Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally non-polar *hrpZ* deletion mutations, truncated HrpZ fragments, and *hrmA* mutations

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**Summary**

*Pseudomonas syringae* pv. *syringae*, like many plant pathogenic bacteria, secretes a ‘harpin’ protein that can elicit the hypersensitive response (HR), a defensive cellular suicide, in non-host plants. The harpin-encoding *hrpZ* gene is located in an operon that also encodes Hrp secretion pathway components and is part of the functional cluster of *hrp* genes carried on cosmid pHIR11 that enables saprophytic bacteria like *Escherichia coli* and *Pseudomonas fluorescens* to elicit the HR in tobacco leaves. We have constructed functionally non-polar *hrpZ* deletion mutations, revealing that HrpZ is necessary for saprophytic bacteria carrying pHIR11 to elicit a typical HR, whereas it only enhances the elicitation activity of *P. s. syringae*. Partial deletion mutations revealed that the N-terminal 153 amino acids of HrpZ can enable *E. coli* MC4100-(pHIR11) to elicit a strong HR. *hrpZ* subclone products comprising the N-terminal 109 amino acids and C-terminal 216 amino acids, respectively, of the 341 amino acid protein were isolated and found to elicit the HR. *P. fluorescens* (pHIR11 *hrmA::TnphoA*) mutants do not elicit the HR, but cell fractionation and immunoblot analysis revealed that they produce and secrete wild-type levels of HrpZ. Therefore, elicitor activity resides in multiple regions of HrpZ. *P. syringae* produces elicitor(s) in addition to HrpZ, and HrpZ is essential but not sufficient for HR elicitation by saprophytic bacteria carrying pHIR11.

**Introduction**

Many Gram-negative plant pathogens elicit the hypersensitive response (HR) in non-host or resistant plants. The HR is a defensive suicide of plant cells in contact with an incompatible pathogen (an organism pathogenic on another host) and is accompanied by the production of antimicrobial factors and the cessation of pathogen growth (Dietrich *et al.*, 1994; Greenberg *et al.*, 1994; He *et al.*, 1993; Klement, 1982). Bacterial elicitation of the HR is dependent on *hrp* genes, which encode regulatory factors, a protein secretion pathway and extracellular proteins that elicit the HR (Bonas, 1994). The *hrp* genes are so named because mutations in them typically abolish both bacterial elicitation of the HR in non-hosts and pathogenesis in hosts (Lindgren *et al.*, 1986). *hrp* genes are clustered, and some are conserved, perhaps universally, among Gram-negative plant pathogens that cause eventual necrosis in their hosts (Bonas, 1994). These pathogens include *Pseudomonas syringae*, *Pseudomonas* (Burkholderia) *solanacearum*, *Xanthomonas campestris*, *Erwinia amylovora*, *Erwinia* stewartii and *Erwinia* chrysanthemi. The most conserved *hrp* genes encode components of a type III protein secretion pathway that is also used by animal pathogenic *Yersinia*, *Shigella* and *Salmonella* species to secrete virulence proteins (Van Gijsegem *et al.*, 1993).

In several plant pathogenic bacteria, the *hrp* genes have been shown to be involved in the production and secretion of protein elicitors of the HR. The proteins share several properties: they are glycine-rich, cysteine-lacking, heat stable and do not possess an N-terminal signal peptide that would target them to the Sec pathway. Despite these general similarities, the elicitor proteins produced by different groups of plant pathogenic bacteria are dissimilar in their amino acid sequence, and mutations in their encoding genes reveal different phenotypes.

The *E. amylovora* harpin was the first of these elicitors to be discovered, and mutations in the *hrpN* gene abolish the ability of *E. amylovora* to elicit the HR in non-host tobacco or to elicit disease in highly susceptible pear fruit (Wei *et al.*, 1992). In contrast, the *P. solanacearum* PopA1 protein...
also elicits the HR in tobacco, but PopA mutants retain their ability to elicit the HR in this and other non-host plants and to incite disease in tomato (Arlat et al., 1994). Mutations in \textit{hrp\textsubscript{N}cass} of the host-promiscuous pathogen \textit{E. chrysanthemi} have an intermediate phenotype of abolished HR elicitation and reduced frequency of infection (Bauer et al., 1994). Therefore, the relative contribution of these elicitors to plant–bacterium interactions varies.

\textit{P. syringae} typically causes leaf spots and other necroses on aerial plant parts and is divided into more than 40 pathovars (Hirano and Upper, 1990). As observed by Klement and co-workers (Klement, 1963; Klement et al., 1964) during the discovery of the bacterial HR, \textit{P. syringae pv. syringae} (many strains of which are pathogenic in bean) elicits the HR in tobacco leaves, whereas \textit{Pseudomonas syringae pv. tabaci}, the agent of wildfire of tobacco, multiplies for several days and incites a delayed, progressive necrosis in tobacco. The \textit{hrp} genes were discovered in \textit{P. syringae} (Lindgren et al., 1986; Niepold et al., 1985), and the ability of one strain, \textit{Pseudomonas syringae pv. syringae} 61, to elicit the HR was shown to be minimally conferred by a 25 kb cluster of \textit{hrp} genes: cosmids pHIR11, which carries this cluster, enables the saprophytes \textit{Pseudomonas fluorescens} and \textit{Escherichia coli} to elicit the HR in tobacco and other plants (Huang et al., 1988).

The harpin-encoding \textit{hrp} gene is located within the pHIR11 \textit{hrp} cluster (He et al., 1993). Complementation analyses involving merodiploids with \textit{TnphoA} insertions in pHIR11 and in the \textit{P. syringae} 61 genome had suggested that group XII (\textit{hrpZ}) is required for the HR and pathogenicity phenotypes of \textit{P. syringae} 61. Furthermore, a \textit{hrpH} mutant, which cannot secrete HrpZ, fails to elicit the HR in tobacco leaves (He et al., 1993; Huang et al., 1992). However, sequence analysis of the DNA flanking \textit{hrpZ} has revealed that the gene is part of a polycistronic operon and that downstream genes (in complementation group XI) encode components of the secretion pathway (Huang et al., 1995; Preston et al., 1995). \textit{TnphoA} insertions in the \textit{P. syringae} \textit{hrp} genes apparently allow some expression of downstream genes (thus permitting the delineation of complementation groups at the cistron level), but explicitly non-polar mutations and complementation tests with \textit{hrpZ} subclones are now required to establish rigorously the role of HrpZ in plant interactions.

Genetic analysis of the role of HrpZ is further complicated by the possibility that certain mutations in the gene may fail to destroy elicitor activity. The initial isolation of \textit{hrpZ} yielded a fortuitous subclone encoding a truncated HrpZ lacking the first 125 amino acids of the 341 amino acid protein (He et al., 1993). The truncated protein is heat stable, readily purified and elicits the HR in tobacco and several other plants. Available restriction sites permitted the construction of several subclones producing additional deletion derivatives altered in this C-terminal portion of HrpZ, but the smaller fragments were not heat stable and could only be tested for elicitor activity in crude lysates.

