unknown function (Deng et al. 1998); and HrcQ_B, a protein having similarity to SpaO, a hypervariable, type III-secreted protein from *Salmonella enterica* (Li et al. 1995). Regulatory proteins and proteins with multiple membrane-spanning domains were the most conserved, and the *hrpZ*-encoded harpin

Fig. 1. Schematic representation of *Pseudomonas syringae* pv. *tomato* DC3000 Hrp pathogenicity island (not drawn to scale). Arrows indicate direction of transcription, boxes indicate *hrp* promoters, and names are given below putative transcriptional units. **A**, Predicted functions for *hrp/hrc* genes. **B**, Percent similarities of *hrp/hrc* genes from DC3000 to orthologs in *P. syringae* pv. *syringae* 61. **C**, Clones used as sequencing templates.

was intermediate. Of the 27 predicted proteins that were compared, 7 had less than 80% similarity and 3 had less than 60% similarity.

The *hrp/hrc* gene cluster from *P. syringae* pv. *syringae* 61 enables Δ *hrp/hrc* mutant CUCPB5114 to elicit the HR in tobacco and snap bean.

The entire *hrp/hrc* gene cluster (*hrpK* through *hrpR*) was deleted from *P. syringae* pv. *tomato* strain DC3000 by homologous recombination of a Ω Cm^r cassette that was flanked by DC3000 sequences bordering *hrpK* and *hrpR*, and the deletion in mutant CUCPB5114 was confirmed by probing a DNA gel blot with an internal fragment of the region deleted (Fig. 2).

To determine whether CUCPB5114 had lost the ability to elicit the HR in the nonhost plants tobacco or snap bean, bacteria were infiltrated into leaves at a concentration of 1×10^8 CFU/ml. As predicted, no visible macroscopic tissue collapse was observed (Fig. 3, panels 2A and 2B). In contrast, wild-type DC3000 caused tissue collapse within 12 h after inoculation (Fig. 3, panels 1A and 1B). The type III secretion system (*hrp/hrc* cluster) of strain 61 carried on plasmid pCPP2071, a *hopPsyA* mutant derivative of pHIR11, restored HR elicitation ability to CUCPB5114 in tobacco and snap bean (Fig 3, panels 3A and 3B).

The *hrp/hrc* gene cluster from *P. syringae* pv. *syringae* 61 enables Δ *hrp/hrc* mutant CUCPB5114 to produce disease symptoms in tomato but not in *Arabidopsis* Col-0 or snap bean.

We evaluated whether CUCPB5114 and CUCPB5114-(pCPP2071) could cause disease symptoms on tomato (strain DC3000 host plant) and snap bean (strain 61 host plant) (Fig. 4). A low level of bacteria (1×10^4 CFU/ml) was introduced into tomato leaves by vacuum infiltration and into snap bean leaves by syringe infiltration. CUCPB5114 was unable to cause disease symptoms on tomato or snap bean (Fig. 4, panels 2A and 2C), whereas CUCPB5114(pCPP2071) caused bacterial speck symptoms only on tomato (Fig 4, panels 3A and 3C). Similarly, wild-type strain DC3000 caused bacterial speck symptoms in tomato only (Fig. 4, panels 1A and 1C), and strain 61 caused brown spot disease symptoms only in snap bean (Fig. 4, panels 4A and 4C). It is noteworthy that the lesions produced by CUCPB5114(pCPP2071) were smaller and lacked chlorotic halos in contrast to those produced by DC3000.

DC3000 is also a pathogen of *Arabidopsis*; therefore, we tested CUCPB5114 and CUCPB5114(pCPP2071) for their ability to cause disease symptoms in susceptible Col-0 plants following vacuum infiltration of bacteria at 1×10^4 CFU/ml.

Fig. 2. The *hrp/hrc* region deleted from the *Pseudomonas syringae* pv. *tomato* DC3000 genome (not drawn to scale). Arrows indicate direction of transcription, hatched areas depict *hrc* genes, gray areas represent regions flanking the *hrp/hrc* cluster. **A**, Schematic drawing illustrating the replacement of the *hrp/hrc* gene cluster from *P. syringae* pv. *tomato* DC3000 by the CAT (Cm^r) cassette from pCPP3132. **B**, Genetic organization of pCPP2071, a derivative of pHIR11 (*hrp/hrc* gene cluster from *P. syringae* pv. *syringae* 61). The X denotes a TnphoA insertion into *hopPsyA* (*hrmA*). **C**, DNA gel blot used to confirm the deletion of the *hrp/hrc* gene cluster from *P. syringae* pv. *tomato* DC3000. Wild-type (wt) or CUCPB5114 (Δ) were digested with *EcoRI*, *HindIII*, and *BglIII* and probed with an internal ≈ 700 -bp fragment (bar above *hrpR*) in panel A.

