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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*::Tn*phoA*) 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.**

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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 $hrpN_{Ech}$ of the host-promiscuous pathogen *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.

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, *P. syringae* pv. *syringae* (many strains of which are pathogenic in bean) elicits the HR in tobacco leaves, whereas *Pseudomonas syringae* pv. *tabaci*, the agent of wildfire of tobacco, multiplies for several days and incites a delayed, progressive necrosis in tobacco. The *hrp* genes were discovered in *P. syringae* (Lindgren *et al*., 1986; Niepold *et al*., 1985), and the ability of one strain, *Pseudomonas syringae* pv. *syringae* 61, to elicit the HR was shown to be minimally conferred by a 25 kb cluster of *hrp* genes: cosmid pHIR11, which carries this cluster, enables the saprophytes *Pseudomonas fluorescens* and *Escherichia coli* to elicit the HR in tobacco and other plants (Huang *et al*., 1988).

The harpin-encoding *hrpZ* gene is located within the pHIR11 *hrp* cluster (He *et al*., 1993). Complementation analyses involving merodiploids with Tn*phoA* insertions in pHIR11 and in the *P. s. syringae* 61 genome had suggested that group XII (*hrpZ*) is required for the HR and pathogenicity phenotypes of *P. s. syringae* 61. Furthermore, a *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 *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). Tn*phoA* insertions in the *P. s. syringae 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 *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 *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 *hrpZ* deletion mutations. We report here that experiments involving these constructs and a mutation in *hrmA*, which is required for *P. fluorescens*(pHIR11) but not *P. s. 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 *P. s. syringae* 61, (iii) *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

Construction of functionally non-polar mutations in the pHIR11 hrpZ

Two types of mutation in *hrpZ* that would permit explicit expression of downstream genes in the *hrpZ* operon were constructed (Fig. 1). In the first, a 0.4 kb *Bsa*BI fragment was deleted from *hrpZ* in pCPP2980, a subclone of pHIR11 carrying a 5.4 kb $hrpRSAZBC^{+}$ fragment in pUC18. The mutation removed nucleotides 461 to 869 of *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 *E. coli* C2110 ($PolA^{ts}$), which does not support effective replication of ColE1 replicons at 37°C. The Δ*hrpZ461-869* derivative of pHIR11, pCPP2985, was transferred by triparental mating into *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 (*nptII*) cassette lacking a rho-independent transcription terminator. This was used to construct mutations in which downstream genes are expressed from the *nptII* promoter and was blunt-end ligated into pCPP2980 derivatives in which either the 0.4 kb *Bsa*BI or 0.8 kb *Bst*XI fragments internal to *hrpZ*

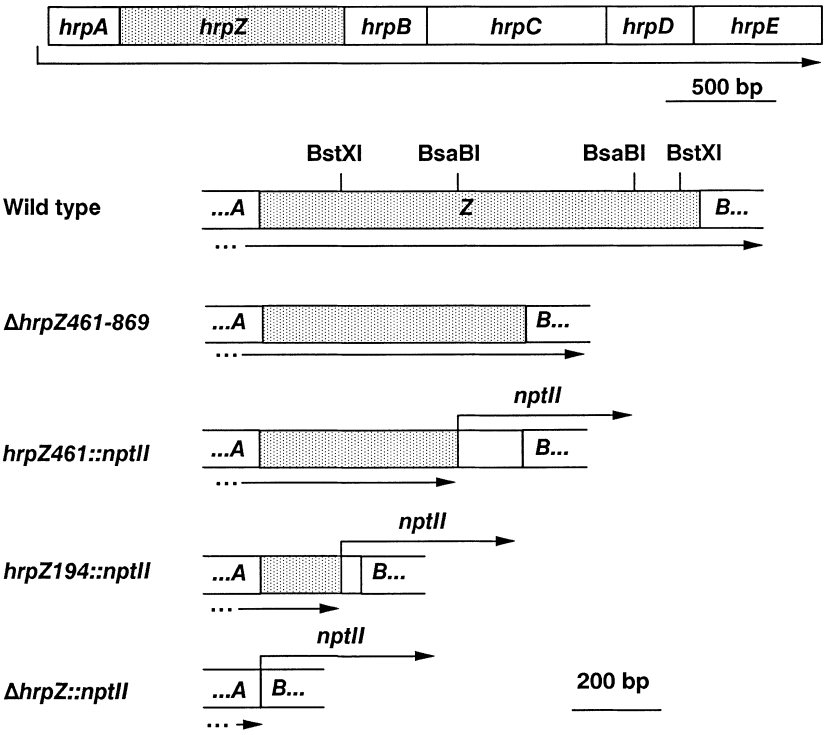

Fig. 1. Construction of functionally non-polar *hrpZ* mutations in pHIR11. The location of *hrpZ* within the operon is depicted at the top. Restriction sites used to construct deletions are shown above the wild-type *hrpZ* ORF (shaded box). Arrows below each diagram denote transcription from the native promoter. The arrows marked *nptII* indicate transcription from the promoter in the *nptII* cassette.