To explore more rigorously the structural requirements for elicitor activity and the role of HrpZ in the HR, we generated subclones encoding a variety of truncated HrpZ derivatives that could be purified by use of an N-terminal His-6 tag, and we constructed a variety of \textit{hrpZ} deletion mutations. We report here that experiments involving these constructs and a mutation in \textit{hrmA}, which is required for \textit{P. fluorescens}(pHIR11) but not \textit{P. syringae} to elicit the HR (Heu and Hutcheson, 1993; Huang et al., 1991), have yielded a series of unexpected findings concerning the role of HrpZ in elicitation of the HR: (i) the C-terminal portion of HrpZ is not the only region with elicitor activity in the protein, (ii) HrpZ is not the only HR elicitor secreted by \textit{P. syringae} 61, (iii) \textit{hrpZ} expression in trans interferes with the secretion of any HrpZ from cells, and (iv) HrpZ can be secreted without eliciting the HR. A preliminary account of portions of this work has been published (Collmer et al., 1994).

Results

\textbf{Construction of functionally non-polar mutations in the pHIR11 \textit{hrpZ}}

Two types of mutation in \textit{hrpZ} that would permit explicit expression of downstream genes in the \textit{hrpZ} operon were constructed (Fig. 1). In the first, a 0.4 kb \textit{Bs}a\textit{BI} fragment was deleted from \textit{hrpZ} in pCPP2980, a subclone of pHIR11 carrying a 5.4 kb \textit{hrpRSAZBC}\textsuperscript{+} fragment in pUC18. The mutation removed nucleotides 461 to 869 of \textit{hrpZ}, resulting in a 137 amino acid deletion. Because the deleted nucleotides do not change the reading frame, the product has the N-terminal 153 and C-terminal 51 amino acids of HrpZ. The mutation was introduced into pHIR11 by successive homologous recombination events occurring at stringent and permissive temperatures fostered by \textit{E. coli C2110} (PolA\textsuperscript{ts}), which does not support effective replication of ColE1 replicons at 37°C. The \Delta\textit{hrpZ461-869} derivative of pHIR11, pCPP2985, was transferred by triparental mating into \textit{E. coli MC4100}, a strain in which pHIR11 is particularly effective in eliciting the HR.

The second type of mutation employed a 1.5 kb neomycin phosphotransferase II (\textit{ntpl}) cassette lacking a rho-independent transcription terminator. This was used to construct mutations in which downstream genes are expressed from the \textit{ntpl} promoter and was blunt-end ligated into pCPP2980 derivatives in which either the 0.4 kb \textit{Bs}a\textit{BI} or 0.8 kb \textit{Bs}a\textit{XI} fragments internal to \textit{hrpZ}}
were deleted. The 0.4 kb BsaBI deletion yielded a hrpZ461::nptII mutant that produced only the N-terminal 153 amino acids of HrpZ. The 0.8 kb BstXI mutation yielded a hrpZ194::nptII mutation that produced only the N-terminal 64 amino acids of HrpZ. To delete the entire hrpZ open reading frame (ORF) we performed oligonucleotide-directed, polymerase chain reaction (PCR) amplification of flanking sequences, followed by insertion of the nptII cassette. The mutations were subsequently recombined into pHIR11 using E. coli strain C2110. All mutations were confirmed by PCR amplification using oligonucleotides corresponding to sequences flanking the deletions.

E. coli MC4100(pHIR11) hrpZ mutants producing C-terminally truncated HrpZ fragments still elicit a strong HR, but a ΔhrpZ mutant does not

E. coli MC4100 cells carrying pHIR11 derivatives producing truncated forms of HrpZ were examined for their ability to elicit the HR following infiltration into tobacco leaves at a concentration of 5 × 10⁸ cells ml⁻¹. Although cultivar Xanthi plants were grown under similar conditions from week to week, we observed significant variation in their responses to the bacteria. Figure 2 shows representative results, and repeated tests permitted delineation of four phenotypic classes: I, no significant difference from wild type with respect to timing and completeness of collapse; II, collapse delayed (but within 48 h) or partially reduced relative to wild type if lower levels of inoculum used; III, very delayed (after 48 h) spotty collapse in approximately 10% of the plants, with most plants showing no response; IV, no collapse ever observed upon repeated assays. The ΔhrpZ461-869 and hrpZ461::nptII mutants, E. coli MC4100(pCPP2985) and E. coli MC4100(pCPP2297), respectively, were class I mutants, although occasionally the collapse elicited by the hrpZ461::nptII mutant was less complete. The hrpZ194::nptII mutant, E. coli MC4100- (pCPP2986), was class II; the ΔhrpZ::nptII mutant, E. coli MC4100(pCPP5024), was class III; and the hrpH::TnphoA mutant E. coli MC4100(pCPP2089) provided a class IV reference (not shown). Thus, HrpZ appears to be the major, if not sole, elicitor of the HR encoded by pHIR11, and the N-terminal half of the protein must contain elicitor information.

A ΔhrpZ::nptII mutation marker-exchanged into P. s. syringae reduces but does not abolish HR elicitation activity

The ΔhrpZ::nptII mutation was marker-exchanged into the P. s. syringae genome to produce mutant 61-5024. The construction of the mutation was confirmed by the kanamycin-resistant (Km⁶), tetracycline-sensitive (Tc⁵) phenotype of 61-5024, DNA gel blot analysis and failure of the mutant to produce immunoblot-detectable HrpZ (data not shown). The mutant was analysed for its ability to elicit...
the HR in tobacco leaves at three levels of inoculum. Wild-type cells elicited a typical HR at all three levels of inoculum (Fig. 3). Mutant 61-5024 failed to elicit an HR at $2 \times 10^7$ cells ml$^{-1}$ but elicited an HR indistinguishable from that of the wild type at $5 \times 10^8$ cells ml$^{-1}$. Therefore, the $\Delta hrpZ::nptII$ mutation had a class II phenotype in P. s. syringae 61. This suggests that HrpZ contributes significantly to the ability of the bacterium to elicit the HR in tobacco leaves, but the protein is not essential, and P. s. syringae 61 must produce at least one additional HR elicitor.