Surprisingly, neither CUCPB5114 nor CUCPB5114 (pCPP2071) caused disease symptoms in Arabidopsis (Fig. 4, panels 2B and 3B). As expected, wild-type DC3000 caused disease symptoms in Arabidopsis, but strain 61 did not (Fig. 4, panels 1B and 4B).

To determine if CUCPB5114(pCPP2071) was able to deliver effector proteins into Arabidopsis, we infiltrated CUCPB5114-(pCPP2071) with or without pAVRB-FLAG2 into ecotype Col-0 plants at concentrations of 1×10^6 , 1×10^7 , and 1×10^8 CFU per ml. Plasmid pAVRB-FLAG2 constitutively expresses AvrB, which is hypersensitively recognized by Arabidopsis ecotypes that carry the *RPM1* resistance gene. We observed confluent collapse of infiltrated leaf tissue with CUCPB5114-(pCPP2071) at 1×10^8 CFU per ml and with CUCPB5114-(pCPP2071)/pAVRB-FLAG2 at both 1×10^7 and 1×10^8 CFU per ml (data not shown). CUCPB5114 failed to elicit necrosis in Arabidopsis Col-0 at any of the concentrations tested. Thus, although the pCPP2071 Hrp system fails to restore pathogenicity in Arabidopsis to CUCPB5114, it does enable the mutant to deliver at least some effector proteins, as indicated by hypersensitive necrosis.

The *hrp/hrc* gene cluster from *P. syringae* pv. *syringae* 61 enables Δ *hrp/hrc* mutant CUCPB5114 to grow in tomato but not in Arabidopsis.

Growth of wild-type DC3000, CUCPB5114, and CUCPB5114(pCPP2071) was monitored in tomato and Arabidopsis following inoculation with bacteria at a concentration of 1×10^4 CFU/ml via vacuum infiltration (Fig. 5). Time points were taken at 1 h post inoculation (day 0) and at day 4. Both DC3000 and CUCPB5114(pCPP2071) multiplied several logs in tomato; however, only DC3000 multiplied in Arabidopsis.

CUCPB5114(pCPP2071) is virulent on tomato PtoS plants and avirulent on tomato PtoR plants.

We next addressed the race-cultivar specificity of CUCPB5114(pCPP2071). We first confirmed by immunoblot analysis of cell pellet and supernatant fractions (van Dijk et al. 1999) that CUCPB5114(pCPP2071), but not CUCPB5114, was able to secrete AvrPto (data not shown). We then addressed whether CUCPB5114(pCPP2071) interacts in the same manner as wild-type DC3000 with tomato Rio Grande plants that either possess or lack *Pto* resistance (Fig. 6). DC3000 is avirulent on PtoR tomato, apparently because of an interaction between *Pto* and *AvrPtoB* inside plant cells (Kim et al. 2002). The nearly isogenic tomato line Rio Grande PtoS, which lacks

Pto resistance, was used as a positive control for virulence. Wild-type DC3000 and CUCPB5114 were used as additional controls. None of the bacterial strains tested produced bacterial speck symptoms in PtoR plants (Fig. 6, panels 1B–3B). Both DC3000 and CUCPB5114(pCPP2071) caused bacterial speck symptoms in PtoS plants (Fig. 6, panels 1A and 3A), but CUCPB5114 did not (Fig. 6, panel 2A). However, as observed in Figure 4, the lesions produced by CUCPB5114(pCPP2071) appeared to be smaller and were accompanied by substantially less chlorosis than DC3000.

pCPP2071 enhances the virulence of Δ *hrp/hrc* mutant CUCPB5114 but decreases the virulence of wild-type DC3000 on Arabidopsis plants expressing NahG.

To further explore the basis for the inability of CUCPB5114(pCPP2071) to cause disease in Arabidopsis, we inoculated plants expressing NahG with DC3000, DC3000(pCPP2071), and CUCPB5114(pCPP2071). Transgenic NahG plants produce salicylate hydroxylase, which reduces levels of salicylic acid, a signaling compound that is important in defense responses against *P. syringae* (Delaney et al. 1994). NahG plants inoculated with CUCPB5114(pCPP2071) produced disease symptoms on some leaves and grew in planta significantly better than CUCPB5114 (Fig. 7). In contrast, pCPP2071 reduced the growth of DC3000 in planta and strongly reduced virulence. To further explore this negative effect of pCPP2071 on DC3000 growth in planta, we inoculated Arabidopsis Col-0 and tomato cv. Rutgers with the test strains. In all plants, the presence of pCPP2071 strongly reduced the growth of DC3000 (Fig. 7).