were deleted. The 0.4 kb *Bsa*BI deletion yielded a *hrpZ461*::*nptII* mutant that produced only the N-terminal 153 amino acids of HrpZ. The 0.8 kb *Bst*XI 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 5×10^8 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 Δ*hrpZ461-869* 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 Δ*hrpZ*::*nptII* mutant, *E. coli* MC4100(pCPP5024), was class III; and the *hrpH*::Tn*phoA* 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 Δ*hrpZ*::*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 kanomycin-resistant (Km^R), tetracycline-sensitive (Tc^S) 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

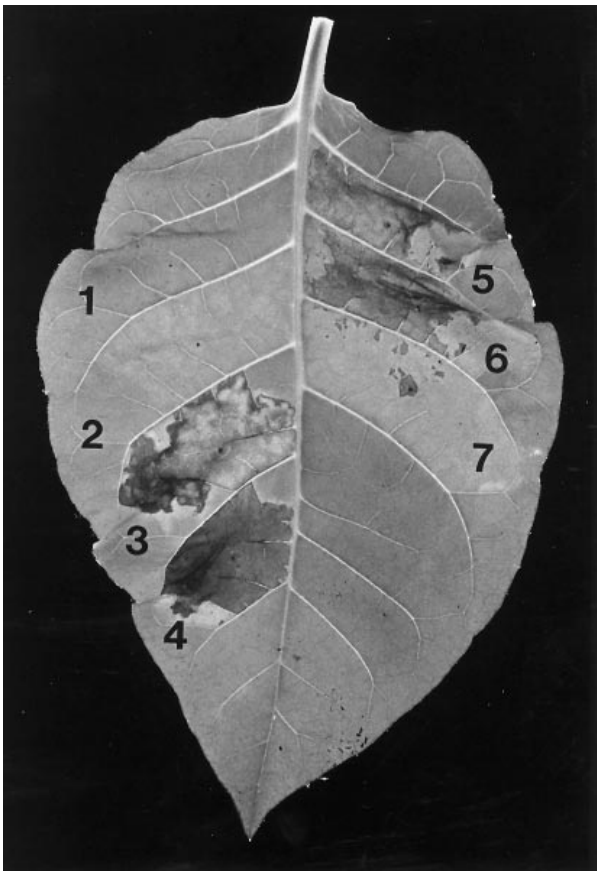

Fig. 2. Tobacco leaf showing ability of *E. coli* MC4100(pHIR11) *hrpZ* mutants producing truncated HrpZ fragments to elicit the HR. *E. coli* cultures were grown overnight in King's B medium, then harvested by centrifugation and resuspended in 20 mM MES pH 5.6, at an OD_{600} of 0.8 (5×10^8 cells ml^{-1}), and infiltrated into tobacco leaf panels. The leaf was photographed with incident light 3 days later. Areas: 1, buffer only; 2, *E. coli* MC4100; 3, *E. coli* MC4100(pHIR11); 4, *E. coli* MC4100(pCPP2985 Δ*hrpZ461-869*); 5, *E. coli* MC4100(pCPP2297 *hrpZ461*::*nptII*); 6, *E. coli* MC4100(pCPP2986 *hrpZ194*::*nptII*); and 7, *E. coli* MC4100(pCPP5024 Δ*hrpZ*::*nptII*).