To confirm that the $\Delta hrpZ::nptII$ mutation in E. coli MC4100(pCPP5024) and P. s. syringae 61-5024 was functionally non-polar, we sought to complement the mutation with a subclone carrying hrpZ but not genes downstream in the same operon. pCPP2984 carried the hrpZ ORF under control of the nptII promoter in pML122. Unexpectedly, pCPP2984 failed to restore HR elicitation activity to E. coli MC4100(pCPP5024) (data not shown). Furthermore, the presence of the plasmid reduced rather than enhanced the HR elicitation activity of P. s. syringae 61-5024 (Fig. 3). This suggested that hrpZ expressed in trans might interfere with HrpZ production. To test this...
directly, we compared the production and localization of HrpZ in *P. s. syringae* and *P. s. syringae* (pCPP2984). Figure 4 shows that HrpZ was localized primarily in the supernatant of *P. s. syringae* but exclusively in the cell-bound fraction of *P. s. syringae* (pCPP2984). HrpZ secretion by *P. s. syringae* 61 was also blocked when the cells carried pCPP2301 (hrpAZ<sup>+</sup>) or pNCHU7 (hrpRSAZ<sup>−</sup>B<sup>−</sup>C<sup>−</sup>) (data not shown). Therefore, expressing hrpZ and hrpA, a candidate chaperone, together in trans did not overcome this interference. Furthermore, since hrpZ is expressed from its native promoter in a lower-copy-number vector in pNCHU7 and produces levels of immunoblot-detectable HrpZ comparable to that of induced *P. s. syringae* (data not shown), this interference effect is not the result of gross overproduction of the protein.

**HR elicitation activity can be restored to a P. s. syringae 61 hrpC mutant by pCPP5024 (ΔhrpZ::nptII) but not by pCPP3001 (hrpZ::Ωsp<sup>−</sup>)**

Because we could not complement ΔhrpZ::nptII mutations with a hrpZ subclone, we devised an alternative test of the functional non-polarity of the mutation. hrpC is downstream of hrpZ in the same operon and is required for HrpZ secretion and HR elicitation by both *P. s. syringae* and *P. fluorescens* (pH119) (Huang et al., 1991; 1995). If the ΔhrpZ::nptII mutation is functionally non-polar, then a pH119 derivative carrying it should complement a *P. s. syringae* hrpC mutation, whereas a pH119 derivative carrying an explicitly polar hrpZ::interposon mutation should not. An Ωsp<sup>−</sup> HindIII fragment was ligated into the HindIII site at nucleotide 408 of the hrpZ ORF in a hrpRSAZ<sup>−</sup>B<sup>−</sup>C<sup>−</sup> pUC18 derivative to produce pCPP3000. The mutation was introduced into pH119 by using *E. coli* C2110. The failure of the resulting plasmid, pCPP3001, to restore the HR phenotype to *P. s. syringae* 61–79 (data not shown) confirmed that hrpC and hrpZ are in the same transcriptional unit. However, HR-elicitation activity was restored by pCPP5024. Therefore, genes downstream of the ΔhrpZ::nptII mutation must be expressed, and the HR-deficient phenotype of *E. coli* MC4100- (pCPP5024) can be attributed to the HrpZ deficiency alone.

**Construction of hrpZ subclones producing fragments of HrpZ reveals that elicitor activity resides in multiple regions of HrpZ**

The HR-positive phenotype of *E. coli* MC4100 carrying pCPP2985 (ΔhrpZ<sup>2461−869</sup>) or pCPP2297 (hrpZ<sup>2461−nptII</sup>) suggested that the N-terminal portion of HrpZ possessed elicitor activity. Since the overlapping C-terminal fragment of the protein encoded by pSYH5 had previously been shown to possess elicitor activity (He et al., 1993), we constructed additional hrpZ deletion derivatives to determine if elicitor activity was localized to the overlap or resided in more than one region of the protein. Oligonucleotide primers, PCR and available restriction sites were used to produce the various internal fragments of hrpZ shown in Fig. 5A. These fragments were subcloned into pQE30/32 (Qiagen) to generate N-terminal translational fusions with vector sequences encoding a His-6 peptide. The peptide tag facilitated partial purification of His-6-HrpZ fragments from *E. coli* DH5α cell lysates by affinity chromatography on Ni-NTA columns (Janknecht et al., 1991). The production of the predicted HrpZ deletion fragments by the subclones was confirmed by staining immunoblotted SDS gels of the purified fragments with Coomassie brilliant blue (Fig. 5B,C).

The protein fragments were infiltrated into tobacco leaves at a concentration of 0.1 to 0.5 µg ml<sup>−1</sup>. Elicitor activity was displayed by all of the HrpZ fragments tested but by none of the control fragments (Fig. 5A). It is particularly noteworthy that the products of pSYH5 (C-terminal 216 amino acids) and pCPP2999 (N-terminal 109 amino acids) represent non-overlapping fragments of the protein. Nevertheless, they produce a hypersensitive-like collapse of tobacco leaf tissue that can be prevented by prior treatment with protease or by co-infiltration with lanthanum chloride, sodium vanadate or cycloheximide (He et al., 1993; data not shown). Therefore, the elicitor activity of HrpZ is not confined to one region of the protein.

**Structural analysis of the HrpZ sequence reveals several repeated features**

To search for similarities between non-overlapping HrpZ
fragments that might underlie their common elicitor activity, we analysed HrpZ for the presence of several relevant structural features. The results are shown in Fig. 6, with the data in the first three panels in alignment with the scale at the top. A Kyte and Doolittle (1992) hydrophilicity plot revealed that HrpZ has a relatively even distribution of short hydrophobic regions, with only one of them, between residues 316 and 335, having a high probability of being a membrane-spanning region (Fig. 6A). PROTEINPREDICT (Rost and Sander, 1993) analysis revealed nine highly probable $\alpha$-helices. Inspection of helical wheel and Eisenberg plots (Eisenberg et al., 1984) indicated that six of these $\alpha$-helices have substantial amphipathic character (Fig. 6B). There are direct, identical repeats of three sequences containing four or more amino acids (Fig. 6B). Additional repeats of similar sequences were found through a DNAStar MEGALIGN DotPlot diagonal self comparison of HrpZ, using a threshold similarity of 32%. Most notably, a region near the C-terminus of HrpZ shows at least 32% identity to stretches of 20 to 30 amino acids in four other, evenly spaced regions of the protein. To further analyse these regions of self similarity, we used Pileup to align the region running from residues 288 through 320 with the regions (adjusted to be of identical length) that were identified as similar. A PrettyPlot of the Pileup displays the similarity between the regions (Fig. 6C). No consensus sequence could be discerned from the Pileup, and for no two fragments was the percentage identity high enough to indicate significant homology (Sander and Schneider, 1991). Four of these regions possess all or parts of the LAKSM and QTGT repeats, but the GGGLGTP repeat regions self-aligned independently. In summary, these analyses suggest that the two non-overlapping HrpZ fragments with elicitor activity possess similar structural features, but they do not reveal any structural signature indicative of that activity.

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**Fig. 5.** Construction of internal subclones of *hrpZ*, isolation of HrpZ fragments and results of HR elicitor assays of these fragments and control proteins.

