The Complete *hrp* Gene Cluster of *Pseudomonas syringae* pv. *syringae* 61 Includes Two Blocks of Genes Required for Harpin_{Pss} Secretion that Are Arranged Colinearly with *Yersinia ysc* Homologs

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Pseudomonas syringae pv. syringae 61 contains a 25-kb hrp cluster that is sufficient to elicit the hypersensitive response (HR) in nonhost plants. Previous studies have shown that mutations in complementation groups VIII, IX, and XI in the hrp cluster abolished the ability of the bacterium to cause the HR. The sequence of a 3.7-kb SmaI-SstI fragment covering groups VIII and IX now reveals five open reading frames (ORFs) in the same transcript, designated as hrpU, hrpW, hrpO, hrpX, and hrpY, and predicted to encode proteins of 14,795, 23,211, 9,381, 28,489, and 39,957 Da, respectively. The hrpU, hrpW, hrpO, hrpX, and hrpY genes are homologous and arranged colinearly with the yscQ/spa33/spaO, yscR/spa24/spaP, vscS/spa9/spaO, vscT/spa29/spaR, and vscU/spa40/spaS genes of Yersinia spp., Shigella flexneri, and Salmonella typhimurium, respectively. These proteins also show similarity to Fli/Flh proteins of Bacillus and enteric bacteria. The Ysc and Spa proteins are involved in the secretion of virulence factors, like the Yop and Ipa proteins. Fli/Flh proteins are involved in flagellar biogenesis. The sequence of a 2.9-kb EcoRV-EcoRI DNA fragment containing mainly group XI revealed five ORFs, designated hrpC, hrpD, hrpE, hrpF, and *hrpG*, predicted to encode proteins of 29,096, 15,184, 21,525, 7,959, and 13,919 Da, respectively. The first three genes belong to an operon conaining hrpZ, which encodes an extracellular protein that elicits the HR. hrpF and hrpG are two potential ORFs upstream of hrpH in the hrpH operon. HrpC is homologous to Yersinia YscJ, Pseudomonas solanacearum HrpI, Xanthomonas campestris pv. vesicatoria HrpB3, and Rhizobium fredii NolT. HrpE is similar to YscL of Yersinia spp. P. s. pv. syringae 61 Hrp proteins are most similar to Ysc proteins among those homologs. TnphoA insertions in hrpC, hrpE, hrpW, hrpX, and hrpY abolished the ability of P. s. pv. syringae 61 to secrete HrpZ (harpin_{Pss}), as determined by immunoblot analysis of cell-bound and culture supernatant fractions. Thus, many of the proteins required for flagellar biogenesis and virulence protein secretion in plant and animal pathogens may have a common ancestry.

Higher plants invaded by an incompatible pathogen (a microorganism that is pathogenic on a different plant) often produce the hypersensitive response (HR), a defense-associated response characterized by the death of plant cells at the site of infection (Klement 1982). The ability of bacteria to elicit the HR in plants is controlled by *hrp* genes, which are so named because they are required for both the HR (on nonhost plants) and pathogenicity (on host plants) (Lindgren et al. 1986). The *hrp* genes, first demonstrated in *Pseudomonas syringae*, are now known to be widespread and conserved in phytopathogens in the genera *Pseudomonas*, *Xanthomonas*, and *Erwinia*, and are typically clustered in the bacterial genome or on a large plasmid (Lindgren et al. 1986; Boucher et al. 1987; Arlat et al. 1991; Beer et al. 1991; Bonas et al. 1991; Bonas

Potential biochemical functions of hrp gene products in the elicitation of the HR are now being revealed. In particular, the initial sequencing of a portion of the hrp genes in P. solanacearum, X. campestris pv. vesicatoria, and P. syringae pv. syringae revealed homologies with components of the type III protein secretion pathway in animal pathogens in the genera Yersinia, Shigella, and Salmonella (Fenselau et al. 1992; Gough et al. 1992; Huang et al. 1992; Van Gijsegem et al. 1993). In phytopathogens, this pathway controls the secretion of several proteinaceous elicitors of the HR, for example, the products of the E. amylovora hrpN (Wei and Beer 1993), P. syringae hrpZ (He et al. 1993), and P. solanacearum popA genes (Arlat et al. 1994). The E. amylovora harpin, the first of these elicitors to be reported, is required for the pathogen to elicit the HR in nonhost tobacco leaves or to cause disease in susceptible pear fruit (Wei et al. 1992). The P. solanacearum PopA1 protein also elicits the HR in tobacco and resistant petunia lines, but popA mutants retain their ability to elicit the HR or disease in appropriate plants (Arlat et al. 1994). These elicitor proteins differ in primary sequence, but they have several features in common: They are glycine rich, heat stable, lack an N-terminal signal peptide, and are secreted to the medium in a hrp-dependent manner (He et al. 1993; Wei et al. 1992, 1993; Arlat et al. 1994).

