The HrpZ Proteins of Pseudomonas syringae pvs. syringae, glycinea, and tomato Are Encoded by an Operon Containing Yersinia ysc Homologs and Elicit the Hypersensitive Response in Tomato but not Soybean

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The Pseudomonas syringae pathovars are composed of host-specific plant pathogens that characteristically elicit the defense-associated hypersensitive response (HR) in nonhost plants. P. syringae 61 secretes an HR elicitor, harpin_HrpZ (HrpZ_Fad), in a hrp-dependent manner. An internal fragment of the P. syringae 61 hrpZ gene was used to clone the hrpZ locus from P. syringae glycinea race 4 (bacterial blight of soybean) and P. syringae tomato DC3000 (bacterial speck of tomato). DNA sequence analysis revealed that hrpZ is the second ORF in a polysaccharic operon. The amino acid sequence identities of HrpZ_Fad/HrpZ_Fad and HrpZ_Fad/HrpZ_Fad were 79 and 63%, respectively. Although none of the HrpZ proteins showed significant overall sequence similarity with other known proteins, HrpZ_Fad contained a 24-amino acid sequence that is homologous with a region of the PopA1 elicitor protein of the tomato pathogen, Pseudomonas solanacearum GMI1000. hrpA, the upstream ORF, was highly divergent. The amino acid sequence identities of HrpA_Fad/HrpA_Fad and HrpA_Fad/HrpA_Fad were 91 and 28%, respectively, and no HrpA sequence showed similarity to known proteins. In contrast, the predicted products of the downstream ORFs in P. syringae 61 and P. syringae tomato, hrbB, hrbC, hreb, and hreF showed varying levels of similarity to those of yscI, yscJ, yscK, and yscL. These are colinearly arranged genes in the virC locus of Yersinia spp., which are involved in the secretion of the Yop virulence proteins via the type III pathway. The similarity of the Ysc proteins was generally stronger in comparisons with the P. syringae tomato Hrp proteins. The HrpZ proteins were purified by heat denaturation of contaminating proteins followed by ammonium sulfate fractionation, hydrophobic chromatography, and gel electrophoresis. All three HrpZ proteins elicited the HR in tomato, whereas none of them elicited significant necrosis in soybean. The results indicate that HrpZ is encoded in an operon containing some of the genes involved in its own secretion and suggest that HrpZ structure does not directly determine bacterial host range.

Phytopathogenic strains of Pseudomonas syringae cause two patterns of necrosis when the bacteria invade a plant. On a susceptible ("compatible") host, a necrotic lesion often develops over a period of days, with necrosis spreading as the bacteria multiply and the plant becomes diseased. On a resistant or nonhost plant, a localized cellular necrosis is induced within 24 to 48 h, and bacterial multiplication is inhibited. This was first reported by Klement (1963; Klement et al. 1964), who observed that when high concentrations of pathogenic bacteria are infiltrated into an incompatible plant they elicit a visible necrosis which is limited to the infiltrated area. This reaction, called the hypersensitive response (HR), involves localized cell death and production of anti-microbial compounds at the site of pathogen invasion (Bonas 1994). The ability of P. syringae and other nonmutogenic, gram-negative, bacterial pathogens to elicit the HR is governed by hrp genes. Typical Hrp mutants are pleiotropically defective in planta: They do not elicit the HR in nonhosts and they fail to multiply and cause disease in host plants (Lindgren et al. 1986). Clusters of hrp genes have been identified in many gram-negative phytopathogenic bacteria (Bonas 1994). A 25-kb hrp cluster from P. syringae 61 is sufficient to confer the tobacco HR phenotype, but not the pathogenic phenotype on nonpathogenic bacteria (Huang et al. 1988). Hrp genes have also been cloned and characterized extensively from P. syringae NPS3121, P. solanacearum GMI1000, Xanthomonas campestris pv. vesicatoria 75-3, and Erwinia amylovora Ea321 (Lindgren et al. 1986; Boucher et al. 1987; Beer et al. 1991; Bonas et al. 1991). Certain hrp genes are widely conserved among these pathogens, and several encode components of a protein secretion pathway that is similar to the type III pathway used by Yersinia, Shigella, and Salmonella spp. to secrete extracellular proteins involved in animal pathogenesis (Van Gijsenberg et al. 1993). One activity of the hrc-encoded secretion pathway in phytopathogenic bacteria is the secretion of proteinaceous elicitors of the HR, which are also encoded by hrp genes.

The first hrp-encoded elicitor characterized was harpin from E. amylovora (Wei et al. 1992). Similar elicitors have since been isolated from other bacteria, including P. syringae 61, P. solanacearum GMI1000, and E. chrysanthemi.

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EC16 (He et al. 1993; Arlat et al. 1994; Bauer et al. 1994). Proteins in this family of elicitors share several general characteristics. They are glycine rich, heat-stable, lack cysteine, and appear highly susceptible to proteolysis. They lack an N-terminal signal peptide, but they are secreted to the bacterial milieu. Their expression and secretion is dependent on hrc genes. The biological role of these proteins in pathogenesis has not yet been determined, but the purified proteins can induce an HR on a nonhost plant such as tobacco. However, there are significant differences in the organization of the elicitor operons and the activity of the elicitors, which suggest that the Erwinia harpins, the P. syringae hrcZ product and the P. solanacearum popA product may represent three distinct classes of elicitors. In this work we will refer to the P. s. pv. syringae elicitor as HarpZp vs. HarpinN (He et al. 1993). This distinction is supported by the weak similarity of the amino acid sequences of the four proteins, with the only exception being the C-terminal halves of the Erwinia harpins (Bauer et al. 1994).

The location of known elicitor genes in reference to the hrc cluster varies in P. s. pv. syringae, P. solanacearum, and E. amylovora. harpN and harpZ are contiguous or within the hrc cluster, whereas popA lies outside (although near) the P. solanacearum hrc cluster (Wei et al. 1992; He et al. 1993; Arlat et al. 1994). There are no genes downstream of the elicitor gene in either the hrcN or the popA operons, which means that mutations in the elicitor genes do not have a polar effect on the Hrp phenotype, and mutant construction is straightforward. In contrast, mutagenesis and complementation studies of the hrc cluster from P. s. pv. syringae 61 have indicated that hrcZ lies upstream of at least one other hrc gene within an operon (Huang et al. 1991; Xiao et al. 1992).

In E. amylovora and E. chrysanthemi, harpins have been demonstrated to be sufficient and necessary to elicit the HR, and mutation of hrcN in E. amylovora has shown that HarpinN is required for pathogenesis (Wei et al. 1992). However hrcN mutants of E. chrysanthemi can establish infections, albeit at a significantly reduced frequency, which suggests that HarpinN is important but not essential for pathogenesis (Bauer et al. 1995). In contrast, a popA mutant of P. solanacearum is fully pathogenic on susceptible hosts, indicating that PopA1 is not required for pathogenesis (Arlat et al. 1994).

These elicitors may play a role in controlling the host specificity exhibited by E. amylovora and plant pathogenic pseudomonads such as P. syringae and P. solanacearum. However, it is difficult to compare the activity of HarpZp vs and HarpinN in host and nonhost plants because legumes and roaceous plants, the hosts of P. s. pv. syringae 61 and E. amylovora Ea321, respectively, respond poorly to preparations of any of these elicitor proteins (Wei et al. 1992; He et al. 1993). PopA1 from P. solanacearum does appear to act in a host-specific manner, inducing an HR on resistant lines of petunia and the nonhost tobacco, but not on susceptible lines of petunia or tomato (Arlat et al. 1994). This phenotype is similar to that of avr genes, but PopA1 is distinct from known Avr proteins in eliciting the HR directly on resistant plants. HarpinN elicits an HR on some compatible hosts of E. chrysanthemi, but in contrast to the other three bacteria E. chrysanthemi is a broad-host-range pathogen and the activity of HarpinN may not be representative of elicitor activity in a highly host-specific system (Bauer et al. 1995).