Fig. 3. Relative ability of *Pseudomonas syringae* pv. *tomato* strains DC3000, CUCPB5114, and CUCPB5114(pCPP2071) to elicit the hypersensitive response in tobacco and bean leaves following infiltration of bacteria at 1×10^8 CFU/ml. Panels and strains: **A**, tobacco cv. Xanthi; **B**, snap bean cv. Eagle; **1**, *P. syringae* pv. *tomato* DC3000; **2**, CUCPB5114; **3**, CUCPB5114(pCPP2071). The leaves were photographed using polarized lighting 12 to 24 h after inoculation.

Fig. 4. Relative ability of *Pseudomonas syringae* pv. *tomato* strains DC3000, CUCPB5114, and CUCPB5114(pCPP2071) and *P. syringae* pv. *syringae* 61 to produce disease symptoms in tomato, Arabidopsis spp., and bean. Panels and strains: **A**, tomato cv. Rutgers; **B**, *A. thaliana* ecotype Col-0; **C**, snap bean cv. Eagle; **1**, *P. syringae* pv. *tomato* DC3000; **2**, CUCPB5114; **3**, CUCPB5114(pCPP2071); **4**, *P. syringae* pv. *syringae* 61. The leaves were photographed using polarized lighting 5 to 7 days after inoculation. Inoculum was delivered to tomato and Arabidopsis leaves by vacuum infiltration of 1×10^4 CFU/ml and to bean leaves by infiltration with a blunt syringe of 1×10^5 CFU/ml.

Plasmid pCPP2071 is lost from CUCPB5114 during growth in tomato leaves.

To determine the stability of pCPP2071 in CUCPB5114 during growth in tomato leaves, we inoculated tomato cv. Rutgers with CUCPB5114(pCPP2071) as described for Figure 4 and then tested over the course of 4 days for retention of pCPP2071 by plating bacteria released from comminuted leaf disks on agar medium containing or lacking tetracycline (Fig. 7). Within 24 h, half of the bacteria had lost the plasmid and, after 4 days, only 4% of CUCPB5114 cells retained the plasmid (Fig. 8).

DISCUSSION

We chose to test the role of the Hrp (type III protein secretion) system in determining the host range of *P. syringae* by investigating *P. syringae* pvs. *tomato* and *syringae* because these are particularly divergent pathovars and their hosts, tomato and bean, respectively, are in different plant families (Manceau and Horvais 1997). By complementing a $\Delta hrp/hrc$ mutation in *P. syringae* pv. *tomato* DC3000 with a plasmid carrying the orthologous genes from *P. syringae* pv. *syringae* 61, we replaced the type III secretion machinery of a tomato pathogen with that of a bean pathogen. Despite the substitution, the DC3000 derivative retained its ability to grow and produce disease in tomato and to elicit the HR in bean. Furthermore, the complemented DC3000 mutant retained its ability to interact differentially with host tomato cultivars differing in a single *R* gene. These observations provide further support for the concept of diverse *P. syringae* strains carrying a universal type

III secretion system that can function to deliver any subset of a potentially large pool of effector proteins to a wide range of plants. According to this concept, host range at both the pathovar-species and race-cultivar levels is determined by the subset of effectors produced by a given strain.

The pathogenic abilities of CUCPB5114(pCPP2071) differed from the parental strain DC3000 in two significant ways. The symptoms in tomato, particularly the chlorotic halos around necrotic lesions, were greatly reduced, and CUCPB5114(pCPP2071) failed to grow or produce any visible response at normally infective levels of inoculum in *Arabidopsis*, which is susceptible to wild-type DC3000. In seeking an explanation for these differences, three factors must be considered: (i) a comparison of the complete *hrp/hrc* gene clusters of strains DC3000 and 61 revealed several significant differences; (ii) copy number effects and eventual loss of pCPP2071 likely impact the interaction; and (iii) manipulation of the Hrp system may alter the production of coronatine, which is responsible for the chlorosis and contributes to the basic pathogenicity of DC3000 in *Arabidopsis*.