The *hrp/hrmA* genes of *P. s.* pv. *syringae* strain 61 are clustered in a 25-kb region of the genome and constitute a minimum genetic unit for bacterial elicitation of the HR in tobacco leaves, as demonstrated by the ability of *Pseudo*-

monas fluorescens and Escherichia coli cells carrying cosmid pHIR11 to elicit the HR (Huang et al. 1988, 1991). DNA sequence and function analyses have revealed that the hrp cluster on pHIR11 encodes a dedicated regulatory system, a protein secretion pathway, and the HrpZ (harpin_{Pss}) elicitor protein. The HrpRS proteins presumably control the expression of other hrp genes through a cascade mediated by HrpL (Xiao et al. 1994). The HrpRS proteins are members of the NtrC family of regulatory proteins (Grimm and Panopoulos 1989; Xiao et al. 1994), and HrpL appears to be an alternative sigma factor (Xiao et al. 1994; Xiao and Hutcheson 1994). The amino acid sequences of HrpH, HrpI, and HrpJ4 reveal significant similarity with components of the type III pathway in animal pathogens, HrpI and HrpJ4 also show significant similarity with flagellar biogenesis proteins, and HrpH has been shown to be required for P. s. pv. syringae to secrete HrpZ to the medium (Huang et al. 1992, 1993; He et al. 1993; Lidell and Hutcheson 1994). The pHIR11 hrp/hrmA cluster is organized into eight transcriptional units, as determined by the combined results of complementation analyses. Tn5-gusA1 mutagenesis, and DNA sequence data (Huang et al. 1991 and 1992; Xiao et al. 1992; Xiao and Hutcheson 1994). DNA sequences have been reported for approximately half of the pHIR11 hrp/hrmA genes, which are in the operons hrmA (Heu and Hutcheson 1993), hrpK, hrpL (Xiao et al. 1994), *hrpJ* (Huang et al. 1993; Lidell and Hutcheson 1994), hrpU (the first two ORFs) (Lidell and Hutcheson 1994), hrpH

(last ORF) (Huang et al. 1992), hrpZ (second ORF) (He et al. 1993), and hrpRS (Xiao et al. 1994). This has left 12 possible genes in the hrp cluster uncharacterized.

In this study, we further investigate the hrpU, hrpH, and hrpZ operons. The nucleotide sequences were determined for the 2.9-kb EcoRV-EcoRI fragment containing part of the hrpZ (hrpC, hrpD, and hrpE) and hrpH (hrpG and hrpF) operons and the 3.7-kb SmaI-SstI fragment containing part of the hrpU operon (hrpU, hrpW, hrpO, hrpX, and hrpY). The hrpC, hrpD, and hrpE gene products were confirmed by using a T7 RNA polymerase expression system, and Western blots of cell fractions probed with anti-harpin antibodies indicated that HrpC, HrpE, HrpW, HrpX, and HrpY are required for the secretion of HrpZ. In conjunction with an accompanying study focusing on the first three ORFs of the hrpZ operons of P. s. pv. syringae 61 and two other P. syringae pathovars (Preston et al. 1995), this work completes the inventory of genes required for gram-negative bacteria to elicit the HR in plants and reveals a particularly close relationship between the hrp genes of P. syringae and two blocks of ysc genes in Yersinia spp.

RESULTS

Nucleotide sequence of the 3.7-kb SmaI-SstI fragment.