In previous work we cloned and characterized the hrcZ gene from P. s. pv. syringae 61, a weak pathogen of bean, and demonstrated with Southern and immunoblotting that other pathovars of P. syringae contain homologs of this gene (He et al. 1993). This supported the hypothesis that HrpZ represents a family of elicitors common to all pathogenic strains of P. syringae. We report here the isolation of homologs of HarpZp vs from two other experimentally important pathovars of P. syringae-P. s. pv. tomato and P. s. pv. glycinea. Examining HrpZ from these three pathovars enabled us to look within this family of elicitors for variations in sequence and activity which could indicate a role in host range determination. In addition, we characterized the two genes flanking hrcZ in P. s. pv. syringae and P. s. pv. glycinea and the entire hrcZ operon of P. s. pv. tomato. In conjunction with an accompanying paper (Huang et al. 1995), this completes the sequence of the P. s. pv. syringae 61 hrc genes carried on pHI-RII and provides clues to the function of the genes downstream of hrcZ.

A preliminary account of portions of this work has been published (Collmer et al. 1994).

RESULTS

Cloning hrcZ from P. s. pv. tomato and P. s. pv. glycinea.

We previously used Southern hybridization to demonstrate that both P. s. pv. glycinea race 4 and P. s. pv. tomato DC3000 contain sequences homologous to a 0.75 kb BstXI internal fragment of hrcZ from P. s. pv. syringae (He et al. 1993). The same probe was used to screen genomic libraries of P. s. pv. glycinea and P. s. pv. tomato. The libraries were constructed in E. coli DH5α by inserting 8- to 12-kb fragments from partial Sau3AI digests of genomic DNA into the BamHI site of pUC19. The screen identified two plasmids with inserts of approximately 10 kb: pCPP2201 (P. s. pv. toma-to) and pCPP2200 (P. s. pv. glycinea). The same BstXI fragment was used to probe a Southern blot of pCPP2201 and pCPP2200 digested with BamHI, EcoRI, and PstI. The probe identified two PstI fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200, respectively (Fig. 1). The two PstI fragments were cloned into the PstI site of pBluescript II SK(-) (Stratagene, La Jolla, CA) in E. coli DH5α to create the plasmids pCPP2202 to pCPP2205, with the inserts in both orientations with respect to the lac promoter. Cell lysates of E. coli DH5α containing pCPP2203 (hrcZp vs in the vector promoter orientation) and pCPP2202 (hrcZp vs in the vector promoter orientation) induced an HR on tobacco, but those from cells containing pCPP2205 (hrcZp vs in the opposite orientation of the vector promoter) and pCPP2204 (hrcZp vs in the opposite orientation of the vector promoter) did not. HR activity was retained after incubating the lysate for 10 min at 100°C and removing denatured proteins by centrifugation. Sensitivity to heat treatment is a characteristic feature of previously isolated HR elicitors. Proteins in the lysates were separated on an SDS-polyacrylamide gel, transferred to an Immobilon-P membrane and immunoblotted with antibodies raised against purified HrpZp vs. Cross-reacting proteins of a similar size to the HrpZp vs were observed and provisionally named HrpZp vs, HrpZp vs (Fig. 2, lanes 2 and 4).

The intensity of the HrpZp vs and HrpZp vs bands was quite low in comparison to the band for HrpZp vs, expressed from pSYH10 in E. coli DH5α (Fig. 2, lane 1). This implied either
that expression was low due to the distance of the cloned gene from the lac promoter or that HrpZ_{Psy} and HrpZ_{Psy} did not hybridize strongly to the antibodies. A band corresponding to HrpZ_{Psy} from pSYH10 could be clearly seen on a Coomassie-stained gel, but the bands for HrpZ_{Psy} and HrpZ_{Psy} were indistinct, which implies that low expression was a primary reason for the low signal. In an attempt to improve the level of expression of HrpZ_{Psy} and HrpZ_{Psy} we subcloned EcoRI-BamHI fragments containing the inserts from pCPP2202 and pCPP2203 behind the T7 promoter of pET21(+)- in E. coli BL21(DE3) to create the plasmids pCPP2206 and pCPP2207.

The T7 promoter enabled a moderate improvement in protein expression (Fig. 2, lanes 3 and 5).

A common arrangement of ORFs in the hrpZ operons of P. s. pv. syringae, P. s. pv. glycinea, and P. s. pv. tomato revealed by DNA sequence analysis.

Previously, we determined the complete nucleotide sequence of hrpZ from P. s. pv. syringae by sequencing a 1.4-kb subclone of pHIR1 (a cosmid containing the entire hrp cluster from P. s. pv. syringae) (He et al. 1993). In addition, analysis of the complementation groups and transcriptional

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*Fig. 1. Physical maps of the hrpZ regions from Pseudomonas syringae pv. syringae 61, P. s. pv. glycinea race 4, and P. s. pv. tomato DC3000 and clones used in this study. Open boxes represent sequenced ORFs; filled boxes represent unsequenced DNA. Direction of transcription is indicated by the arrows. Key restriction sites within the sequenced regions are indicated, along with the subclones used in this study. The 0.75-kb BstXI fragment from hrpZ_{Psy} used as a probe for hrpZ genes in other pathovars is also shown. Restriction endonuclease abbreviations: A, AgeI\textsuperscript{*}; B, BglII; Bx, BstXI\textsuperscript{*}; E, EcoRI; EV, EcoRV\textsuperscript{*}; F, PstI; S, SacI; Sa, SstIII\textsuperscript{*}; SI, SalI\textsuperscript{*}. \* Not all sites are shown.*
units of pHIR11 using TnphoA and Tn5-gusA1 mutagenesis (Huang et al. 1991; Xiao et al. 1992) suggested that hrpZ lay within an operon, upstream of at least one other hrp gene. Further subclones of pHIR11 were used to determine the sequence of the entire hrpZ operon (this study, Huang et al. 1995). We also determined the sequence of (i) the 2.2- and 2.4-kb PstI subclones from pCPP2201 (hrpZ$_{pp}$) and pCPP2200 (hrpZ$_{pp}$), (ii) an overlapping 3.7-kb SacI-EcoRI subclone from pCPP2201 (designated pCPP2209), and (iii) part of an overlapping 3.6-kb subclone from pCPP2200 (designated pCPP2208), as shown in Figure 1. This yielded the sequence of the entire P. s. pv. tomato hrpZ operon and the first half of the P. s. pv. glycinea operon. The sequenced region of P. s. pv. syringae and P. s. pv. tomato extends from hrpS (Xiao et al. 1994), through the hrpZ operon to the beginning of the hrpH operon (Huang et al. 1992), demonstrating that the organization of this region of the hrp cluster is conserved in both pathovars.

Codon preference analysis of the DNA sequence, using P. s. pv. syringae codon usage data, predicted that hrpZ was the second of six ORFs, all oriented in the same direction, an arrangement conserved in P. s. pv. tomato and at least the first four ORFs of P. s. pv. glycinea. The sequence of the noncoding DNA is shown in Figure 3. Five of the six ORFs have clear potential ribosome binding sites. The fifth ORF has a putative ribosome binding site in P. s. pv. syringae, but the site in P. s. pv. tomato is less clear, the initiation codon shown being selected by alignment with the ORF in P. s. pv. syringae. In the absence of recognizable terminator elements downstream of the first five ORFs it seems likely that the six ORFs represent a single operon, transcribed from upstream of the first ORF. The five predicted ORFs were provisionally named hrpA through hrpE, as shown in Figures 1 and 3.

A hrp/avr promoter consensus sequence lies upstream of the hrpZ operons of the three P. syringae pathovars.