Three proteins, HrpA, HrcQ_B, and HrpF are strikingly divergent in strains DC3000 and 61. Given the proposed need for Hrp machinery components to function universally with a pool of interchangeable effectors and the likely need to function in multiprotein complexes containing conserved Hrp components, the divergence of these three proteins must be driven by some variable factor, which most likely involves interaction with host components at the bacterium–host interface. Indeed, the most variable of these proteins is the HrpA pilin, which is known to be extracellular and is suspected to penetrate the plant cell wall (Brown et al. 2001; Jin et al. 2001; Roine et al. 1997). Whether HrcQ_B is secreted by *P. syringae* is not known, but the protein is a homolog of SpaO, which is secreted by *Salmonella enterica* (Li et al. 1995). Little is known about

Fig. 5. Relative ability of *Pseudomonas syringae* pv. *tomato* strains DC3000, CUCPB5114, and CUCPB5114(pCPP2071) to grow in tomato and *Arabidopsis* leaves. *P. syringae* pv. *tomato* DC3000 (dark gray hatching), CUCPB5114 (light gray hatching), and CUCPB5114(pCPP2071) (diamond pattern) were vacuum infiltrated into tomato cv. Rutgers and *Arabidopsis thaliana* ecotype Col-0 as follows. Inoculum was delivered to tomato and *Arabidopsis* leaves by vacuum infiltration of 1×10^4 CFU/ml. Data presented are the average of three leaf discs taken at 0 and 4 days post inoculation. Error bars indicate the standard deviation.

Fig. 6. Relative ability of *Pseudomonas syringae* pv. *tomato* strains DC3000, CUCPB5114, and CUCPB5114(pCPP2071) to cause disease in tomato cv. Rio Grande plants differential for *Pto*. Panels and strains: **A**, tomato cv. Rio Grande PtoS; **B**, tomato cv. Rio Grande PtoR; **1**, *P. syringae* pv. *tomato* DC3000; **2**, CUCPB5114; **3**, CUCPB5114(pCPP2071). Inoculum was delivered to tomato leaves by vacuum infiltration of 1×10^4 CFU/ml. The leaves were photographed using polarized lighting 5 to 7 days after inoculation.

HrpF except that the strain 61 protein is required for all Hrp phenotypes, and it shows intriguing similarity to the DC3000 HrpA protein (Deng et al. 1998). HrpP is another candidate extracellular protein because it has an intermediate level of divergence, similar to that of HrpZ, which is known to be secreted. HrcQ_A and HrcT also have intermediate levels of divergence, but the presence of multiple membrane-spanning domains in HrcT argues against its secretion and direct interaction with the host.

Our data suggest that none of these candidate extracellular factors determines the parasitic compatibility underlying host specificity. *P. syringae* strains typically are compatible (grow parasitically and produce disease) in host plants and are in-

compatible (elicit the HR) in nonhosts or resistant cultivars of the host. However, several *P. syringae* strains that are compatible with a given host may vary in virulence on that plant, and some strains produce null reactions (neither pathogenesis nor HR) in test plants. For example, *P. syringae* pv. *phaseolicola* produces a null response in *Arabidopsis* unless transformed with an *avr* gene that is recognized by *Arabidopsis*, in which case it elicits the HR (Yu et al. 1993). One interpretation of our observation that CUCPB5114(pCPP2071) has reduced virulence in tomato and a null interaction with *Arabidopsis* unless infiltrated at high concentrations is that inefficient delivery of effectors may be a factor in null interactions. According to this hypothesis, an inefficient Hrp system may function adequately

Fig. 7. Ability of pCPP2071 to enhance the virulence of *Pseudomonas syringae* pv. *tomato* $\Delta hrp/hrc$ mutant CUCPB5114 on NahG *Arabidopsis* but decrease the virulence of wild-type DC3000 on *Arabidopsis* Col-0 and tomato. Bacteria were vacuum infiltrated into leaves at a concentration of 1×10^5 CFU/ml. The inoculum for panels A (NahG) and B (Col-0) were: 1, *P. syringae* pv. *tomato* DC3000; 2, DC3000(pCPP2071); 3, CUCPB5114; 4, CUCPB5114(pCPP2071). Arrows indicate representative leaves showing symptoms. Panel C shows individual leaves with symptoms: 1, DC3000 on Col-0; 2, DC3000 on NahG; 3, CUCPB5114 on NahG; 4, CUCPB5114(pCPP2071) on NahG. The leaves were photographed using polarized lighting 5 days after inoculation. Panel D shows population increases in planta 3 days after inoculation of indicated plants with DC3000 (hatching), DC3000(pCPP2071) (solid), CUCPB5114 (stipple), and CUCPB5114(pCPP2071) (stripes). Data represent the average of three leaf discs sampled 3 days after inoculation of *Arabidopsis* spp. at 1×10^4 CFU/g of tissue and tomato at 5×10^2 CFU/cm². Error bars indicate the standard deviation.

to support HR elicitation but not pathogenesis, and *Arabidopsis* may be more recalcitrant to the *P. syringae* Hrp injector than tomato. Given this hypothesis and the divergence of the HrpA pilin proteins of strains DC3000 and 61, it would be interesting to see if the replacement of *hrpA* alone would give DC3000 the same *Arabidopsis* null interaction phenotype as CUCPB5114(pCPP2071). Nevertheless, it is important to note that pCPP2071 did not appear to render the DC3000 mutant incompatible with *Arabidopsis*, as indicated by the failure of CUCPB5114(pCPP2071) to elicit the HR in *Arabidopsis* at 10^7 CFU/ml unless carrying *avrB*.