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 2×10^7 cells ml^{-1} but elicited an HR indistinguishable from that of the wild type at 5×10^8 cells ml^{-1}. Therefore, the Δ*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.

hrpZ *expressed* in trans *in* P. s. syringae *61 or 61-5024 interferes with HrpZ secretion*

To confirm that the Δ*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

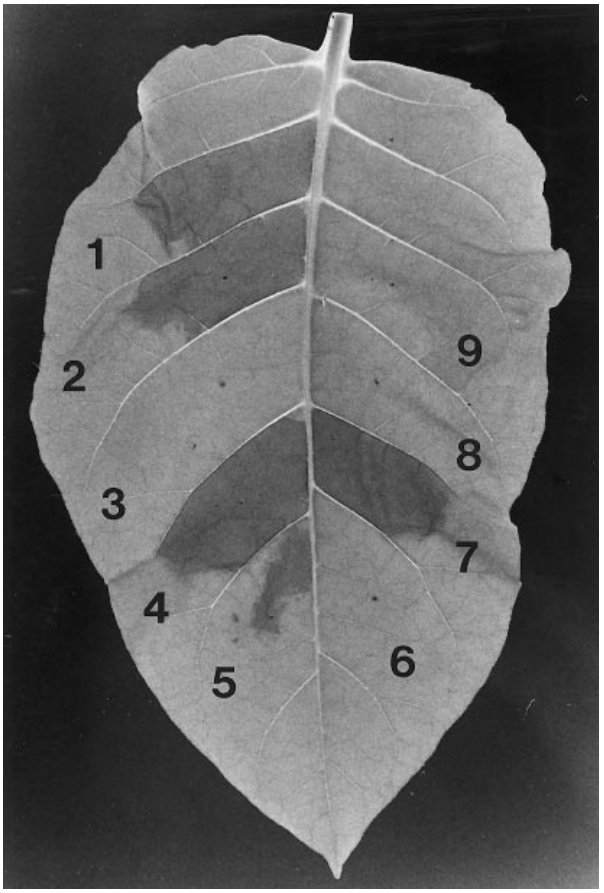

Fig. 3. Tobacco leaf showing residual ability of *P.s. syringae* 61-5024 (Δ*hrpZ*::*nptII*) to elicit the HR and the complementation interference effect of pCPP2984 (*hrpZ*$^+$). Bacterial inoculum was prepared as described for Fig. 2, except that leaf panels were infiltrated with bacteria at three concentrations (cells ml^{-1}): 1–3, 5×10^8; 4–6, 1×10^8; 7–9, 2×10^7. The leaf was photographed 2 days later with cross-polarized transillumination to enhance visualization of necrotic areas. Areas: 1, *P.s. syringae* 61; 2, *P.s. syringae* 61-5024; 3, *P.s. syringae* 61-5024(pCPP2984); 4, *P.s. syringae* 61; 5, *P.s. syringae* 61-5024; 6, *P.s. syringae* 61-5024(pCPP2984); 7, *P.s. syringae* 61; 8, *P.s. syringae* 61-5024; and 9, *P.s. syringae* 61-5024(pCPP2984).

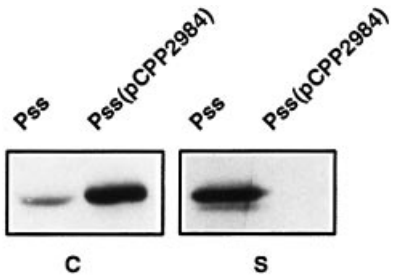

Fig. 4. Immunoblot showing that *hrpZ* expression *in trans* prevents secretion of any HrpZ from *P.s. syringae* 61. Cultures of *P.s. syringae* 61 (Pss) with and without pCPP2984 were grown in Hrp-minimal medium to an OD_{600} of 0.8 and then separated into cell-bound (C) and supernatant (S) fractions by centrifugation. Soluble proteins were released from the cell-bound fraction by sonication, and then all fractions were subjected to SDS–PAGE and immunoblot analysis with anti-HrpZ antibodies and secondary antibodies conjugated with alkaline phosphatase.

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*$^+$) or pNCHU7 (*hrpRSAZBC*$^+$) (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^r)

Because we could not complement Δ*hrpZ*::*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*(pHIR11) (Huang *et al*., 1991; 1995). If the Δ*hrpZ*::*nptII* mutation is functionally non-polar, then a pHIR11 derivative carrying it should complement a *P. s. syringae hrpC* mutation, whereas a pHIR11 derivative carrying an explicitly polar *hrpZ*::interposon mutation should not. An Ω*sp*^{r} *Hin*dIII fragment was ligated into the *Hin*dIII site at nucleotide 408 of the *hrpZ* ORF in a *hrpRSAZBC*$^+$ pUC18 derivative to produce pCPP3000. The mutation was introduced into pHIR11 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 Δ*hrpZ*::*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 (Δ*hrpZ461-869*) or pCPP2297 (*hrpZ461*::*nptII*) 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^{-1}. 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