The conserved sequence GGAACCC—16bp—CCACNN lies 50 bp upstream of the initiation codon of hrpA in all three pathovars (Fig. 3). This motif has been identified in the promoter regions of many avr and hrp genes (Innes et al. 1993; Shen and Keen 1993), and appears to be involved in positive regulation by HrpL, a putative alternative sigma factor which is itself positively regulated by HrpK and HrpS (Xiao and Hutcheson 1994). HrpL is a member of a family of alternative sigma factors, many of which are involved in secretion of extracellular factors in response to environmental stimuli (Lonetto et al. 1992). The presence of this promoter motif further supports the suggestion that the six ORFs form a single transcriptional unit which is regulated in a hrp-dependent manner. This motif can also be found beyond hrpE, upstream of hrpFGH in P. s. pv. syringae and P. s. pv. tomato, as indicated at the bottom of Figure 3, suggesting that the latter three ORFs form an independent hrp-regulated transcriptional unit in these two pathovars.

Comparison of the HrpZ proteins of the three P. syringae pathovars.

The predicted amino acid sequences for HrpZ from each of the three pathovars are aligned in Figure 4. Although the proteins migrate slightly anomalously on an SDS polyacrylamide gel, the relative sizes of the estimated molecular weights correspond to the predicted values, with HrpZ$_{pp}$ being the largest of the three proteins (36.5 kDa), followed by HrpZ$_{ps}$ (35.3 kDa) and HrpZ$_{m}$ (34.7 kDa). Amino-terminal sequencing of the first 10 to 15 residues of purified HrpZ$_{pp}$ and HrpZ$_{ps}$ confirmed the predicted initiation codons of both proteins, which aligned with the start codon of HrpZ$_{pp}$ as shown in Figures 3 and 4. The proteins expressed in E. coli appear to be the same size as those recovered from the supernatants of P. s. pv. glycinea and P. s. pv. tomato, indicating that the cloned gene is intact and that there are no large post-translational modifications or deletions of HrpZ taking place in P. syringae but not in E. coli.

The amino acid sequence of HrpZ$_{pp}$ is quite highly conserved with respect to HrpZ$_{ps}$, having 87% similarity and 79% identity. HrpZ$_{pp}$ is less conserved with respect to the other two proteins, with 75% similarity and 63% identity to HrpZ$_{ps}$. However, the physical features of HrpZ$_{pp}$ and HrpZ$_{ps}$ are almost identical to those reported for HrpZ$_{ps}$ (He et al. 1993). All three are glycine-rich proteins lacking cysteine and tyrosine. HrpZ$_{pp}$ is the most glycine rich, being 15.7% glycine. The proteins lack the hydrophobic signal sequence used to target proteins for secretion via the Sec export pathway (Pugsley 1989). Analysis of the amino acid sequence fails to identify any obviously significant secondary structure, which is consistent with their sensitivity to proteases, and supports the suggestion that they adopt a fairly open structure in aqueous solution.

In our previous analysis of HrpZ$_{ps}$ (He et al. 1993), we noted the presence of two sets of short, direct repeats. Only one of these repeats, GGGLGTP, is conserved in the three proteins, with the substitution of a serine for threonine in the first repeat of both HrpZ$_{pp}$ and HrpZ$_{ps}$. The significance of these repeats, if any, is unknown. A database search with each of the three proteins using the BLAST algorithm (Alschul et

Fig. 2. Immunoblot showing expression of cloned hrpZ in E. coli. Cultures were grown in LM to an OD$_{600}$ of 0.8 to 1.0 at 30°C, collected by centrifugation and resuspended in 5 mM MES, pH 5.5. For lanes 3, 5 and 7, T7 expression was induced with 1 mM IPTG when the cells reached an OD$_{600}$ of 0.6-0.8 prior to collection. The cells were disrupted by sonication, and the crude lysate was partially purified by removal of the insoluble fraction after incubating the samples at 100°C for 10 min. SDS-loading buffer was added and the samples were incubated at 100°C for 2 min. The proteins were resolved by SDS-polyacrylamide gel electrophoresis. Following electrophoresis, the proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA), probed with anti-HrpZ$_{ps}$ antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase. Lanes: 1, E. coli DH5α (pSY110 (HrpZ$_{pp}$); 2, E. coli DH5α (pCPP2200/HrpZ$_{pp}$); 3, E. coli BL21(DE3) (pCPP2206/HrpZ$_{pp}$); 4, E. coli DH5α (pCPP2203/HrpZ$_{pp}$); 5, E. coli BL21(DE3) (pCPP2207/HrpZ$_{pp}$); 6, E. coli DH5α (pBluescript II); 7, E. coli BL21(DE3)(pET21a).
### Fig. 3. Nucleotide sequences of the noncoding regions of the hrpZ operon from *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *glycinia*, and *P. syringae* pv. *tomato*. The sequences flanking the six ORFs of the hrpZ operon were aligned using the PILEUP algorithm (Genetics Computer Group). For *P. syringae* pv. *syringae* and *P. syringae* pv. *tomato* the sequence extends from immediately downstream of *hrpS* to the end of the operon. For *P. syringae* pv. *glycinia* the sequenced region terminates at the beginning of *hrpC*. The proposed initiation and termination codons are highlighted for each ORF. The *hrpA/hrpF* consensus sequence upstream of *hrpA* and *hrpF* are marked by double lines, with the conserved nucleotides in bold and the putative ribosome binding sites for each ORF underlined. A short inverted repeat upstream of *hrpZ* is also indicated with dashed arrows.
al. 1990) did not find significant homology to any other bacterial proteins, with the exception of a single, glycine rich region found only in HrpZ\(_{P_{1}}\) (Fig. 4). This stretch of 24 amino acids has homology at both the nucleotide and amino acid level to a region of the host-specific elicitor PopA1 from \(P.\) solanacearum, as shown at the bottom of Figure 4. There is no overall similarity of the amino acid and nucleotide sequences of HrpZ to the HR elicitors characterized from \(E.\) amylovora, \(E.\) chrysanthemi, and \(P.\) solanacearum except to a degree accounted for by their similar composition.

![Alignment of the protein sequences of HrpA and HrpZ from Pseudomonas syringae pv. syringae, P. s. pv. glycinea, and P. s. pv. tomato were aligned using the PILEUP algorithm (Genetics Computer Group). The alignment of a unique glycine rich region of HrpZ\(_{P_{1}}\) with a homologous region of PopA1 from \(P.\) solanacearum is also shown.](image-url)
The predicted HrpA protein of *P. s. pv. tomato* differs substantially from that of *P. s. pv. syringae* and *P. s. pv. glycinea*.

The first ORF of the *hrpz* operon starts 50 bp downstream of the conserved *hrpzP* promoter motif, as shown in Figure 3. The predicted product is a small (11 kDa), hydrophilic protein with a hydrophobic N-terminus. An alignment of the amino acid sequences from all three pathovars is shown in Figure 4. Although the predicted sequences of HrpA from *P. s. pv. syringae* and *P. s. pv. glycinea* are highly conserved, with 92% similarity and 91% identity to each other, HrpA from *P. s. pv. tomato* is quite divergent, having only 42% similarity and 28% identity to HrpA from *P. s. pv. syringae*. The presence of a ribosome binding site and the highly conserved character of HrpA in two of the three pathovars supports the hypothesis that HrpA is translated. T7 polymerase-dependent expression of *hpA* (described below) provides further evidence for production of a HrpA protein. Cell lysates of *E. coli* expressing only HrpA did not elicit the HR on tobacco (data not shown), which suggests that it does not contribute directly to the HR. The role of HrpA in the bacteria is unknown, and it shows no significant homology to any previously characterized proteins.