The copy number and instability in planta of pCPP2071 are other factors that must be considered. Although the pCPP2071 vector, pLAFR3, is a low copy number plasmid (Schmidhauser et al. 1988), the presence of multiple copies of Hrp regulator and secretion machinery genes could alter the deployment of effector proteins. More certain is the loss of the Hrp system because of plasmid instability in the absence of antibiotic selection during growth in planta. Given this, it is noteworthy that CUCPB5114(pCPP2071) grew as well as DC3000 in tomato despite the loss of the *hrpL/hrc* cluster from 50% of the bacteria in day 1 and from 96% of the bacteria by the end of day 4. Previous experiments involving antibiotics infiltrated into infected leaf tissue had demonstrated that only a relatively brief period of bacterial RNA and protein synthesis is needed for the effective delivery of Avr proteins that elicit the HR (Huynh et al. 1989; Puri et al. 1997). Our observation that bacterial speck symptoms developed in tomato despite rapid loss of the plasmid-borne Hrp system from a substantial fraction of the bacterial population suggests that the parasitic benefits of effector proteins delivered early in the interaction persist during prolonged pathogen development. It would be interesting to further test this hypothesis by rendering production of the Hrp system even more transient by recloning the *P. syringae* pv. *syringae* 61 *hrpL/hrc* cluster into a plasmid that is even less stable during bacterial growth in planta or by manipulating the expression of the HrpL alternative sigma factor.

Diminished coronatine production also may be a key factor in the reduced ability of CUCPB5114(pCPP2071) to produce disease symptoms in tomato or to produce any reaction in *Arabidopsis*. Coronatine is produced by five pathovars of *P. syringae* and is structurally and functionally similar to methyl jasmonate, a plant signaling compound involved with developmental processes, wound responses, and resistance to pathogens that (unlike *P. syringae*) are broad-host-range necrotrophs (Bender et al. 1999; Norman et al. 1999; Thomma et al. 1998). Complex regulatory interactions between the Hrp and coronatine biosynthesis systems may occur in *P. syringae* pv. *tomato* DC3000. For example, an *hrcC* mutant produced chlorosis in tomato leaves, which is indicative of coronatine production, following spraying with inoculum at 1×10^6 CFU/ml. However, as expected, the mutant failed to grow in tomato or produce necrotic lesions (Peñaloza-Vázquez et al. 2000). On the other hand, miniTn5gus mutagenesis has revealed that coronatine biosynthesis genes are activated by the HrpL alternative sigma factor (Fouts et al. 2002). The dramatically reduced chlorosis associated with CUCPB5114(pCPP2071) in tomato suggests that the coronatine biosynthesis system is poorly expressed in this strain. Furthermore, the reduction in the size of lesions produced by CUCPB5114(pCPP2071) is similar to the phenotype of *P. syringae* pv. *tomato* PT23 mutants deficient in coronatine production (Bender et al. 1987). It is also noteworthy that a coronatine-deficient DC3000 mutant was unable to grow or produce lesions when dip-inoculated into *Arabidopsis* leaves (Mittal and Davis 1995).

Coronatine sensitivity in *Arabidopsis* is mediated by *COI1*, and *coi1* mutants have increased resistance to *P. syringae* (Feys et al. 1994). COI1-mediated resistance to bacterial growth is dependent on salicylic acid, as indicated by the wild-type growth of *P. syringae* pv. *tomato* DC3000 in *coi1-20* NahG *Arabidopsis* (Kloek et al. 2001). Because of our suspicion that pCPP2071 reduced bacterial coronatine production, we tested the virulence of CUCPB5114(pCPP2071) in NahG transgenic *Arabidopsis*. Although there was a small increase in growth in planta and symptoms were produced, the most striking finding was that pCPP2071 strongly reduced the virulence (in both *Arabidopsis* and tomato) of wild-type DC3000. Direct measurement of the production of coronatine or the expression of coronatine biosynthesis genes will be important in exploring the basis for the reduced virulence of strains carrying pCPP2071. However, the *hrpL/hrc* deletion mutant and the different phenotypes of the complemented mutant in tomato and *Arabidopsis* should provide new tools for dissecting the molecular interactions of the model strain DC3000 with its two model hosts.

MATERIALS AND METHODS

Bacterial strains, plasmids, oligonucleotides, plant cultivars, and media.