A. Subclones producing HrpZ fragments are aligned with the full-length *hrpZ* ORF of pSYH10. pSYH10 and pSYH5 contain *hrpZ* sequences in pBluescript SK–, and products were partially purified by heat denaturation of contaminating proteins as described (He et al., 1993). pCPP2991, pCPP2992 and pCPP2999 contain *hrpZ* sequences in pOE30/32. The products have an N-terminal His-6 tag and were purified by Ni-NTA chromatography and infiltrated into tobacco leaf panels at a concentration of 0.1 to 0.5 $\mu$g ml$^{-1}$ in 20 mM MES pH 5.6. The products of pQE32 (N-terminal His-6-tagged oligopeptide), pQE16 (C-terminal His-6-tagged mouse DHFR) and pJA16 (N-terminal His-6-tagged *P. s. glycinea* AvrB) were similarly prepared and infiltrated. Leaf symptoms were scored 24 h after infiltration: + denotes complete collapse of the infiltrated area; – denotes no collapse.

B. The relative purity of HrpZ fragment preparations was analysed by electrophoresis through an SDS 10% polyacrylamide gel followed by staining with Coomassie brilliant blue R-250.

C. Identification of the major band in each preparation above as a HrpZ fragment was confirmed by immunoblotting a polyacrylamide gel run in parallel with the one shown in (B) and visualizing HrpZ with anti-HrpZ antibodies as in Fig. 4.
P. fluorescens cells carrying pHIR11 hrmA :: TnphoA derivatives secrete HrpZ but fail to elicit the HR in tobacco leaves

Although the hrpZ :: nptII mutant E. coli MC4100-(pCPP5024) did not elicit the confluent necrosis typical of the HR within 24 h in tobacco leaves, it occasionally elicited a much delayed, spotty necrosis (Fig. 2). This raised the possibility that pHIR11 may harbour another, albeit secondary, elicitor of necrosis. hrmA warranted attention because it is located at the extreme left end of the hrp cluster in a location occupied by an avr gene in P. s. phaseolicola (Mansfield et al., 1994) and it is necessary for HR elicitation activity in P. fluorescens(pHIR11) but not P. s. syringae 61 (Heu and Hutcheson, 1993; Huang et al., 1993). To explore the role of hrmA in HrpZ deployment, we used immunoblots to determine the relative production and localization of HrpZ in P. fluorescens cultures carrying pHIR11 and two hrmA :: TnphoA derivatives, pCPP2070 and pCPP2071 (Huang et al., 1991). We used P. fluorescens rather than E. coli (MC4100) because only the former, like P. s. syringae 61, efficiently secretes most of the HrpZ to the medium in late-log cultures. This enabled us to examine definitively the HrpZ secretion phenotype of strains containing hrmA mutations. Surprisingly, HrpZ was synthesized and secreted by both mutants as well as by the wild-type P. fluorescens(pHIR11) (Fig. 7). In contrast, mutations in hrpL completely inhibited HrpZ synthesis by P. fluorescens(pHIR11), and those in hrpH blocked HrpZ secretion (data not shown). Therefore, in the absence of hrmA, wild-type levels of extracellular HrpZ appear insufficient for P. fluorescens(pHIR11) to elicit the HR in tobacco leaves. Moreover, in contrast to the occasionally leaky class III phenotype of the hrpZ mutation, hrmA mutations are class IV, completely abolishing the ability of P. fluorescens and E. coli MC4100 cells carrying pHIR11 derivatives to elicit any visible response in tobacco leaves.

Discussion

Typical hrp mutations produce bacteria that are pleiotropi...
cally defective in the in planta behaviour that distinguishes pathogens from saprophytes (Bonas, 1994). In P. s. syringae 61 this has been shown for hrp genes now known to be involved with protein secretion and the expression of secretion pathway genes, suggesting that extracellular proteins are key to the pathogenic abilities of the bacterium (He et al., 1993; Huang et al., 1991; 1995; Lidell and Hutcheson, 1994; Xiao et al., 1994). HrpZ is the only protein presently known to be secreted in a hrp-dependent manner by this bacterium (He et al., 1993), and a hrpZ::TnphoA (complementation group XII) mutant is unable to elicit the HR (Huang et al., 1991). However, our present data indicate that (i) genetic analysis of hrpZ is complicated by complementation interference effects and the presence of elicitor information in multiple regions of the protein, (ii) HrpZ is not the only HR elicitor protein produced by P. s. syringae 61, and (iii) extracellular HrpZ production by P. fluorescens(pHIR11), although necessary for HR elicitation, is not sufficient for it. We discuss below hrpZ genetic analyses, the structure and function of HrpZ, and possible relationships between hrpZ, hrmA and avr genes.

In contrast to the HR-elicitor proteins produced by E. amylovora and P. solanacearum, HrpZ is encoded by a gene within an operon containing other hrp genes. Significantly, four hrp genes downstream from hrpZ show varying levels of similarity in their predicted products to genes in Yersinia, Shigella and Salmonella spp. that encode components of the type III secretion pathway (Huang et al., 1995; Preston et al., 1995). Thus, the polar effects of typical transposon insertions in hrpZ should disrupt the secretion of any proteins that travel this pathway. But unexpectedly, many TnphoA insertions in P. s. syringae 61 hrp genes are evidently not polar since they permitted the identification of 12 complementation groups that now appear to be arranged in only seven operons, including those groups corresponding to hrpZ and hrpC in the hrpZ operon (Huang et al., 1991; 1995; Xiao et al., 1992). These complementation analyses involved insertions in the pHIR11 hrp cluster that were in trans to those in the P. s. syringae chromosome, and it is possible that some TnphoA mutations in the chromosome are significantly polar unless the affected operon is hyperexpressed by the presence of positive regulators in trans. The recent discovery that hrl is an alternative sigma factor suggests that such hyperexpression may occur when pHIR11 derivatives are present in P. s. syringae (Xiao et al., 1994; Xiao and Hutcheson, 1994).

Because of these concerns, we constructed a series of functionally non-polar mutations in which portions of hrpZ were deleted and replaced with an nptII cassette lacking a rho-independent transcription terminator. The expression of secretion pathway genes, such as hrpC, downstream of the cassette was confirmed by two observations. First, a ΔhrpZ::nptII pHIR11 derivative could complement a P. s. syringae hrpC mutation, whereas a hrpZ::Δsp mutant could not. Second, a P. s. syringae hrpZ::nptII mutant still elicited the HR, whereas P. s. syringae hrpC mutants do not (Huang et al., 1991; 1995). The latter observation is particularly compelling because it involves expression of the chromosomal hrpC in the absence of additional copies of any hrp genes, thus diminishing the potentially confounding effects of hyperexpression.