**T7 expression studies.**

To confirm the production of proteins corresponding to the two sets of newly cloned *hrpA* and *hrpz* genes, the BglII-PstI fragments from *P. s. pv. glycinea* and *P. s. pv. tomato* were subcloned into pET21(+), and the products specifically labelled by T7 promoter/polymerase-dependent expression in *E. coli* BL21(DE3) cells incubated with [35S]-methionine (Studier et al. 1990). Radiolabeled proteins in the cell lysate were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 5). Lysates of cells containing pCPP2211 displayed unique bands which corresponded well with the predicted molecular weight of HrpA (11.5 kDa) and were consistent with the previously observed mobility of HrpZ (Fig. 5, lane 2). Lysates of cells containing pCPP2210 contained bands corresponding to HrpZ (36 kDa) and HrpA (11 kDa) (Fig. 5, lane 3). No HrpB band was visible in the products of pCPP2211 (Fig. 5, lane 2), but this could potentially be attributed to the omission of cysteine, which is not required for HrpA and HrpZ synthesis, from the amino-acids added to the reaction mixture. T7 expression of HrpB was independently confirmed for both *P. s. pv. syringae* and *P. s. pv. tomato* using a 0.84-kb PstI-AgeI fragment of pHR11 and the 3.7-kb SacI-EcoRI fragment from pCPP2209, subcloned into LITMUS28 to construct the plasmids pCPP2303 and pCPP2304. T7 expression in *E. coli* BL21(DE3) cells was performed as outlined above and in Figure 5. In each case a protein of about 13 kDa was observed, which corresponds well with the predicted molecular weight of HrpB from each of the two pathovars (data not shown). In an accompanying study Huang et al. (1995) have confirmed the production of proteins corresponding to HrpC, HrpD, and HrpE from *P. s. pv. syringae* 61. The similarities between the three pathovars suggest that the equivalent ORFs in *P. s. pv. glycinea* and *P. s. pv. tomato* also encode proteins. However, when we independently confirmed the production of HrpD from *P. s. pv. syringae* 61 using a 1.3-kb SalI-SacI subclone from pHR11 cloned into pT7-7 (pCPP2305) our results suggested the use of an alternative initiation codon to make a larger (21 kDa) HrpD protein (data not shown). In the absence of a strong ribosome binding site at either of the putative initiation codons, the exact size of HrpD remains uncertain.

**The four ORFs downstream of *hrpz* show varying similarities to *Yersinia* Ysc proteins.**

The *hrpC*, *hrpD*, and *hrpE* genes downstream of *hrpz* in *P. s. pv. syringae* 61 have been sequenced and the products identified using T7 polymerase-dependent expression (Huang et al. 1995). Two of the predicted proteins, HrpC and HrpE, were shown to be homologous to the proteins YscJ and YscL, respectively, which are encoded in the *virC* operon of *Yersinia enterocolitica* and are involved in the type III secretion pathway (Michels et al. 1991). Homologs of YscJ have also been found in the *hrp* clusters of several other phytopathogenic bacteria, including *P. solanacearum* and *X. campestris* (Fennessey et al. 1992; Gough et al. 1992). Additional homologs are *Salmonella typhimurium* IT10 and *Rhizobium fredii* NIO1 (Jones et al. 1989; Meinhardt et al. 1993). The same four downstream ORFs are found in *P. s. pv. tomato*, and the partial sequence of the operon from *P. s. pv. glycinea* confirms the presence of the first two of these ORFs, *hpPB* and *hpC*, in this pathovar (Fig. 6).

HrpB is fairly conserved in all three pathovars, as shown by the alignment presented in Figure 6. It encodes a small serine-rich protein of approximately 13 kDa. BLAST searches using HrpB from either *P. s. pv. syringae* or *P. s. pv. glycinea* identified no significant homologues, but a search using HrpB from *P. s. pv. tomato* identified similarity to the *Yersinia* protein, YscL. YscL is 115 amino acids long, thus slightly shorter than HrpB (127 amino acids). *yscJ* lies immediately upstream of *yscJ* in the *virC* operon, which suggests that the downstream ORFs of the *hrpz* operon might be colinear with a region of the *virC* operon.

![Fig. 5. T7 polymerase-dependent expression and radiolabeling of HrpA and HrpZ. T7 promoter/polymerase expression was carried out using the pET21(+)-vector system in *E. coli* BL21(DE3). Cells were grown in LGM to an OD of 0.5, then centrifuged and resuspended in M9 minimal medium supplemented with 0.01% amino acids (lacking methionine and cysteine), glucose and thiamine. Cells were incubated at 30°C for 3 h and then induced with 1 mM IPTG for 10 min, followed by incubation with rifampicin at 300 μg/ml for 30 min. Cells were incubated with 10 μCi [35S]-methionine for 10 min, lysed in SDS-loading buffer, and the proteins were separated by SDS-polyacrylamide electrophoresis and visualized by autoradiography.](image-url)
The apparent colinear arrangement of this group of hrp and ysc genes led us to inspect the P. s. pv. syringae and P. s. pv. tomato HrpD proteins for possible similarity to the Yersinia spp. YscK proteins. The similarity between the HrpD of P. s. pv. syringae and Y. pseudotuberculosis was the highest, with 28% of the amino acids identical and 57% similar. The HrpD and YscK proteins are of similar overall composition, and they lack any predicted transmembrane segments. However, there is a striking discrepancy between the sizes of the two proteins. HrpD is only 133 amino-acids long, whereas YscK from Y. pseudotuberculosis is 209 amino acids long. From the T7 experiments described above it is important to note that in the absence of a strong ribosome binding site, the precise initiation codon of the hrpD ORF is uncertain; it is conceivable that hrpD actually initiates immediately downstream of hrpC, at the ATG codon which overlaps the stop codon of hrpC, which would yield a predicted protein of 176 amino acids for HrpZp or 175 amino acids for HrpZpE, in an arrangement similar to that of the yscJ and yscK ORFs in Yersinia spp. However, this codon and all other potential initiation codons upstream of the one we have chosen lack ribosome binding sites, and the pattern of codon usage suggests that the intergenic region is not translated.

Although the similarities between HrpB/YscI, HrpD/YscK, and HrpE/YscL are lower than those involving HrpC/YscJ, the similarities of HrpB/YscI and HrpE/YscL are clearly in-

Fig. 6. Alignment of the protein sequences of HrpB from Pseudomonas syringae pv. syringae, P. s. pv. glycinea, and P. s. pv. tomato, and HrpC, HrpD and HrpE from P. s. pv. syringae and P. s. pv. tomato with YscI, YscJ, YscK, and YscL from Y. enterocolitica and Y. pseudotuberculosis (Michiels et al. 1991; Rimplainen et al. 1992). (continued on next page)
dicative of probable homology as based on a difference between the scores for the optimized and the average of 100
random Gap alignments being at least 5 times the standard deviation for the randomized alignments (Doolittle 1986).
The scores for HrpD/YscK lie at the margin of significance by this measure. However, the varying levels of similarity are consisent with the divergence observed between Hrp proteins from different P. syringae pathovars and between Ysc proteins from different Yersinia spp. The results for HrpB,C and E lend support to the weak homology of HrpD to YscK and suggest that hrbB, hrbC, hrbD, and hrbE are collinear with yscI, yscJ, yscK, and yscL.

In a recent report, Van Gijsemeg et al. (1995) observe that the P. solanacearum GMI1000 hpr cluster also encodes homolos of YscJ and YscL but not YscS and YscK. It is possible that with relatively divergent Hrp sequences, similarities with Ysc proteins may be found only after examining the sequences from several plant pathogens. It is interesting to note that there is no ORF following hrbE that is homologous to the protein encoded by the final gene of the virC operon, YscM. However, the hrbZ operon lies immediately upstream of the hrbH operon (Fig. 1), and HrpH is a homolog of YscC, a secretion protein which lies upstream of yscJKL within the virC operon (Michiels et al. 1991). This suggests that a sig-

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**Fig. 6. (continued from preceding page)**
significant proportion of the virC operon is conserved in P. syringae, albeit in a rearranged form. Eckhardt (1978) gels of total DNA, Southern-blotted and probed with a 0.75-kb BsrXI internal fragment of hrpZ<sub>PsP</sub>, suggested that the hpr genes are chromosomal in all three strains of P. syringae studied, rather than being plasmid-borne as are the hpr genes of P. solanacearum GMI1000 or the ysc genes of Yersinia spp. (Van Gijsegem et al. 1993; data not shown). The homologies of the hprZ operons are summarized in Table I.