Bacterial strains, plasmids, oligonucleotides, and plant cultivars used in this study are given in Table 1. *E. coli* strains were grown in Terrific Broth at 37°C for the propagation of plasmids (Tartof and Hobbs 1987). *Pseudomonas* strains were grown in King's B (KB) medium at 25°C (King et al. 1954). The following concentrations were used for antibiotics and chromogenic substrates: ampicillin (Ap), 100 µg/ml; chloramphenicol (Cm), 20 µg/ml; gentamicin (Gm), 10 µg/ml; kanamycin (Km), 50 µg/ml; rifampicin (Rp), 50 µg/ml; spectinomycin (Sp), 50 µg/ml; tetracycline (Tc), 10 µg/ml; trimethoprim (Tp) 100 µg/ml; cycloheximide (Cx), 2 µg/ml; 5-bromo-4-chloro-3-indolyl phosphate (Xphos), 20 µg/ml; and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal), 20 µg/ml.

Fig. 8. Loss of pCPP2071 (Tc') from CUCPB5114 during growth in tomato leaves. Percent plasmid retention was calculated as the number of CFU/cm² plated on media containing tetracycline divided by the number of CFU/cm² plated on media lacking tetracycline, multiplied by 100. Data points reflect the average of three leaf discs per time point.

General DNA manipulations and enzymes.

Standard procedures were used for DNA cloning (Sambrook et al. 1989). All ligation products were transformed into *E. coli* DH5 α by an RbCl₂ heat shock method (Hanahan 1985). *Pseudomonas* genomic DNA was harvested using DNA NOW reagent (Biogentex, Seabrook, TX, U.S.A.). Polymerase chain reaction (PCR) was performed using Tfl DNA polymerase (Epicentre Technologies, Madison, WI, U.S.A.) under standard conditions. Restriction enzymes were purchased from New England Biolabs (Beverly, MA, U.S.A.). DNA ligase was purchased from Life Technologies (Rockville, MD, U.S.A.). The Klenow fragment of DNA Polymerase I was purchased from United States Biochemical Corporation (Cleveland, OH, U.S.A.). Purification of DNA from agarose gels was accomplished using a Prep-A-Gene Purification Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Digested genomic DNA was separated through a 0.7% Agarose IV (Amresco, Solon, OH, U.S.A.) agarose gel in 1 $\times$ Tris-borate EDTA and transferred to a Nytran Plus membrane using a Turboblotter (Schleicher & Schuell, Keene, NH, U.S.A.). La-

beling of the DNA probe (gel-purified 813-bp *Xho*I fragment from pCPP2501) was accomplished using a Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA, U.S.A.), and [α -³²P]CTP (3,000 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston). Prehybridization and hybridization was performed using Puregene HYB-9 Hybridization Solution following the manufacturer's recommendations (Gentra Systems, Minneapolis, MN, U.S.A.). Unbound probe was washed off the membrane under high stringency conditions (one 15-min wash at room temperature in 1 $\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate) per 0.2% sodium dodecyl sulfate (SDS) followed by four 15-min washes at 65°C in 0.4 $\times$ SSC per 0.2% SDS.

DNA sequencing, contig assembly, and bioinformatics.

The *hrpU* operon, *hrpO*, and half of *hrcN* of *P. syringae* pv. *tomato* DC3000 were sequenced from plasmid pCPP3152. The rest of the *hrpJ* operon and *hrpL* were sequenced from plasmid pCPP3091 and its subclone, plasmid pCPP3138. The sequence of the remainder of the *hrp/hrc* cluster was previously obtained

Table 1. Bacterial strains, plasmids, DNA oligonucleotides, and plant cultivars used in this study