Unfortunately, further evidence for non-polarity based on complementation with hrpZ subclones was unattainable because the expression of hrpZ in trans prevented the secretion of any hrpZ from the cells of P. s. syringae or P. fluorescens(pHIR11). This complementation interference effect was observed with hrpZ, hrpAZ and hrpRSAZBC subclones, but not with pHIR11. Since secretion of the Yop and Ipa proteins via the type III pathway by Yersinia and Shigella spp. involves specific chaperones (Menard et al., 1994; Wattiau et al., 1994; Wattiau and Cornels, 1993), this raises the possibility that even partially uncoupling the expression of hrpZ from that of a chaperone results in blocked secretion. The observed linkage of several yop and syc (chaperone) genes led us to investigate hrlA as a possible chaperone for hrpZ. However, expression of the gene in trans, along with hrpZ, had no observable effect on HrpZ secretion. It is possible that the pHIR11 hrp cluster harbours a chaperone gene outside of the hrpZ operon. Alternatively, HrpZ overproduction may cause cytoplasmic aggregation or the cell may possess regulatory mechanisms which enforce co-ordinated secretion of HrpZ and other disease proteins.

Given the evidence that our nptII-marked mutations were functionally non-polar, the class III virtually HR+ phenotype of the ΔhrpZ::nptII mutation in E. coli MC4100(pCPP5024) suggests that HrpZ is the sole or major elicitor protein produced by the pHIR11 hrp cluster. Consequently, the HR+ phenotype of the hrpZ461::nptII mutation in MC4100(pCPP2297) suggests that the N-terminal 153 amino acids of HrpZ must possess elicitor activity. Interestingly, since hrpH or hrpC mutations in E. coli MC4100- (pHIR11) or P. fluorescens(pHIR11) block HrpZ secretion and elicitation of the HR, elicitor activity must be dependent upon secretion, and the N-terminal part of the protein must be competent for secretion. Independent evidence that the N-terminal portion of HrpZ possesses elicitor activity was obtained by observing that a purified His-6-tagged fragment containing the N-terminal 109 amino acids of HrpZ elicited a tissue collapse that was indistinguishable from that elicited by full-length HrpZ or a fragment containing the C-terminal 216 amino acids. It is important to note that our analysis of HrpZ frag-

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ments was limited to those that could be isolated, and the results differed somewhat from those of a previous study involving crude lysates from cells expressing hrpZ internal subclones (He et al., 1993). The previous results indicated that some HrpZ fragments lack elicitor activity, whereas all of the fragments in the present study were active. This follows, at least in part, from our failure to obtain stable His-6-tagged products corresponding to many of these hrpZ subclones. However, for pSYH32 we were able to obtain a stable His-6-tagged equivalent from pCPP2991. This product was active, whereas E. coli DH5α(pSYH32) lysates were not. Because the levels of candidate elicitors that were infiltrated into leaves in the previous work could not be effectively controlled, we feel that our current results, based on known amounts of purified fragments, are more definitive. Importantly, since all of the stable fragments we analysed were active, it is possible that there are additional non-overlapping fragments with elicitor activity.

The search for a structural signature for regions with elicitor activity was unsuccessful. The N-terminal 109 amino acid fragment and the non-overlapping C-terminal 216 amino acid fragment share a generally similar structure, regions of self-similarity and heat stability (J. R. Alfano, unpublished). However, elicitor activity is not associated with any consensus sequence, pairs of direct repeats or probable membrane-spanning regions. Nevertheless, because the various HrpZ fragments are unlikely to be acting as enzymes, elicitor activity must be determined directly by structural features. Whether these features are involved in interaction with the plant cell membrane, protein receptors or some novel effect on the cell wall is difficult to predict from the primary sequence. Two recent studies indicate the potential challenge. A 10 amino acid oligopeptide derived from E. coli haemolysin was found to be sufficient for transmembrane pore formation and haemolytic activity (Oropeza et al., 1992), and a 13 amino acid oligopeptide derived from a Phytophthora mega-sperrma glycoprotein was sufficient for the receptor-mediated defence elicitation activity of the protein (Nurnberger et al., 1994). Because many of the smaller fragments of HrpZ appear to be degraded in the bacterial cell and internal redundancy may mask the phenotype of site-directed mutations, identifying such elicitor-active oligopeptides within HrpZ may be difficult.

The role of HrpZ in elicitation of the HR by living bacteria is enigmatic. Although HrpZ is necessary for saprophytic bacteria carrying pHIR11 to elicit a typical HR, and the purified protein has elicitor activity, strains carrying hrmA mutations elicit no necrosis despite secreting wild-type levels of HrpZ. This suggests that HrmA and HrpZ are both required for pHIR11-dependent bacterial elicitation of the HR.

Several observations suggest that hrmA may function like an avr gene that interacts with an uncharacterized tobacco resistance gene. (i) Like individual avr genes (and unlike typical hrp genes) hrmA is present in relatively few strains of P. syringae (Heu and Hutcheson, 1993). (ii) The location occupied by hrmA in P. s. syringae 61 is occupied by avrPphE in P. s. phaseolicola 1302A (Mansfield et al., 1994). (iii) hrmA is not required for P. s. syringae 61 (which likely carries other avr genes) to elicit the HR in tobacco, while it is required for saprophytes carrying pHIR11 to elicit the HR (Huang et al., 1991). (iv) Whereas the hrpJ, U, H, and Z operons control HrpZ secretion and are necessary for HR elicitation activity in both P. fluorescens (pH11) and P. s. syringae 61 (He et al., 1993; Huang et al., 1995; Lidell and Hutcheson, 1994; A. O. Loniello and A. Collmer, in preparation), and the hrpL, S and R products are positive regulators necessary for hrpZ expression and HR elicitation activity (Huang et al., 1991; Xiao et al., 1994; Xiao and Hutcheson, 1994), hrmA has no obvious role in HrpZ regulation or secretion. (v) The incompatible interactions of P. fluorescens (pH11) with tobacco are formally analogous to the incompatible interactions of, for example, P. s. glycinea races with differential soybean cultivars: elicitation of the HR is determined not by the distribution of hrpZ, which is probably present in all P. syringae strains, but by the distribution of avr genes. (vi) Like typical avr genes, the contribution of hrmA to elicitation of the HR is observable only in a Hrp+ bacterium (Dangl, 1994; Keen, 1990); lysates of E. coli cells with highly expressed hrmA subclones fail to elicit the HR (He et al., 1993; data not shown).