**Overexpression, purification, and biological assay of HrpZ<sub>P</sub> and HrpZ<sub>PsP</sub>**

Partially purified lysates of E. coli expressing HrpZ<sub>P</sub> and HrpZ<sub>PsP</sub> elicited a clear HR on tobacco while control lysates of *E. coli* containing vector alone did not. However, the activity of the cell lysates on the two host plants was more ambiguous. Soybean is generally unreactive to cell lysates from either pathogen, while tomato is quite sensitive and sometimes weakly reactive not only to cell lysates of *E. coli* expressing HrpZ, but also to control lysates of *E. coli* containing vector alone. To accurately evaluate the biological properties of HrpZ from each of the two pathovars, it was necessary to purify HrpZ. It was also necessary to ascertain that the HR observed on tobacco was due solely to HrpZ and not to the products of either of the two flanking ORFs, HrpA and HrpB, since HrpA and a fusion protein of HrpB were being expressed in addition to HrpZ by the original hprZ<sub>PsP</sub> and hprZ<sub>PsP</sub> clones.

As a first step towards purifying HrpZ, we attempted to increase the level of expression. From the sequence of the PsP clones encoding hprZ it was clear that long stretches of DNA encoding hprA and the 3′ end of hprS (1,144 bp in hprZ<sub>PsP</sub>, pCPP2202 and 809 bp in hprZ<sub>PsP</sub> pCPP2203) separated hpr<sub>S</sub> from the lac promoter in pBluescript II. A series of deletions of the 5′ end of the hprZ<sub>PsP</sub> clone were constructed using the Erase-a-Base system (Promega), bringing the lac promoter within 100 bp of the hprZ initiation codon, and removing hprA. Although cell lysates expressing the deleted clones retained HR eliciting activity, they did not show a substantial increase in gene expression. Searching for an explanation for this behavior we identified a number of potential contributing factors. The first possibility was the presence of a cis-acting sequence contained in the 100 bp remaining upstream of hprZ<sub>PsP</sub>. Using a terminator analysis program we identified a 9-bp inverted repeat located between hprA and hprZ (Fig. 3). Although this repeat lacks the AT-rich sequence downstream which is characteristic of many terminators, it is possible that its presence encourages premature transcription termination. Similar repeats, albeit with weaker secondary structure, can be found upstream of hprZ<sub>PsP</sub> and hprZ<sub>PsP</sub>. A second factor contributing specifically to the low expression of hprZ<sub>PsP</sub> may be the absence of a strong ribosome binding site. Finally, there could be factors related to the proteins themselves, such as a lack of stability.

To eliminate possible cis-acting sequences and to obtain clones of hprZ<sub>P</sub> and hprZ<sub>PsP</sub> that lack hprA and hprB, the hprZ genes from both pathovars were amplified by PCR, directly cloned into pBluescript II and transformed into *E. coli* DH5α. pF'lac<sup>+</sup>. We obtained significantly increased expression of HRP<sub>ZPsP</sub> using the plasmid pCPP2255 (Fig. 7), but unexpectedly, overexpression of HrpZ<sub>P</sub> appeared to be deleterious to the cells, and plasmids recovered from transformants often showed rearrangements. To maximize expression of HrpZ<sub>PsP</sub> under these conditions, we introduced subclones containing the gene behind the T7 promoter of pET21(+)(Novagen, Madison, WI). Unlike the lac promoter, the T7 promoter is less sensitive to distance effects, and expression of HrpZ<sub>P</sub> in *E. coli* BL21(DE3), with pET21(+) as the vector, resulted in increased expression as shown in Figures 2 and 8. Expression in BL21(DE3) also allowed us to retain almost complete repression of hprZ until induction with IPTG. Good expression of HrpZ<sub>P</sub> was achieved using the plasmid pCPP2211 in *E. coli* BL21(DE3).

The quality of the samples obtained following partial purification of the lysates by heat treatment was quite variable. To ensure removal of the majority of the contaminating proteins and to obtain a more concentrated sample of protein, we further purified HrpZ by ammonium sulphate precipitation and hydrophobic chromatography, which as indicated in Figure 8, yielded a distinct band on a Coomassie-stained gel. Purified, active HrpZ could then be obtained by electrophoresis from excised gel slices. This procedure was also used to isolate

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**Table I. Homologies of Pseudomonas syringae pv. syringae hprZ operon proteins with proteins from other P. syringae pathovars and Yersinia spp.**

<table>
<thead>
<tr>
<th>P. s. pv. syringae</th>
<th>HrpA (108&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>HrpZ (341)</th>
<th>HrpB (124)</th>
<th>HrpC (268)</th>
<th>HrpD (133)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>HrpE (192)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. s. pv. glycinea</td>
<td>(108)</td>
<td>(345)</td>
<td>(124)</td>
<td>(268)</td>
<td>(133)</td>
<td>(193)</td>
</tr>
<tr>
<td>P. s. pv. tomato</td>
<td>91/92&lt;sup&gt;c&lt;/sup&gt;</td>
<td>79/87</td>
<td>94/96</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. s. pv. tomato</td>
<td>(108)</td>
<td>(370)</td>
<td>(124)</td>
<td>(268)</td>
<td>(133)</td>
<td>(193)</td>
</tr>
<tr>
<td>Y. enterococitica</td>
<td>28/42</td>
<td>63/75</td>
<td>68/80</td>
<td>90/95</td>
<td>76/87</td>
<td>76/87</td>
</tr>
<tr>
<td>Y. pseudotuberculosis</td>
<td></td>
<td></td>
<td>22/45&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22/48</td>
<td>22/46</td>
<td></td>
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<td></td>
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<td>22/45&lt;sup&gt;d&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td>21/44</td>
<td>38/60</td>
<td>23/49</td>
<td>22/46</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of amino acids in the protein is given in parentheses.

<sup>b</sup> Percent identical and similar amino acids in comparison with the P. s. pv. syringae protein.

<sup>c</sup> The first pair of values are the percent identical and similar amino acids in comparison with the P. s. pv. syringae protein; the second are in comparison with P. s. pv. tomato.

<sup>d</sup> The data presented are for the shorter of the two potential ORFs encoding hprD. The larger versions of the HrpD proteins of P. s. pv. syringae and P. s. pv. tomato would be respectively 175 and 176 amino acids long with 74/84% identity/similarity to each other.
HrpZ from the supernatants of P. s. pv. tomato and P. s. pv. glycinea grown in hrp-inducing minimal media (Fig. 9).
Preparations of the purified HrpZ proteins from P. s. pv. syringae, glycinea, and tomato, at a concentration of ≥20 μM in MES buffer, were infiltrated into the leaves of tobacco, soybean, and tomato. The three proteins elicited a collapse involving >50% of the infiltrated tissue in tobacco and tomato leaves that developed within 18 h and was typical of the HR elicited by incompatible P. syringae strains, but they caused no visible reaction in soybean. It is worth noting that tobacco and tomato plants vary substantially in their sensitivity to harpin preparations. For example, some leaves on sensitive tomato plants will respond to 2 to 5 μM HrpZ, but ≥20 μM is required for consistent results. Furthermore, unlike tobacco, tomato plants that have responded hypersensitively to a HrpZ preparation do not respond to subsequent infiltrations of the elicitor. The spurious necroses sometimes observed were deduced to result from mechanical damage incurred during infiltration or the infiltration of preparations contaminated with salts or containing high concentrations of vector control E. coli lysates. These necroses developed much more quickly (within 4 to 6 h), and were much weaker and patchier than the confluent HR elicited by HrpZ. The fact that the HR induced by HrpZ in tomato and tobacco is an active response of host tissue was confirmed by coinfiltration of either sodium vanadate at \(5 \times 10^{-3}\) M or lanthanum chloride at \(1 \times 10^{-3}\) M. Each of these two inhibitors of plant metabolism completely inhibited the HR elicited by HrpZ preparations from each of the three pathovars but not the necrosis caused by the other factors mentioned.