Designation	Relevant characteristic	Source or (reference)
<i>Escherichia coli</i> DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>end1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> <i>Nal</i> ^r	Life Technologies, Inc., Grand Island, NY, U.S.A.
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	Rp ^r derivative of DC52 (wild-type pv. <i>tomato</i> race 0), pathogen of tomato and <i>Arabidopsis</i> spp.	Cuppels 1986
pv. <i>tomato</i> CUCPB5114	DC3000 with <i>hrpK-hrpR</i> replaced with Cm ^r cassette	This work
pv. <i>syringae</i> 61	Nx ^r , wild-type, snap bean pathogen	Baker et al. 1987
Plasmids		
pBluescript II SK(+)	Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pCPP2071	Tc ^r , Km ^r , derivative of pHIR11 containing a <i>TnphoA</i> insertion within <i>hopPsyA</i> (<i>hrmA</i>)	Alfano et al. 1997
pCPP2201	Ap ^r , 10.6-kb <i>Sau3A</i> partial fragment from pv. <i>tomato</i> DC3000 (<i>hrpV-hrpR</i>) cloned into <i>Bam</i> HI site of pUCP19	Deng et al. 1998
pCPP2357	Tc ^r , cosmid containing <i>hrpZ</i> operon—conserved effector locus <i>orf5</i> in pCPP47	Charkowski et al. 1998
pCPP2501	Ap ^r , 5.7-kb <i>Eco</i> RI fragment of pCPP2357 (416 bp of <i>hrpR</i> , <i>CEL orf1</i> , and 2,574 bp of <i>avrE</i>) cloned into <i>Eco</i> RI of pBluescript II SK(+)	Allison K. Conlin
pCPP3091	Ap ^r , 16.6-kb <i>Bam</i> HI fragment from pv. <i>tomato</i> DC3000 (910 bp of <i>VacC</i> , 1,008 bp of <i>hrcN</i>) cloned into <i>Bam</i> HI of pBluescript II SK(+)	James R. Alfano
pCPP3129	Ap ^r , pCPP2501 with 813-bp <i>Xho</i> I fragment containing <i>hrpR</i> removed	This work
pCPP3130	Ap ^r , Cm ^r , pCPP3129 containing Ω Cm cassette from pHP45 Ω Cm	This work
pCPP3131	Tc ^r , pRK415 containing $\approx$ 3-kb of DNA from the EEL region of pv. <i>tomato</i> DC3000	This work
pCPP3132	Tc ^r , Cm ^r , pCPP3131 containing an <i>Eco</i> RI- <i>Kpn</i> I fragment from pCPP3130 (Ω Cm+CEL)	This work
pCPP3138	Ap ^r , 4.9-kb <i>Bam</i> HI/ <i>Bgl</i> III subclone of pCPP3091 (783 bp of <i>hrpJ</i> , 1,008 bp of <i>hrcN</i>) cloned into <i>Bam</i> HI of pBluescript II SK(+)	This work
pCPP3152	Ap ^r , 8.8-kb <i>Bam</i> HI/ <i>Eco</i> RI fragment from pv. <i>tomato</i> DC3000 (256 bp of <i>hrcN</i> , the <i>hrpU</i> operon, and 419 bp of <i>hrpG</i>) cloned into <i>Bam</i> HI/ <i>Eco</i> RI of pBluescript II SK(+)	James R. Alfano
pHP45 Ω Cm	Ap ^r , Cm ^r , pHP45 with the Cm ^r gene from pKT210	Fellay et al. 1987
pR751	Tp ^r , IncP β ,	Jobanputra and Datta 1974
pRK415	Tc ^r , IncP α , pRK404 with polylinker from pUC9	Keen et al. 1988
DNA oligonucleotides		
P205	CACGCCCGGGCTCGGCACTAT	IDT, Coralville, IA, U.S.A.
P206	TGTCTAGAAAAACATGGAAGCAC	IDT, Coralville, IA, U.S.A.
P277	CCGAGTAGAGGAGGGTAAGAT	IDT, Coralville, IA, U.S.A.
P282	CTTACAAACCGGATACAGT	Cornell BioResource Center
P312	CTCATGATTGAATCTCCGC	IDT, Coralville, IA, U.S.A.
P416	GTTTTCACAGTCACGACGTTGTA	IDT, Coralville, IA, U.S.A.
P417	TTGTGAGCGGATAACAATTTC	IDT, Coralville, IA, U.S.A.
Plant cultivars		
<i>Arabidopsis</i> sp.	<i>Arabidopsis thaliana</i> ecotype Col-0	Terrence P. Delaney
<i>Arabidopsis</i> sp.	NahG <i>Arabidopsis thaliana</i> ecotype Col-0	Delaney et al. 1994
Bean	<i>Phaseolus vulgaris</i> L. cv. Eagle	Asgrow Seed Co., Kalamazoo, MI, U.S.A.
Tobacco	<i>Nicotiana tabacum</i> L. cv. Xanthi, nonhost for pv. DC3000	Thomas Hammond
Tomato PtoR	<i>Lycopersicon esculentum</i> Mill. cv. Rio Grande PtoR, contains <i>Pto R</i> gene	Gregory B. Martin
Tomato PtoS	<i>Lycopersicon esculentum</i> Mill. cv. Rio Grande PtoS, lacks <i>Pto R</i> gene	Gregory B. Martin
Tomato Rutgers	<i>Lycopersicon esculentum</i> Mill. cv. Rutgers, host for pv. DC3000	Agway, Ithaca, NY, U.S.A.

from pCPP2201 (Deng et al. 1998). The Erase-A-Base kit from Promega Corporation (Madison, WI, U.S.A.) was used to create nested deletions in these plasmids. These clones then were sequenced with ABI 3100 and ABI 377 DNA sequencers at the Cornell BioResource Center DNA-sequencing facility with universal cycle (P416) and reverse cycle (P417) primers (Table 1). Gaps were filled using primers synthesized by Integrated DNA Technologies (Coralville, IA, U.S.A.). Contig assembly and bioinformatics were conducted using DNASTar software (DNASTar Inc., Madison, WI, U.S.A.). Percent similarity was calculated using the BLAST program (Altschul et al. 1997). An independently obtained draft sequence of the DC3000 genome, including the *hrp/hrc* gene cluster, is also available online.