To further characterize the loci in the *hrpU* operon, the nucleotide sequence of the 3.5-kb *BgIII-SstI* fragment was determined (Fig. 1). This region contains the complementation

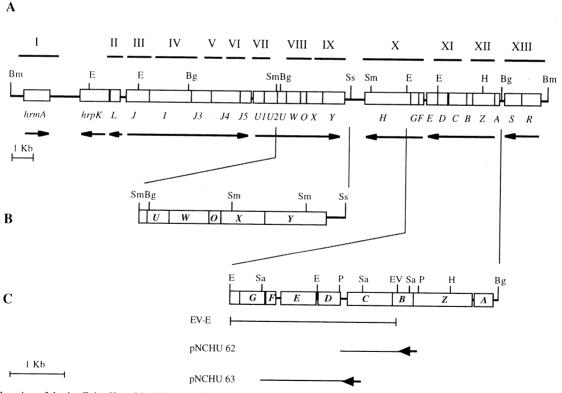


Fig. 1. The location of the hrpZ, hrpH, and hrpU operons in relation to the entire pHIR11 hrp cluster and the regions subcloned for DNA sequencing and T7 polymerase-dependent expression of hrpC, hrpD, and hrpE. A, The complementation groups were assigned as described previously (Huang et al. 1991). The hrp genes are organized into eight transcriptional units, and arrows indicate the direction of transcription (Xiao et al. 1992 and references in Table 3). B, The 3.7-kb SmaI-SstI fragment containing the hrpU, hrpW, hrpO, hrpX, and hrpY genes was sequenced. C, The nucleotide sequence of the 2.9-kb EcoRV-EcoRII fragment containing the hrpC, hrpD, hrpE, hrpF, and hrpG genes was also determined. pNCHU62 was used for T7 promoter-dependent expression of hrpC, and pNCHU63 was similarly used to express hrpD and hrpE. Restriction sites: Bg, BgIII; Bm, BamHI; E, EcoRI; EV, EcoRV H, HindIII; P, PstI; Sa, SalI, Sm, SmaI; Ss, SstI.

groups VIII and IX defined by TnphoA mutagenesis (Huang et al. 1991). Five ORFs were found to extend from the lefthand BglII site to the right-hand SstI site (Fig. 1B). The putative translational initiation site of ORF1 was to the left of the BglII site, downstream of hrpU2, and in the previously reported nucleotide sequence of the adjoining 4.3-kb BglII fragment (Lidell and Hutcheson 1994). Including the relevant portion of the 4.3-kb BglII fragment, the nucleotide sequence of the 3.7-kb SmaI-SstI fragment is shown in Figure 2. The ORFs were predicted to encompass nucleotides 175-573, 600-1223, 1234-1497, 1501-2292, and 2292-3368, and were designated hrpU, hrpW, hrpO, hrpX, and hrpY, respectively. These genes, as well as the previously reported hrpU1 and hrpU2 genes, appear to form one transcriptional unit since one putative hrp/avr promoter was discerned upstream of the hrpU1 gene (Lidell and Hutcheson 1994; Xiao and Hutcheson 1994). The predicted molecular weight, pI, and ribosome binding sites for these genes are listed in Table 1. Unlike the hydrophilic HrpU protein, HrpW, HrpO, HrpX, and HrpY are predicted to be inner membrane proteins since each of them contains two to four potential membrane spanning domains according to hydrophobicity plots (see Table 3 below).