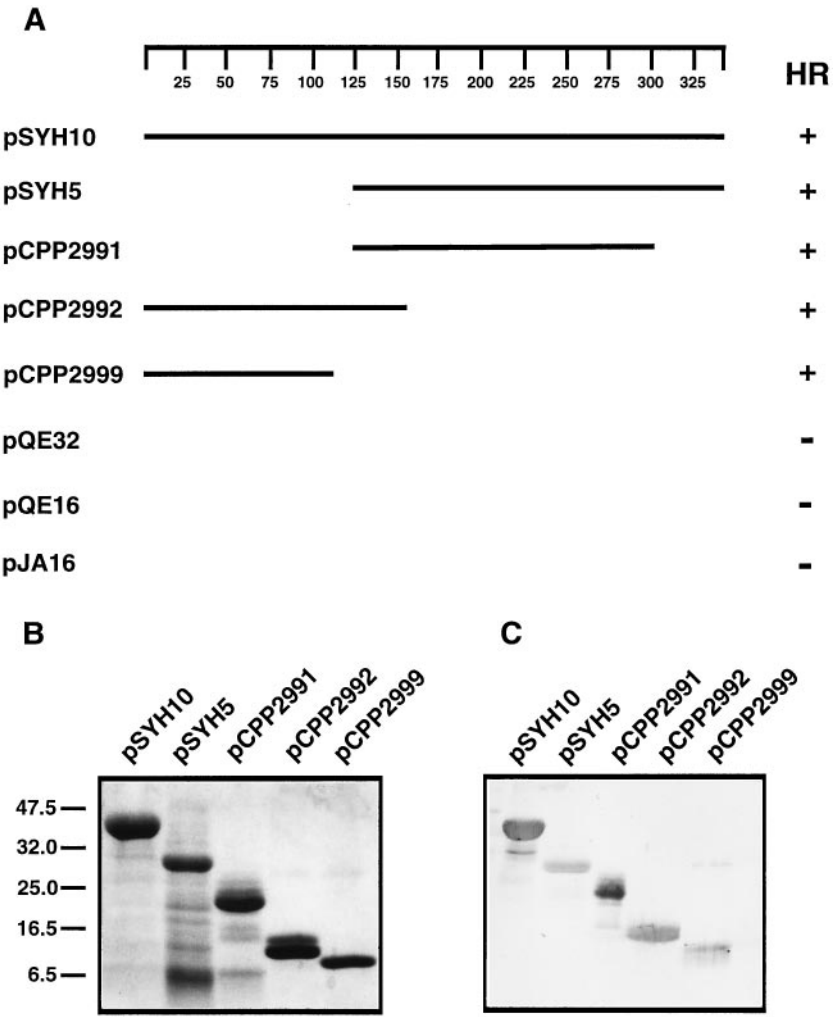

Fig. 5. Construction of internal subclones of *hrpZ*, isolation of HrpZ fragments and results of HR elicitor assays of these fragments and control proteins.
A. Subclones producing HrpZ fragments are aligned with the full-length *hrpZ* ORF of pSYH10. pSYH10 and pSYH5 contain *hrpZ* sequences in pBluescript 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 pQE30/32. 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.

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 α-helices. Inspection of helical wheel and Eisenberg plots (Eisenberg *et al*., 1984) indicated that six of these α-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.