Construction of *hrp/hrc* cluster deletion in *P. syringae* pv. *tomato* DC3000.

To create a sufficient region of homology on the right side of the *hrp/hrc* cluster for allelic exchange of a *hrp/hrc* cluster deletion marked with the chloramphenicol resistance (Ω Cm^r) cassette (Fig. 2A), plasmid pCPP2501 was created by insertion of a 5.7-kb *Eco*RI fragment into pBluescript SK(+) from pCPP2357, which contains the 5' end of *hrpR*, *orfI*, and the 5' end of *avrE* in the conserved effector locus. To ensure complete removal of *hrpK* through *hrpR*, the portion of *hrpR* in pCPP2501 was removed by digestion with *Xho*I, dilution of the cut vector, and religation to create pCPP3129. The Cm^r cassette from pHP45 Ω Cm (Fellay et al. 1987) was cloned into *Xho*I-digested/Klenow-polished pCPP3129 by ligation with the *Bam*HI-digested/Klenow-polished omega fragment to create pCPP3130.

To obtain a sufficient region of homology on the left side (exchangeable effector locus) (Alfano et al. 2000) of the *hrp/hrc* cluster for homologous recombination, a 3.0-kb region was amplified from pCPP3091 via PCR using primers P205 and P206. This PCR product was digested with *Sma*I and *Xba*I and ligated to pRK415, which was first digested with *Bam*HI, Klenow polished, and subsequently digested with *Xba*I to create pCPP3131. The left border region of homology and the right border- Ω Cm construct were joined in pCPP3131 by digestion with *Eco*RI and *Kpn*I and ligation to create pCPP3132 (Fig. 2B).

CUCPB5114 then was constructed as follows. pCPP3132 was conjugated into *P. syringae* pv. *tomato* DC3000 via triparental mating with DH5 α (pCPP3132) and DH5 α (pRK2013) and transconjugants were selected on KB medium supplemented with rifampicin and chloramphenicol. Single colonies were isolated and mixed with *E. coli* HB101(pR751) to produce *P. syringae* pv. *tomato* DC3000(pCPP3132/pR751). Selection for pR751 and the Ω Cm^r cassette resulted in the exchange of the *hrp/hrc* cluster for the Ω Cm^r cassette and loss of pCPP3132 due to IncP plasmid incompatibility. The deletion of the *hrp/hrc* cluster was verified by DNA gel blot hybridization as described above.

Plant assays.

Tobacco plants, used for HR assays, were grown under greenhouse conditions and transferred to the laboratory where they were kept at room temperature with 12 h of supplemental lighting from a 500 W halogen light source. Tomato plants were transferred from the greenhouse to a plant growth chamber held at a constant temperature of 22°C with a 12-h photoperiod. Arabidopsis and snap bean plants were started and maintained in the same 22°C growth chamber. *Pseudomonas* strains were prepared for infiltration by suspending 24-h cultures grown on KB plates in 10 mM morpholinoethanesulfonic acid (MES), pH 5.5, and adjusting to an optical density at 600

nm of 0.3 (equivalent to 1×10^8 CFU/ml). Tobacco leaves were infiltrated using this concentration of bacteria as described (Bauer et al. 1994). Snap bean was infiltrated by this method at a concentration of 1×10^5 CFU/ml. Arabidopsis and tomato plants were infiltrated using vacuum infiltration (27 inHg for 5 min) at a concentration of 1×10^4 CFU/ml in 10 mM MES, pH 5.5, 0.004% Vac-in-Stuff (Silwet L-77) (Lehle Seeds, Round Rock, TX, U.S.A.). Plants were covered with clear plastic bags containing holes to maintain a high level of humidity. A #2 cork-borer was used to collect three leaf disks of 0.5 cm diameter for each time point in growth assays. Each leaf disk was added to 0.5 ml of 10 mM MES, pH 5.5, crushed with a plastic pestle, and serially diluted. Samples were plated onto KB media supplemented with the appropriate antibiotics plus cycloheximide and grown at room temperature.

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