Alternatively, hrmA has been proposed to be a positive regulatory factor because its introduction fails to convert P. s. glycinea race 4 to incompatibility in a range of soybean differential cultivars, and preliminary work suggested that strains with hrmA mutations had reduced hrpJ promoter activity (Heu and Hutcheson, 1993). However, the lack of a major effect of hrmA mutations on HrpZ secretion argues against such a regulatory role, and it is perhaps not surprising that P. s. syringae 61, which is a weak pathogen of legumes, would harbour an avr gene that interacts with a resistance gene in tobacco but not soybean. In the absence of differential tobacco cultivars segregating for such a resistance gene, demonstration that HrmA is an Avr analogue awaits deeper understanding of the defining biochemical characteristics of Avr proteins.

If HrmA is indeed an Avr analogue, the requirement of HrpZ for its activity may have general significance. Although many models can be conjured for this requirement, the three simplest are that (i) Avr proteins and harpins give the plant two different signals, these signals normally act synergistically in eliciting the HR and artificially high levels of exogenous harpins (but not Avr proteins) can overcome the two-signal requirement; or (ii) HrpZ is necessary for the effective delivery of Avr.
proteins (the actual elicitors) to, or into, plant cells, just as YopD is necessary for the delivery of YopE into mammalian cells by Yersinia spp. (Rosqvist et al., 1994; Sory and Cornelis, 1994); or (iii) Avr proteins act by modifying harpins or altering their deployment in a host-differential manner. Although this leaves uncertain the biological significance of the elicitor activity of HrpZ and its fragments (and the actual role in pathogenesis), it is worth recalling that the only bacteria known to elicit the HR are plant

Table 1. Strains and plasmids.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
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<tr>
<td>E. coli DH5α</td>
<td>supE44 ΔlacI169 (φ80 lacZΔM15)</td>
<td>Hanahan (1983); Life Technologies</td>
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<td></td>
<td>λ recA1 endA1 gyrA96 thi-1 relA1, NalR</td>
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<tr>
<td>DH5αF′ lacI8</td>
<td>F′ proAB lacI8 lacZΔM15 z2f′-Tn5 supE44 ΔlacI169 (φ80 lacZΔM15)</td>
<td>Life Technologies</td>
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<td></td>
<td>λ recA1 endA1 gyrA96 thi-1 relA1, NalR</td>
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<td>C2110</td>
<td>PolA NalR</td>
<td>Kahn and Hanawalt (1979)</td>
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<tr>
<td>MC4100</td>
<td>F− Δ(lac)U169 araD136 relA rpsL thi-1 smR</td>
<td>Oliver and Beckwith (1981)</td>
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<tr>
<td>P. fluorescens 55</td>
<td>NalR</td>
<td>Huang et al. (1988)</td>
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<tr>
<td>P. s. syringae 61</td>
<td>Wild type; spontaneous NalR</td>
<td>Huang et al. (1988)</td>
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<td>61–79</td>
<td>hrpC::TnphoA</td>
<td>H.-C. Huang (1988)</td>
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<td>61–5024</td>
<td>HrpZ::nptII</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmid</td>
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<tr>
<td>pML122</td>
<td>Broad-host-range IncQ vector for expression from nptII promoter, GmR</td>
<td>Labes et al. (1990)</td>
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<td>pQE30/32</td>
<td>His-6 translational fusion vector, ApR</td>
<td>Jorgensen et al. (1979)</td>
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<td>pRZ102</td>
<td>ColE1 carrying Tn5</td>
<td>This work</td>
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<td>pCPP2988</td>
<td>Bluescript vector II SK— carrying 1.5kb HindIII–SalI fragment from pRZ102 with nptII lacking terminator</td>
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<td>pRK415</td>
<td>Broad-host-range vector, unstable in absence of selection, TcR</td>
<td>Keen et al. (1988)</td>
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<td>pRK2013</td>
<td>Tra−, KmR</td>
<td>Rukin and Ausubel (1981)</td>
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<td>pHR11</td>
<td>P. s. syringae 61 hrpC cluster in pLAFR3</td>
<td>Huang et al. (1988)</td>
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<td>pNCHU7</td>
<td>5.4 kb BamHI–EcoRI fragment carrying hrpRSA2BC in pCPP30</td>
<td>H.-C. Huang (1988)</td>
</tr>
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<td>pCPP2980</td>
<td>5.4 kb BamHI–EcoRI hrpRSA2BC′ fragment from pNCHU7 subcloned in pUC18</td>
<td>This work</td>
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<td>pCPP2981</td>
<td>Derivative of pCPP2980 with 0.4 kb BsaBI fragment deleted; ΔhrpZ461–869</td>
<td>This work</td>
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<td>pCPP2982</td>
<td>Derivative of pCPP2980 with nptII in BsaBI site, hrpZ461::nptII</td>
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<td>pCPP2987</td>
<td>Derivative of pCPP2980 with nptII in BamHI site; hrpZ184::nptII</td>
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<td>pCPP2985</td>
<td>ΔhrpZ461–869 pHrI11</td>
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Table 1. Continued.

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<th>Characteristics</th>
<th>Reference</th>
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<td>pCPP2297</td>
<td>hrpZ461::nptII pHrI11</td>
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<td>pCPP5986</td>
<td>hrpZ194::nptII pHrI11</td>
<td>This work</td>
</tr>
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<td>pCPP2312</td>
<td>PCR product with 2.7 kb of DNA 5′ of hrpZ in pUC18</td>
<td>This work</td>
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<td>pCPP2313</td>
<td>PCR product with 2.0 kb of DNA 3′ of hrpZ in pUC18</td>
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<td>pCPP2315</td>
<td>ΔhrpZ::nptII mutant with nptII flanked by inserts from pCPP2312 and pCPP2313 in pUC18</td>
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<td>pCPP2316</td>
<td>Insert from pCPP2315 recloned in pRK415</td>
<td>This work</td>
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<td>pCPP5024</td>
<td>ΔhrpZ::nptII pHrI11</td>
<td>This work</td>
</tr>
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<td>pCPP2984</td>
<td>hrpZ in BamHI–Xhol fragment from pSYH10 expressed from nptII promoter in pML122</td>
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<td>pCPP2301</td>
<td>hrpAZ in pML122</td>
<td>This work</td>
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<td>pCPP3000</td>
<td>5.4 kb BamHI–EcoRI fragment carrying hrpRSA2BC from pCPP2980 with ΩspR in hprZ HindIII site; in pUC18 derivative lacking HindIII site</td>
<td>This work</td>
</tr>
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<td>pHP45Ω</td>
<td>ΩspR</td>
<td>Prentki and Krisch (1984)</td>
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<tr>
<td>pCPP3001</td>
<td>pHrI11 with ΩspR in HindIII site in hrpZ</td>
<td>This work</td>
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<td>pSYH10</td>
<td>hrpZ in Bluescript Sau3A fragment encoding C-terminal 216 amino acids of HrpZ in Bluescript</td>
<td>He et al. (1993)</td>
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<td>pSYH5</td>
<td>pQE30 derivative producing HrpZ 126–300 with N-terminal His-6</td>
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<td>pCPP2991</td>
<td>pQE32 derivative producing HrpZ 1–153 with N-terminal His-6</td>
<td>This work</td>
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<td>pCPP2992</td>
<td>pQE32 derivative producing HrpZ 1–109 with N-terminal His-6</td>
<td>This work</td>
</tr>
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<td>pCPP2999</td>
<td>pQE32 derivative producing HrpZ 1–109 with N-terminal His-6</td>
<td>This work</td>
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<td>pAVR1</td>
<td>Carries P. s. glycinea avrB, KmR</td>
<td>Tamaki et al. (1988)</td>
</tr>
<tr>
<td>pJA16</td>
<td>pQE32 derivative producing AvrB with N-terminal His-6</td>
<td>This work</td>
</tr>
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<td>pCPP2070</td>
<td>pHrI11 hrmA1::TnphoA</td>
<td>Huang et al. (1991)</td>
</tr>
<tr>
<td>pCPP2071</td>
<td>pHrI11 hrmA2::TnphoA</td>
<td>Huang et al. (1991)</td>
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pathogens (or recombinant derivatives thereof) and that harpin-like proteins are the only products of these bacteria known to have elicitor activity (Collmer and Bauer, 1994). Therefore, even if observable under artificial conditions only, the HR-eliciting activity of HrpZ is likely to hold important clues to the molecular basis of bacterial plant pathogenicity.