**DISCUSSION**

We have used the P. s. pv. syringae 61 hrpZ gene to isolate the hrpZ locus from P. s. pv. glycinea race 4 and P. s. pv. tomato DC3000. Characterization of the hrpZ genes, products, and flanking DNA of these three pathovars has revealed the structure of the hrpZ operon, the relative variation among ORFs within the operon, the presence of genes downstream of hrpZ that are colinear with a block of genes involved with Yersinia virulence protein secretion, and the presence in HrpZα of a sequence related to a sequence in the PopA1

![Fig. 8. Overexpression and purification of HrpZα. Cultures were grown to an OD\(_{600}\) of 0.6 and induced with 1 mM IPTG. HrpZα was then partially purified from the cell lysate in a three-step process: first, by heat-treatment at 100°C as previously described, then by precipitation with ammonium sulphate at 30 to 45% saturation, and finally by binding to a hydrophobic resin (phenyl-sepharose) at 30% ammonium sulphate. A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, E. coli BL21(DE3)pET21++; 2, E. coli BL21(DE3)pCPP2211. B, Immunoblot of the samples shown in A, probed with anti-HrpZα antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.](image1)

![Fig. 9. Purification of HrpZα from hrp-induced Pseudomonas syringae pv. tomato. Cells were grown in King’s broth (KB) at 30°C and then resuspended in hrp-inducing minimal medium (Huynh et al. 1989) and incubated at room temperature overnight. Cells were removed by centrifugation and the supernatant heat-treated at 100°C for 10 min. Proteins in the supernatant were precipitated with ammonium sulphate at the percent saturations indicated. Proteins were desalted, concentrated, and resuspended in 5 mM MES using Centricon-10 tubes (Amicon). A, Coomassie stained SDS-polyacrylamide gel. Lanes: 1, supernatant extracted with Stratagene resin (Stratagene); 2, heat-treated supernatant extracted with Stratagene resin (Stratagene); 3, to 20% ammonium sulphate fraction; 4, 20 to 30% ammonium sulphate; 5, 30 to 40% ammonium sulphate; 6, 30 to 45% ammonium sulphate. B, Immunoblot of the samples shown in A, probed with anti-HrpZα antibodies and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.](image2)
protein of the tomato pathogen \textit{P. solanacearum} GMI1000. We also observed that purified HrpZ$_{Pa}$ was at least as effective as HrpZ$_{Pa}$ and HrpZ$_{Ps}$ in eliciting an HR-like necrosis in the leaves of tomato, a host of \textit{P. s. pv. tomato} DC3000, whereas none of the HrpZ preparations elicited significant necrosis in soybean, the host of \textit{P. s. pv. glycinea}.

The HrpZ proteins of three \textit{P. syringae} pathovars.

A comparison of the sequences of the three HrpZ proteins with each other and with HR elicitors characterized from other bacteria indicates that the HrpZ proteins represent a distinct family of elicitors that is conserved among \textit{P. syringae} pathovars. The amino acid sequences of the three proteins are sufficiently similar to reveal their relatedness, but (with the exception of a sequence within HrpZ$_{Pa}$), they show no significant relatedness to elicitor proteins from other bacteria. Interestingly, \textit{hpaZ} is the second most divergent ORF in the \textit{hpaZ} operons of \textit{P. s. pv. syringae} and \textit{P. s. pv. tomato}, with only 63\% of the predicted amino acids being identical. Nevertheless, HrpZ$_{Pa}$, HrpZ$_{Ps}$, and HrpZ$_{Ps}$ are indistinguishable in several biological and physical properties. They have the same effect on different plants (discussed below), and they are heat stable, glycine-rich, and devoid of cysteine and tyrosine. The lack of tyrosine is a feature they differentially share with the \textit{P. solanacearum} PopA1 protein but not the \textit{Erwinia} harrins. This property has been speculated to allow the protein to avoid the H$_2$O$_2$-mediated cross-linking of tyrosine residues that may occur in plant cell walls during defense responses (Bradley et al. 1992; He et al. 1993).

Interestingly, a 24 amino acid, glycine-rich stretch of HrpZ$_{Ps}$ shows homology to part of PopA1, as does the cognate nucleotide sequence. The region of homology between HrpZ$_{Pa}$ and PopA1 corresponds exactly to the insertion in HrpZ$_{Ps}$. The insertion of this element within HrpZ$_{Ps}$ sequences that are otherwise similar among the three HrpZ proteins suggests horizontal transfer and a common ancestry with PopA1. Because the host range of \textit{P. solanacearum} overlaps with that of \textit{P. s. pv. tomato}, it is tempting to speculate that this region has some particular significance to pathogenesis on tomato, although, as discussed below, this is not obvious from the different effects of the two proteins on tomato.

The presence of this insert in active HrpZ$_{Pa}$ is another indicator of the apparent plasticity of structure/function relationships in these glycine-rich elicitor proteins. That significant changes to the structure of these proteins does not abolish their activity was previously demonstrated when a fortitous \textit{hrpZ$_{Pa}$} clone was found to produce an active derivative of HrpZ missing the N-terminal 125 amino acids, and the popA product was observed to be degraded in culture to an active form missing the N-terminal 93 amino acids (He et al. 1993; Arlat et al. 1994). Clearly the presence of this "additional" internal sequence does not diminish the ability of the protein to elicit the HR. In fact, although it is difficult to make a quantitative assessment, HrpZ$_{Ps}$ may actually be a slightly more potent elicitor of the HR than HrpZ$_{Pa}$.

However, HrpZ$_{Pa}$ appears to differ from the other HrpZ proteins in being deleterious to \textit{E. coli} cells when overexpressed and is possibly more unstable, making it difficult to purify large amounts of the protein. Since the glycine-rich region is the most obvious difference between HrpZ$_{Ps}$ and HrpZ$_{Ps}$, it is possible that it contributes to this phenomenon.

We were able to overcome this problem experimentally by using a tightly regulated T7 promoter/polymerase system, but never obtained quite the same level of expression we achieved with HrpZ$_{Ps}$ and HrpZ$_{Ps}$. However, there remains the obvious question of how HrpZ toxicity is avoided by \textit{P. s. pv. tomato}. One possibility would be that HrpZ is never expressed at levels high enough to affect the bacterium, even when it is induced in plants. Some indirect evidence for this hypothesis is provided by our examination of the DNA upstream of \textit{hpaZ} and \textit{hrpA}. The ORF has a weak ribosome binding site, and we also observed that expression of cloned \textit{hrpZ} from the lac promoter appears to be attenuated by the presence of cis-acting upstream sequences. A 9-bp GC-rich repeat upstream of \textit{hpaZ} may be significant in this regard. Preliminary data from northern blotting experiments also indicate that premature transcription termination may take place when \textit{hrpA}-\textit{hrpZ} clones are expressed in \textit{E. coli} (G. Preston, unpublished).

A second possibility is that the location of the \textit{hpaZ} gene in an operon with secretion genes ensures tight coupling of synthesis and secretion. Genes encoding extracellular proteins and secretion pathway components are often coregulated, but with a few exceptions involving the type I pathway, they do not lie within the same operon (Fath and Kolter 1993). A third possibility is that \textit{P. s. pv. tomato} is more tolerant of high levels of HrpZ than is \textit{E. coli}, or it possesses a means of keeping HrpZ in a nontoxic form while it is in the cell.

Further comparison with the \textit{Yersinia} virulence system presents an intriguing possibility in this regard. It has been shown that secretion of certain "Yops" (the \textit{Yersinia} pathogenicity determinants), involves chaperone proteins, small hydrophilic proteins which help keep the Yop protein in a translocation competent form and help target it for secretion (Wattiau et al. 1994). The genes encoding each chaperone are located adjacent to the gene encoding the corresponding Yop. Given the presence of several small ORFs of undetermined function in the \textit{phir11} \textit{hrp} cluster, it is tempting to speculate that one of them, particularly \textit{hrpA}, might encode a protein with chaperone function. There is a superficial resemblance between HrpA and \textit{Yersinia} chaperones such as SycE. They are all small, hydrophilic, cytoplasmic proteins which lack a signal sequence, but there are no specific homologies. We are now constructing nonpolar mutations to test the role of HrpA in secretion. Preliminary results suggest that HrpA is not required for \textit{E. coli} MC4100 (phir11) to elicit an HR or secrete HrpZ (J. R. Alfano, unpublished), but in chaperone-mediated systems limited secretion of a protein will usually occur even in the absence of its chaperone, so it may be necessary to look quantitatively at secretion and accumulation of HrpZ to assess whether mutations in \textit{hrpA} or other \textit{hrp} genes have an effect.