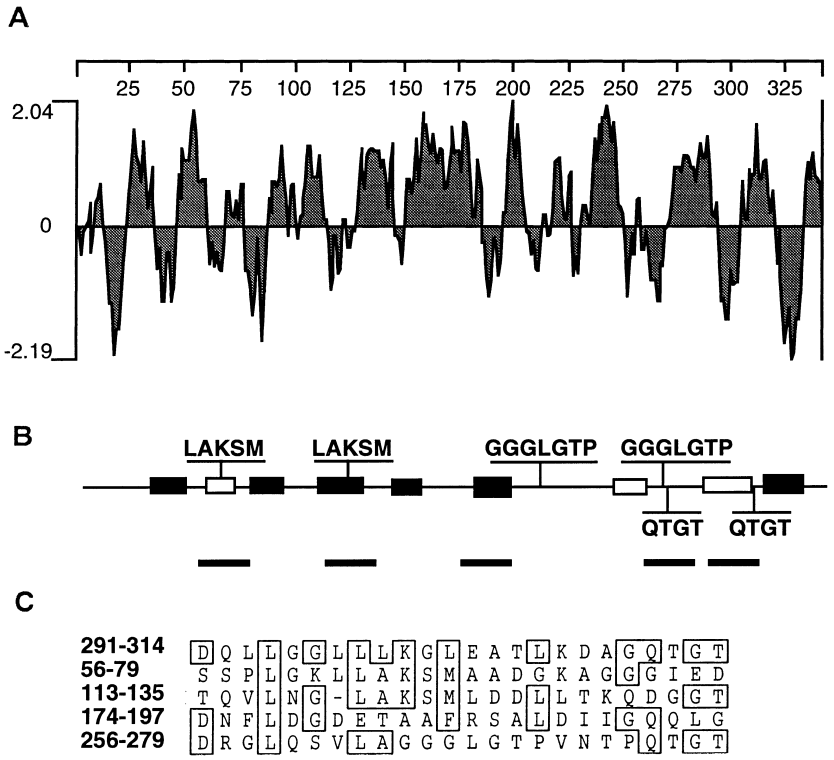

Fig. 6. Structural features of HrpZ.
A. Hydrophilicity plot of HrpZ.
B. Potentially important structural features of HrpZ include α-helices (boxed regions), amphipathic α-helices (black boxes), direct repeats of identical sequences and direct repeats of similar sequences (black bars).
C. PRETTYPLOT alignment of the sequences that show similarity with amino acids 291–314.

P. fluorescens *cells carrying pHIR11* hrmA *::* TnphoA *derivatives secrete HrpZ but fail to elicit the HR in tobacco leaves*

Although the Δ*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*::Tn*phoA* derivatives, pCPP2071 and pCPP2070 (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 Δ*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-

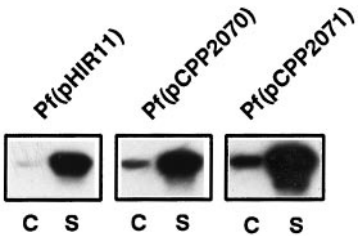

Fig. 7. Immunoblot showing that *P. fluorescens*(pHIR11) *hrmA*::Tn*phoA* mutants secrete HrpZ. Cultures of *P. fluorescens* carrying pHIR11 and two *hrmA*::Tn*phoA* derivatives, pCPP2070 and pCPP2071, were grown in Hrp-minimal medium to an OD_{600} of 0.8 and then separated into cell-bound (C) and supernatant (S) fractions by centrifugation. The distribution of HrpZ in cell fractions was determined as described in Fig. 4.

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*::Tn*phoA* (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 Tn*phoA* 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 Tn*phoA* 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 *hrpL* 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 Δ*hrpZ*::*nptII* pHIR11 derivative could complement a *P. s. syringae hrpC* mutation, whereas a *hrpZ*::Ω*sp*^{r} 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 Cornelis, 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 *hrpA* 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 Δ*hrpZ*::*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 *hrpZ*461::*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-

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 megasperma* 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* (pHIR11) 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* (pHIR11) 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

Table 1. Strains and plasmids.

Designation	Characteristics	Reference
Strain		
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, Nal^R	Hanahan (1983); Life Technologies
DH5αF′*lacI*^{q}	F′*proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15 zzf*::Tn*5 supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*, Nal^R	Life Technologies
C2110	$PolA^{ts}$ Nal^R	Kahn and Hanawalt (1979)
MC4100	F$^-$ Δ(*lac*)*U169 araD136 relA rpsL thi*, Sm^R	Oliver and Beckwith (1981)
P. fluorescens 55	Nal^R	Huang *et al*. (1988)
P. s. syringae		
61	Wild type; spontaneous Nal^R	Huang *et al*. (1988)
61–79	*hrpC*::Tn*phoA*	H.-C. Huang
61–5024	Δ*hrpZ*::*nptII*	This work
Plasmid		
pML122	Broad-host-range IncQ vector for expression from nptII promoter, Gm^R	Labes *et al*. (1990)
pUC18	Cloning vector, Ap^R	Yanisch-Perron *et al*. (1985)
pQE30/32	His-6 translational fusion vector, Ap^R	Qiagen
pRZ102	ColE1 carrying Tn*5*	Jorgensen *et al*. (1979)
pCPP2988	pBluescript vector II SK− carrying 1.5 kb *Hin*dIII–*Sal*I fragment from pRZ102 with *nptII* lacking terminator	This work
pRK415	Broad-host-range vector, unstable in absence of selection, Tc^R	Keen *et al*. (1988)
pRK2013	Tra^+, Km^R	Ruvkin and Ausubel (1981)
pHIR11	*P. s. syringae* 61 *hrp* cluster in pLAFR3	Huang *et al*. (1988)
pNCHU7	5.4 kb *Bam*HI–*Eco*RI fragment carrying *hrpRSAZBC* in pCPP30	H.-C. Huang
pCPP2980	5.4 kb *Bam*HI–*Eco*RI *hrpRSAZBC*$^+$ fragment from pNCHU7 subcloned in pUC18	This work
pCPP2981	Derivative of pCPP2980 with 0.4 kb *Bsa*BI fragment deleted; Δ*hrpZ461-869*	This work
pCPP2982	Derivative of pCPP2980 with *nptII* in *Bsa*BI site, *hrpZ461*::*nptII*	This work
pCPP2987	Derivative of pCPP2980 with *nptII* in *Bst*XI site; *hrpZ194*::*nptII*	This work
pCPP2985	Δ*hrpZ461-869* pHIR11	This work