Experimental procedures

Bacterial strains, plasmids and media

Bacterial strains and plasmids are listed in Table 1. E. coli strains were routinely grown in LM (Hanahan, 1983) or Terrific broth (Sambrook et al., 1989) at 37°C, except MC4100 cultures carrying pHIR11, which were grown at 30°C in King’s B broth (King et al., 1954) prior to infiltration into tobacco plants. P. s. syringae 61 was grown in King’s B broth at 30°C. For in vitro expression of P. s. syringae 61 hrp genes, cultures were grown in the hrp-depressing fructose minimal medium of Huynh et al. (1989) at 30°C. Antibiotics were used at the following concentrations (µg·ml⁻¹): ampicillin, 100; kanamycin, 50; tetracycline, 20; spectinomycin, 50; and gentamicin, 10.

Plant materials

Tobacco plants (Nicotiana tabacum L. cv. Xanthi) were grown in a greenhouse with supplemental metal halide lamp illumination and a photoperiod of 16 h. Infiltration of tobacco leaves with HrpZ preparations or bacteria (in 20 mM MES pH 5.6) was done with needleless syringes.

DNA manipulations

Restriction enzymes and T4 DNA ligase were purchased from either New England Biolabs or Life Technologies, Inc. Recombinant DNA manipulations and PCR reactions were done according to standard protocols (Sambrook et al., 1989; Innis et al., 1990). PCR reactions were done in a total volume of 100 µl using the following concentrations of components: 50 ng template DNA, 0.25 µM each primer, 40 µM deoxynucleotide triphosphates, 1.5 mM MgCl₂, 1× Ultra PCR buffer (Perkin Elmer), and 2.5 units/reaction Ultima DNA Polymerase (Perkin Elmer). The reactions were initiated using a hot start by adding Ampliwax PCR Gem 100 (Perkin Elmer) wax beads to each reaction tube as described in the manufacturer’s instructions. The cycling parameters used were: 94°C 2 min; 30 cycles of 94°C 1 min, 55°C 2 min and 72°C 3 min; and 72°C for 7 min.

Deletion of the hrpZ ORF and replacement with a terminator-less nptII cassette

A 2.7 kb region of DNA upstream of hrpZ was PCR-amplified from pCPP2980 and cloned into the HindIII site of pUC18, constructing pCPP2312, using the primers 5’-ATGAGTGACCTGAGAGGTCGAC-3’ and 5’-ATGAGAATTCCTGGTGACAGAGAGGACCCG-3’, which contained the restriction enzyme sites KpnI and EcoRI, respectively. nptII without its rho-independent transcriptional terminator was isolated from Tn5 carried on pRZ102 (Jorgensen et al., 1979) as a HindIII–Sall fragment and subcloned into pBluescript vector II SK− to produce pCPP2988. The 2.0 kb PCR product was cloned into pUC18 to construct pCPP2313. The nptII cassette and the 2.7 kb PCR product were cloned into pCPP2313, constructing pCPP2315, which lacked hrpZ but contained instead the nptII marker and the flanking 2.7 and 2.0 kb DNA fragments. To introduce this mutation into pHIR11 or into the chromosome of P. s. syringae we used strategies described below.

Construction of partial deletion mutations in hrpZ and recombination of mutations into pHIR11

pCPP2980 contained a hrpRSAZB‘C‘ 5.4 kb BamHI–EcoRI fragment in pUC18. The 408 bp BstXI fragment internal to hrpZ was deleted from pCPP2980 and religated to construct a plasmid designated pCPP2981. pCPP2982 was constructed in the same way as pCPP2981 except that the nptII cassette was blunt-end ligated into pCPP2980, following T4 DNA polymerase treatment, replacing the BstXI fragment with nptII. Similarly, pCPP2987 was constructed by deleting the 780 bp BstXI fragment within hrpZ and replacing it with the nptII gene via blunt-end ligation. The polar hrpZ-Δhrs‘ mutation in pCPP3000 was constructed by excising the 3500 bp HindIII fragment from pHPI40 and ligating it into the HindIII site in phpZ in a pCPP2980 derivative in which the hrpRSAZBC genes had been recloned into a pUC18 derivative in which the HindIII site in the vector was eliminated. The hrpZ mutants carried on pCPP2315, pCPP2981, pCPP2982, pCPP2987 and pCPP3000 were introduced into pHI11 using a strategy based on the principle that ColE1 plasmids cannot replicate in polA-containing E. coli mutants whereas broad-host plasmids such as pHI11 can. Each construct carrying a hrpZ mutation on pUC18 was electroporated into E. coli C2110 and spread on LM plates, selecting for antibiotic markers for both vectors at 37°C. Ap⁻ Te² transformants were transferred to 5 ml of LM medium containing tetracycline and grown at 30°C for 4 days. A small amount of culture was transferred daily to fresh LM medium. 1:1000 dilutions of the final transfers were plated and Te⁻ colonies picked to LM plates containing tetracycline with and without ampicillin. DNA was isolated from Ap⁺ colonies and screened for the presence of the appropriate mutation by restriction site analysis and PCR. Triparental matings were used to move pHIR11 hrpZ mutants from E. coli C2110 to E. coli MC4100 or from MC4100 into P. s. syringae 61. The helper plasmid was pRK2013; host DH5α permitted the use of streptomycin to counterselect donor and helper in transfers to E. coli MC4100, whereas E. coli HB101 permitted cassette selection with nalidixic acid in transfers to P. s. syringae 61.
Marker-exchange of the ΔhrpZ::nptII mutation into P. s. syringae 61

The DNA insert contained in pCPP2315 was subcloned into the BamHI–EcoRI sites of pRK415. The resulting subclone, pCPP2316, was electroporated into P. s. syringae 61, selecting for Km and Tc. Loss of plasmid and retention of the nptII marker was achieved by growing cultures of P. s. syringae 61(pCPP2316) and selecting for Km. Fresh cultures were inoculated daily with a small inoculum of the previous over-night culture for 3 days. The final culture was plated onto King’s B agar supplemented with nalidixic acid and kanamycin, with and without tetracycline. Total DNA was made from Tc colonies and used for DNA gel blot analysis and PCR reactions with primers flanking hrpZ to confirm that Tc colonies lacked any hrpZ coding sequence.