The colinear relationship between several \textit{hrp} and \textit{ycs} genes.

From the sequence of the \textit{hrpZ} operon it is clear that the parallels with the \textit{Yersinia} type III secretion pathway extend beyond homologies of individual genes. The four genes downstream of \textit{hrpZ}, \textit{hrpB}-\textit{E}, appear to be arranged colinearly with the region of the \textit{virC} secretion operon from \textit{Yersinia} that encodes YscI-L. The \textit{virC} operon is a large operon containing 13 genes, \textit{yscA}-\textit{yscM}, several of which have been demonstrated to have a role in Yop secretion (Michiels et al. 1991). Of the four \textit{Yersinia} genes with putative ho-
mologs in the hrpZ operon, only yscJ and yscL are known to have a role in secretion. An accompanying paper shows that five more hrp genes, downstream of the hrpH operon, are colinear with the yscQ–U genes in the virB operon of Yersinia (Huang et al. 1995).

It appears that a significant portion of the type III secretion pathway described in Yersinia can be identified in P. syringae, and it seems likely that increasing parallels between the two systems will be found. In both systems the secreted proteins are involved with early events in the interaction with the host, and expression of secretion genes and virulence proteins is tightly coregulated. The secretion pathway seems to function in a similar way, as in both cases secreted proteins lack an N-terminal signal peptide and are not posttranslationally processed.

**HrpZ and host specificity.**

The function of HrpZ in compatible interactions is unclear. A likely role is the release of nutrients to the apoplast. Atkinson and Baker (1987a, 1987b) have proposed that the alkalinization of the apoplast caused by HrpP bacteria (which occurs at a slower rate in compatible interactions) results in the leakage of sucrose and other nutrients to support bacterial growth. One of the key unanswered questions regarding P. syringae HrpZ proteins is their role in host specificity. Compatible interactions leading to disease are distinguished by the absence of the HR. Host-differential elicitor activity would be one way to reconcile the production of HR-eliciting proteins by P. syringae and the phenomenon of host-specific compatibility. The failure of the PopA1 protein to elicit the HR in tomato, a host of P. solanacearum GMI1000, supports this concept (Arlat et al. 1994). Similarly, the isolated P. s. pv. syringae 61 HrpZ protein fails to elicit the HR in bean, although the significance of this is diminished by the fact that bean leaves appear insensitive to any harpins (He et al. 1993).

To further explore this question, we infiltrated all three HrpZ proteins into the leaves of the host plants for each of the pathovars. The host plants of P. s. pv. syringae 61, and P. s. pv. glycinea, bean and soybean, respectively, are uniformly unreactive to HrpZ from both compatible and incompatible pathovars; however, tomato leaves proved to be highly sensitive to all three HrpZ proteins. Thus, our data argue against the hypothesis that host-differential activity of HrpZ proteins controls the host specificity of P. syringae pathovars.

If isolated HrpZpp elicits the HR in tomato, why does P. s. pv. tomato not elicit the HR during pathogenesis? One possibility is that the response of tomato to HrpZpp is qualitatively different from the response to HrpZpp and HrpZpp despite manifestation of the same gross morphology. That is, the necrosis elicited by HrpZpp is fundamentally different from the HR and does not involve associated defenses that stop the pathogen. We are now testing this possibility with probes for HR-specific transcripts. A second possibility is that HrpZpp production is regulated in a host-specific manner. However, hrf is clearly part of the Hrp regulon: hrf expression is transcriptionally linked with genes encoding components of the secretion pathway, the hrf operons in all three of these P. syringae pathovars have virtually the same hrf promoter sequence, and expression of the hrf operon is likely required for pathogenicity. The conserved promoter sequences suggests that the hrf operon is regulated in P. s. pv. glycinea and P. s. pv. tomato by the same nutritional conditions and HrpR, HrpS, HrpL regulatory cascade described for P. s. pv. syringae and P. s. pv. phaseolicola (Grimm and Panopoulos 1989; Rahme et al. 1992; Xiao et al. 1992; Xiao et al. 1994; Xiao and Hutcherson 1994; Grimm et al. 1995). Whether differential expression of the Hrp regulon controls host specificity awaits determination. A third possibility is that the P. syringae pathovars produce host-specific suppressors of defense responses. This is supported by the observation that compatible pathogens do not trigger defense responses in host plants that are elicited by nonpathogens (Jakobek et al. 1993).

It is important to note that our data do not eliminate the possibility that the three HrpZ proteins actually have different activity in host plants when delivered by living bacteria and that the HR observed may be an abnormal response resulting from the presentation of a high concentration of HrpZ in an artificial manner. In that regard, it is interesting that lesions, which appear insensitive to isolated harpins, respond to Hrp recombinant E. coli cells that secrete the same proteins (He et al. 1993). Experiments in which the hrfZ genes of P. syringae pathovars are switched or altered in their patterns of deployment should test more definitively the role of HrpZ in determining host specificity.

In conclusion, we have characterized an operon containing two components of the HrpP system of P. syringae—a block of secretion-related genes that are conserved in eukaryotic pathogen in the genera Pseudomonas, Xanthomonas, Erwinia, Yersinia, Shigella, and Salmonella and a gene encoding an elicitor that is unique to plant pathogens. The elicitors found in the P. syringae pathovars are a subfamily of a larger class that appears to be characteristic of plant pathogens, and which we postulate to have a role in releasing nutrients for bacterial utilization. Our challenge now is to determine how the various components of the Hrp system have been adapted to serve plant parasitism in the face of plant defenses.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.**

Bacteria and plasmids used in this study are shown in Table 2. Pseudomonas were routinely grown in King’s B broth (King et al. 1954) at 30 °C, but for certain experiments the hrfP-depressing minimal medium of Huynh et al. (1989), adjusted to pH 5.5, was used. E. coli was grown in LB (Sambrook et al. 1989) or terrific broth (Tartof and Hobbs 1987). Plasmids were introduced into bacteria by transformation (Sambrook et al. 1989) or electroporation (Gene Pulser, Bio-Rad).

**Plant materials.**

The plants used in this study were tobacco (Nicotiana tabacum L. ‘Xanthii’), tomato (Lycopersicon esculentum Mill. ‘Moneymaker’), and soybean (Glycine max L. ‘Harosoy’). Plants were grown in greenhouse or growth chamber at 23 °C to 25 °C with a photoperiod of 16 to 24 h. Infiltration of plant leaves with HrpZ preparations was performed with blunt syringes as described (Huang et al. 1988).

**DNA analysis and sequencing.**

All DNA manipulations, except where specified, followed standard protocols (Ausubel et al. 1987; Sambrook et al. 1989). The hrfZ region of pHIR11 was subcloned into
pBluescript II (Huang et al. 1995). Two PstI fragments of 2.2 and 2.4 kb from pCPP2201 and pCPP2200, respectively, were subcloned into pBluescript II SK(−) in both orientations. A series of overlapping nested deletions covering both strands was generated for each of the subclones using Erase-a-Base (Promega, Madison, WI). The deletions were sequenced from double-stranded templates using Sequenase version 2.0 (United States Biochemicals, Cleveland, OH) and forward and reverse M13 primers. Sequencing was completed using specific primers synthesized by Integrated DNA Technologies (Coralville, IA). In addition, the 3.7 and 6.5 kb SacI-EcoRI fragments, which overlap the PstI subclones from pCPP2201 and pCPP2200, were also subcloned into pBluescript II SK(−) and sequenced using the ABI 373A DNA sequencer at the Cornell Biotechnology Program DNA sequencing facility and specific primers synthesized by IDT. Nucleotide and derived amino acid sequences were analyzed with the Genetics Computer Group Sequence Analysis Software Package (Devereaux et al. 1984). Homology searches against major sequence databases were done with the BLAST program (Altschul et al. 1990).