Table 1. Continued.

Designation	Characteristics	Reference
pCPP2297	*hrpZ461*::*nptII* pHIR11	This work
pCPP2986	*hrpZ194*::*nptII* pHIR11	This work
pCPP2312	PCR product with 2.7 kb of DNA 5′ of *hrpZ* in pUC18	This work
pCPP2313	PCR product with 2.0 kb of DNA 3′ of *hrpZ* in pUC18	This work
pCPP2315	Δ*hrpZ*::*nptII* mutant with *nptII* flanked by inserts from pCPP2312 and pCPP2313 in pUC18	This work
pCPP2316	Insert from pCPP2315 recloned in pRK415	This work
pCPP5024	Δ*hrpZ*::*nptII* pHIR11	This work
pCPP2984	*hrpZ* in *Bam*HI–*Xho*I fragment from pSYH10 expressed from *nptII* promoter in pML122	This work
pCPP2301	*hrpAZ* in pML122	This work
pCPP3000	5.4 kb *Bam*HI–*Eco*RI fragment carrying *hrpRSAZBC* from pCPP2980 with Ω*sp*^{r} in *hrpZ* **Hin**dIII site; in pUC18 derivative lacking *Hin*dIII site	This work
pHP45Ω	Ω*sp*^{r}	Prentki and Krisch (1984)
pCPP3001	pHIR11 with Ω*sp*^{r} in *Hin*dIII site in *hrpZ*	This work
pSYH10	*hrpZ* in pBluescript	He *et al*. (1993)
pSYH5	*Sau*3A fragment encoding C-terminal 216 amino acids of HrpZ in pBluescript	He *et al*. (1993)
pCPP2991	pQE30 derivative producing HrpZ 126–300 with N-terminal His-6	This work
pCPP2992	pQE32 derivative producing HrpZ 1–153 with N-terminal His-6	This work
pCPP2999	pQE32 derivative producing HrpZ 1–109 with N-terminal His-6	This work
pAVRB1	Carries *P. s. glycinea avrB*, Km^R	Tamaki *et al*. (1988)
pJA16	pQE32 derivative producing AvrB with N-terminal His-6	This work
pCPP2070	pHIR11 *hrmA1*::Tn*phoA*	Huang *et al*. (1991)
pCPP2071	pHIR11 *hrmA2*::Tn*phoA*	Huang *et al*. (1991)