Preparation of HrpZ fragments

The primers 5'-TACGGGTATCTAGTCTCATTTACCA-3' and 5'-AGTAGGCTCTCATGTAAGTCTT-3', which contained BamHI and SacI sites, respectively, were used to PCR-amplify from pSYH10 the 5' portion of hrpZ corresponding to the N-terminal 153 amino acids of HrpZ. The amplified product was subcloned into the BamHI and SacI sites of pQE32 (Qiagen), constructing pCPP2992. pCPP2999 was constructed by excising a PpuMI–PstI fragment from pCPP2992, treating with T4 DNA polymerase and recircularizing the plasmid by blunt-end ligation. This interrupted the hrpZ ORF at the Pfu MI site (nucleotide 327). The primers 5'-ATACGGATCCGATCTTCATCGTTAG-3' and 5'-CTTTAGCTCTCATAGCGCACTTCGCAA-3', which contained BamHI and SacI restriction enzyme sites, respectively, were used to PCR-amplify from pSYH10 nucleotides 378–900 of the hrpZ coding region, corresponding to amino acids 126–300. This PCR product was directionally cloned into the BamHI–SacI sites of pQE30 (Qiagen) to produce pCPP2991. The primers 5'-AGATG- GATCCGCGC- TGCGTCTCGTCGAAA-3' and 5'-ATACACTGACAGAAGCA- TCGAATCTAG-3' were used to PCR-amplify avrB from pAVRB1 (Tamaki et al., 1988), and the product was subcloned into the BamHI–PstI sites of pQE30 using the synthetic restriction sites on the end of each primer to produce pJA16.

E. coli cells carrying pCPP2992, pCPP2999, pCPP2991 or pJA16 produced proteins with an N-terminal His-6 tag, which facilitates purification under non-denaturing conditions using Ni-NTA spin columns. The column manufacturer’s (Qiagen) instructions were modified as follows. E. coli DH5α F' lacI were grown in 5 ml of Terrific broth overnight with shaking at 30ºC. These were used to inoculate 100 ml Terrific broth cultures at an OD600 of 0.1. The cultures were induced with 1 mM IPTG at an OD600 of 0.5 and grown for 5 h at 30ºC. The cells were harvested by centrifugation, resuspended in 2 ml lysis buffer (50 mM Na phosphate pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mg/ml lysozyme, 1 mM phenylmethylsulphonyl fluoride (PMSF; Life Technologies)) and incubated on ice for 20 min. Cells were disrupted by sonication on ice for 2 min each. HrpZ preparations were boiled for 10 min for further purification. Cell debris was removed by microcentrifugation, and supernatants were loaded onto equilibrated Ni-NTA spin columns in 600 µl portions and eluted with a buffer containing 250 mM imidazole, as described by Qiagen. Protein samples were desalted using Biogel P-6 DG chromatography as described by the manufacturer (Biorad). Total protein concentration was measured by the method of Bradford (1976).

Computer analyses of HrpZ protein structure

The HrpZ sequence was analysed for structural features with the DNAStar Lasergene Software Package (Madison, WI), the Genetics Computer Group Sequence Analysis Software Package (Doveraux et al., 1984) and the ProteinPredictor server (Rost and Sander, 1993; Rost et al., 1994). The hydrophilicity analysis (Kyte and Doolittle, 1982) was done using the PROTEAN program from DNAStar with a setting of nine residues to average the hydrophilicity. The PredictProtein server (Rost et al., 1994) was used to identify α-helices within HrpZ. The input was a HrpZ protein alignment file of the HrpZ proteins of P. s. syringae 61, P. s. glycinea race 4 and Pseudomonas syringae pv. tomato DC3000 (Preston et al., 1995) constructed using the GCG program Pileup (Deveraux et al., 1984). We exploited a PredictProtein server feature of rating structure predictions and included in Fig. 6 only α-helices that received a rating of 9 (the highest). The amphipathic character of each putative α-helix was analysed using Helical Wheels and Eisenberg moment plots (Eisenberg et al., 1984), both included in the PROTEAN program from DNAStar. The parameters used for the Eisenberg moments were the default values: residues to average, 11; alpha angle, 100; and beta angle, 170. To detect similar regions within HrpZ we used a DNAStar MEGALIGN DotPlot analysis. The parameters used for the DotPlot were: percent match, 32; minimum windows, 1; and window, 30. The similarities detected using the DotPlot analysis were further analysed by protein alignments using the GCG programs Pileup and PrettyPlot.

Protein analyses

Cell-bound and secreted proteins were isolated from P. syringae or P. fluorescens(pH1R11) cultures that were grown in 5 ml King’s B broth overnight at 30ºC, harvested by centrifugation, resuspended in 10 ml of hrp-derepressing fructose minimal medium (Huynh et al., 1989) at an OD600 of 0.4 and then grown at 30ºC to an OD600 of 0.8. Cells were separated into cell-bound and supernatant fractions by centrifugation, and 1 mM PMSF was added to each supernatant fraction immediately after passage through 0.2 µm filters to remove residual cells. Supernatant fractions were concentrated using Centriprep-10 concentrators (Amicon) to a volume of approximately 1 ml. Cell pellets were resuspended in 1 ml 20 mM MES buffer pH 5.6, and soluble proteins were released by sonication of each sample for 2 min in the presence of 1 mM PMSF. SDS–PAGE was done using standard procedures (Sambrook et al., 1989). For immunoblot analysis, separated proteins were transferred to Immobilon-P transfer membranes (Millipore) and HrpZ was recognized with rabbit polyclonal antibodies raised to HrpZ (He et al., 1993). Goat-anti-rabbit IgG alkaline phosphate conjugate (Sigma Chemical Co.) was used as the secondary antibody. Membrane-
bound secondary antibodies were visualized with either BCIP/NBT tablets (Sigma) or by chemiluminescence using the Western-Light chemiluminescent detection system (Tropix).

Acknowledgements

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References