PCR amplification of hrpZ from P. s. pv. glycinea and P. s. pv. tomato.

The hrpZ genes of P. s. pv. glycinea and P. s. pv. tomato were amplified by PCR from the plasmids pCPP2202 and pCPP2203, respectively. Reactions were performed using the PCR Optimizer kit (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. Reactions were overlaid with mineral oil and incubated in a Hybaid Thermal Reactor (Hybaid, Teddington, U.K.) using these cycle parameters: 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at 55°C, 3 min at 72°C, followed by a final incubation of 7 min at 72°C. The primers used for hrpZp5 were 5'-TACCAGGGTCTCTTTAAGTGGTGTGTTACGC-3' and 5'-TACGCTGACAGTACGTCAGCAAGGACAT-3', and those for hrpZp6 were 5'-TACGATTTACCGCAAGGACATTAACGC-3' and 5'-GGAACGTCAGCAAGGACATTAACGC-3'. All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA), and were designed to introduce a BamHI and a PstI site at the 5' and 3' ends, respectively, of each amplified fragment.

The hrpZp5 fragment from pCPP2202 was successfully amplified in all reaction buffers tested. The hrpZp6 fragment from pCPP2203 was successfully amplified using reaction buffer B (reaction concentration 60 mM Tris-HCl, 15 mM (NH4)2SO4, 2 mM MgCl2, pH 8.5). PCR products of the expected sizes of 1.0 and 1.2 kb were purified from an agarose gel, digested with PstI and BamHI, cloned into pBluescript II, and then transformed into E. coli DH5a F′ lacI, yielding plasmid pCPP2255 carrying hrpZp5. Plasmids containing

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant characteristics*</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>Escherichia coli</td>
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</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Hanahan 1983: Life Technologies, Inc. Grand Island, NY</td>
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<td>DH5α FlacF</td>
<td>F′ proAB supE44 lacZΔM15 zfs:Ts: Kmφ80 lacZΔM15 ΔlacZYA-argF U169 endA1 recA1 hsdR17 (84 m+) deoR thi-1 supE44 ΔlacZΔM15</td>
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<td>BL21(D3)</td>
<td>F′</td>
<td>Novagen</td>
</tr>
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<td>Pseudomonas syringae pv. syringae 61</td>
<td>Wild type</td>
<td>Baker et al. 1987</td>
</tr>
<tr>
<td>pv. glycinea race 4</td>
<td>Wild type</td>
<td>C. J. Baker</td>
</tr>
<tr>
<td>pv. tomato DC3000</td>
<td>Wild type, Rf</td>
<td>D. E. Cuppels</td>
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<tr>
<td>Plasmids</td>
<td></td>
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<td>pBluescript II SK(−)</td>
<td>Cloning vector, Amp′</td>
<td>Novagen</td>
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<td>pUCP19</td>
<td>pUC19 derivative, Amp′</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pET21(+)</td>
<td>T7 transcription vector, Amp′</td>
<td>Novagen</td>
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<td>pET27-5</td>
<td>T7 transcription vector, Amp′</td>
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<tr>
<td>LITMUS28</td>
<td>Cloning vector, Amp′</td>
<td>Tabor and Richardson 1988</td>
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<td>pH1R11</td>
<td>25-kb cosmid containing P. s. pv. syringae 61 hrp cluster</td>
<td>New England Biolabs</td>
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<td>pSYH10</td>
<td>hrpZp5 ORF in pBluescript II</td>
<td>Huang et al. 1988</td>
</tr>
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<td>pCPP2203</td>
<td>0.5-kb PstI-AgeI subclone from pH111, containing hrpB, in LITMUS28</td>
<td>He et al. 1993</td>
</tr>
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<td>pCPP2205</td>
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</tr>
<tr>
<td>pCPP2200</td>
<td>pUCP19 carrying 10-kb partial Sau3A1 fragment of P. s. pv. glycinea DNA with hrpZp5</td>
<td>This study</td>
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<td>pCPP2202</td>
<td>2.4-kb PstI subclone of pCPP2200 in pBluescript II; hrpArg and hrpZp5 in expressed orientation with respect to Ploc</td>
<td>This study</td>
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<td>pCPP2204</td>
<td>As pCPP2202 but with hrpZp5 in reversed orientation to Ploc</td>
<td>This study</td>
</tr>
<tr>
<td>pCPP2206</td>
<td>2.4-kb PstI hrpArg and hrpZp5 subclone from pCPP2202 in pET21(+)</td>
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<td>pCPP2208</td>
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<td>pCPP2255</td>
<td>PCR-amplified hrpZp5 ORF in pBluescript II</td>
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<td>pCPP2221</td>
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<td>pCPP2203</td>
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<td>pCPP2209</td>
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<td>3.7-kb SacI-EcoRI subclone from pCPP2202 in LITMUS28</td>
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<td>pCPP2211</td>
<td>2.0-kb BglII-PstI hrpZp5 subclone from pCPP2203 in pET21(+)</td>
<td>This study</td>
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</tbody>
</table>

* Amp′ = ampicillin resistance; NaF = nalidixic acid resistance; Rf′ = rifampicin resistance.
PCR-amplified hrpZ<sub>eu</sub> were found to be unstable and appeared to promote cell lysis.

**HrpZ purification and analysis.**

HrpZ was purified from *E. coli* as previously described (He et al. 1993) with the following modifications. Cells were lysed in either 5 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.5, or cell lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). For some experiments the supernatant from heat-treated lysate was partially purified after sonication with ammonium sulphate precipitation (25 to 45% saturation), with desalting and concentration being performed with Centricon-10 tubes (Amicon). For experiments requiring highly purified HrpZ expressed in *E. coli* BL21(DE3), the supernatant was further purified by binding to phenyl-Sepharose (Sigma) in the presence of ammonium sulphate (>30% saturation) and elution with 5 mM MES, pH 5.5, followed by electrophoresis through a native 15% polyacrylamide gel. The purified protein was then eluted from excised gel slices using an Elutrap apparatus (Schleicher & Schuell) or from crushed gel slices using a Microtrep separator (Amicon). Protein concentrations were determined using Bio-Rad protein assay solution. HrpZ was also purified from heat-treated supernatants of *P. syringae* grown in hrp-inducing medium (Huynh et al. 1989) by ammonium sulphate precipitation (25 to 45% saturation) and desalting/concentration using Centricon-10 tubes. For infiltration into plant tissue, HrpZ preparations were diluted to various degrees with 5mM MES, pH 5.5. The amino-terminal sequence analyses were performed at the Cornell Biotechnology Program Protein Analysis Facility (HrpZ<sub>eu</sub>) and the University of Kentucky Macromolecule Structure Analysis Facility (HrpZ<sub>eu</sub>).

**T7 expression and labeling of proteins in *E. coli*.

Proteins encoded by the *hrp* operon were expressed in *E. coli* BL21(DE3) by using the pET21(+) T7 expression system (Novagen). Conditions for isopropyl-B-D-thiogalactopyranoside (IPTG) induction of T7 RNA polymerase-dependent expression and labeling with L-[<sup>35</sup>S]methionine were as described by Studier et al. (1990). After being labeled, cells were collected by centrifugation and then resuspended and lysed in SDS-loading buffer and the proteins resolved on an SDS-polyacrylamide gel. Gels were stained, dried and exposed to Kodak X-ray film.

**Nucleotide sequence accession numbers.**

The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers L41861 (*P. syringae* pv. tomato *hrpA*, *hrpZ*, *hrpB*, *hrpC*, *hrpD*, *hrpE*), L41862 (*P. syringae* pv. glycinea *hrpA*, *hrpZ*, *hrpB*), L41863 (*P. syringae* pv. syringae *hrpA*), and L41864 (*P. syringae* pv. syringae *hrpB*).

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**LITERATURE CITED**