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

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*-derepressing fructose minimal medium of Huynh *et al*. (1989) at 30°C. Antibiotics were used at the following concentrations ($\mu g\ ml^{-1}$): 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 $MgCl_2$, 1× Ultma PCR buffer (Perkin Elmer), and 2.5 units/reaction Ultma 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 *Hin*dIII site of pUC18, constructing pCPP2312, using the primers 5′-ATGAAAGCTTGCATGCCTGCAGGTCGAC-3′ and 5′-ATGAAAGCTTATCACAACCTCCTAAAAG-3′. Both primers contained *Hin*dIII sites incorporated at their 5′ ends to facilitate cloning. The product of the above primers spanned from the nucleotides immediately upstream of the start site for *hrpZ* to those upstream of *hrpR*. The 2.0 kb region spanning from immediately downstream of *hrpZ* to within *hrpC* was PCR-amplified from pCPP2980 with the primers 5′-ATGAGGTACCTGACCGACAACCGCCTGA-3′ and 5′-ATGAGAATTCCTGGTGCAGAGAGGAGCG-3′, which contained the restriction enzyme sites *Kpn*I and *Eco*RI, respectively. *nptII* without its rho-independent transcriptional terminator was isolated from Tn*5* carried on pRZ102 (Jorgensen *et al*., 1979) as a *Hin*dIII–*Sal*I 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 *hrpRSAZBC*$^+$ 5.4 kb *Bam*HI–*Eco*RI fragment in pUC18. The 408 bp *Bsa*BI 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 *Bsa*BI fragment with *nptII*. Similarly, pCPP2987 was constructed by deleting the 780 bp *Bst*XI fragment within *hrpZ* and replacing it with the *nptII* gene via blunt-end ligation. The polar *hrpZ*::Ω*sp*^{r} mutation in pCPP3000 was constructed by excising the Ω*sp*^{r} *Hin*dIII fragment from pHP45Ω and ligating it into the *Hin*dIII site in *hrpZ* in a pCPP2980 derivative in which the *hrpRSAZBC* genes had been recloned into a pUC18 derivative in which the *Hin*dIII site in the vector was eliminated.

The *hrpZ* mutations carried on pCPP2315, pCPP2981, pCPP2982, pCPP2987 and pCPP3000 were introduced into pHIR11 using a strategy based on the principle that ColE1 plasmids cannot replicate in *polA*-containing *E. coli* mutants whereas broad-host plasmids such as pHIR11 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^R Tc^R 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 Tc^R colonies picked to LM plates containing tetracycline with and without ampicillin. DNA was isolated from Ap^S 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 counterselection 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 *Bam*HI–*Eco*RI sites of pRK415. The resulting subclone, pCPP2316, was electroporated into *P. s. syringae* 61, selecting for Km^R and Tc^R. Loss of plasmid and retention of the *nptII* marker was achieved by growing cultures of *P. s. syringae* 61(pCPP2316) and selecting for Km^R. Fresh cultures were inoculated daily with a small inoculum of the previous overnight 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^S colonies and used for DNA gel blot analysis and PCR reactions with primers flanking *hrpZ* to confirm that Tc^S colonies lacked any *hrpZ* coding sequence.

Preparation of HrpZ fragments

The primers 5′-TACGGGATCCAGAGTCTCAGTCTTAACA-3′ and 5′-AGTAGAGCTCTCACATGAACTGCGCGATCTT-3′, which contained *Bam*HI and *Sac*I 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 *Bam*HI and *Sac*I sites of pQE32 (Qiagen), constructing pCPP2992. pCPP2999 was constructed by excising a *Ppu*MI–*Pst*I fragment from pCPP2992, treating with T4 DNA polymerase and recircularizing the plasmid by blunt-end ligation. This interupted the *hrpZ* ORF at the *Ppu*MI site (nucleotide 327). The primers 5′-ATACGGATCCGATCTTCTGACCAAGCAG-GAT-3′ and 5′-CGTTGAGCTCCTACAGGCCCTTGAGCAG-CAA-3′, which contained *Bam*HI and *Sac*I 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 *Bam*HI–*Sac*I sites of pQE30 (Qiagen) to produce pCPP2991. The primers 5′-AGATG-GATCCGGC-
TGCGTCTCGTCGAAA-3′ and 5′-ATCACTGCAGAAAG-CAA-
TCAGAATCTAG-3′ were used to PCR-amplify *avrB* from pAVRB1 (Tamaki *et al*., 1988), and the product was subcloned into the *Bam*HI–*Pst*I 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*^{q} were grown in 5 ml of Terrific broth overnight with shaking at 30°C. These were used to inoculate 100 ml Terrific broth cultures at an OD_{600} of 0.1. The cultures were induced with 1 mM IPTG at an OD_{600} of 0.5 and grown for 5 h at 30°C. 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^{-1} lysozme, 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 (Devereaux *et al*., 1984) and the ProteinPredict 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 *Psuedomonas synringae* 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*(pHIR11) cultures that were grown in 5 ml King's B broth overnight at 30°C, harvested by centrifugation, resuspended in 10 ml of *hrp*-derepressing fructose minimal medium (Huynh *et al*., 1989) at an OD_{600} of 0.4 and then grown at 30°C to an OD_{600} 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).

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