....>hrpU2 1 CCCGGGAACAAATCGCCTCGCTACGCCCGGTGACGTGGTGCTGCCGCCGCCGCTGCCGCT 181 ACTGAAGATCTGTATCAGGACGACGTCGAGATGCTCGACGACTATGAAGAACCGGTGCCT T E D L Y Q D D V E M L D D Y E E P V P 241 GAACAGCGTGAACAGCGTGACGACGAATACGCCGAGCACGCATTCGGCTACCGG E Q A D Q Q R D D E Y A E H A F G Y A 301 GACAGCGACGCGGAGCATGAAGACCACCATGAATCGCCGATGCTCGAT D S D A E H E E Q S G D H H E S P M L D TCACTGGACCTGGACCTGCGCTGCGGGGATCTCAGATTGACCCTTGCCGAACTG CTGTGCCACGGTGAGCAGGTGGTTGCCGAAGGCGAACTGGTCGACGTCGAAGGCCGCCTC L C H G E Q V V A E G E L V D V E G R L 541 GGTTTGCAGATTACCCGGCTGGTGGCGCGCTCATGATCATGGAGGGCGTCAACCCGATCA G L Q I T R L V A R S *> hrpW TGCTGGCGCTGTTCTCGGCTCGCTGCTCGATCCCGTTCCTGTTGATCGTCTGCACCG CCTTTCTGAAGATCGCCATGACGCTGCTGATCACCCGCAACGCGATCGGCGTTCAGCAAG A F L K I A M T L L I T R N A I G V Q Q TACCGCCGAACATGGCGTTGTATGGCATCGCCCTGGCCGCGACGATGTTCGTCATGGCCC V P P N M A L Y G I A L A A T M F V M A CGGTGGCGCACGACATACAACAGCGGGTCCATGAACATCGCTGGAACTGAGCAACGCCG ACAAACTGCAGAGCAGCCTCAAAGTGGTCATCGAGCCGCTGCAACGCTTCATGACACGCA D K L O S S L K V V I E P L O R F M T R D K L Q S S L K V V I E P L Q R F M T R ACACCGACCCGGACGTCGTCGCCCACCTGCTGGAAAATACCCAGCGCATGTGGCCCAAGG 1021 S E L Q A G F E I G F L I Y I P F I V I ACCTGATTGTCTCCCAACCTGTTGCTGGCACTGGGCATGCAGATGGTCTCGCCGATGACAC G M F L V V I L T A P P L A V A V L V G 1321 GTGGTCACCTCGCTGCTGCAGGCACTCATGCAGATTCAGGATCAGACATTGCCCTTCGGC V V T S L L Q A L M Q I Q D Q T L P F G 1381 ATCAAACTGGGGGCCGTCGGACTGACCCTGGCCATGACCGGCGTCGGATCGGCGTCGAC 1501 ATGCCCTTCGACGCGCACAGCGCCTTTCAATTCATGCTGGGCATGGGACTGGCAATGGCG M P F D A H S A F Q F M L G M G L A M A 1561 CGGCTGATGCCTGCATGCTGCTGCTGCAAAGGCCCG R L M P C M L L V P A F C F K Y L K G P 1621 TTGCGTTATGCCGTCGTGGCGGTGATGGCGAGCCTCTGCTTCAAATACCTCAAAGGCCCCG R L R Y A V V A V M A M I P A P A I T R A 1681 CTGGAGTCCCTCGACGACAACTGGTTCGCAATCGCGGCCTTCATCAAGGAAGCGGTA L E S L D D N W F A I G G L L I K E A V

Nucleotide sequence of the 2.9-kb *Eco*RV-*Eco*RI fragment.

To characterize the loci in the second half of the *hrpZ* operon and the first half of the *hrpH* operon, we analyzed the nucleotide sequence of the 2.9-kb *Eco*RV-*Eco*RI fragment (internal to the 3.7-kb *HindIII-Eco*RI fragment) (Fig. 1). This region involves the complementation groups X and XI that

Table 1. The features of deduced Hrp proteins

Protein	Amino acids (no.)	M.W (Da)	pI	RBS ^a
HrpU	133	14,795	3.95	GAGGAG
HrpW	208	23,211	5.72	GGAGGG
HrpO	88	9,381	7.64	TGAGG
HrpX	264	28,489	4.70	GGGGT
HrpY	359	39,957	9.14	^b
HrpC	268	29,096	9.33	AGAGG
HrpD	133	15,184	5.57	TGAAG
HrpE	193	21,525	4.61	AGGAA
HrpF	74	7,959	3.79	TGGAG
HrpG	130	13,919	11.21	AGGAGG

a RBS: putative ribosomal binding site

^b RBS can not be determined.

1741	CTCGGCACCCTACTGGGGCTTTTGCTGTATGCGCCGTTCTGGATGTTCGCCTCGGTCGG
	L G T L L G L L L Y A P F W M F A S V G
1801	GCACTGCTCGACAGCCAGCGGGGTGCGCTCAGCGGCGCCAGTTGAACCCGGCGCTGGGC
	A L L D S Q R G A L S G G O L N P A L G
1861	CCTGACGCCACGCCACTGGGCGAATTGTTTCAAGAAACCCTGATCATGCTGGTCATCCTC
	PDATPLGELFOETLIMLVIL
1921	ACCGGGGGGCTATCGCTGATGACTCAGATCATCTGGGACAGCTACAGCGTCTGGCCTCCG
	TGGLSLMTQIIWDSYSVWPP
1981	ACGGCCTGGCTGCCGGCATGAACGCTGGCGGCCTGGATGTATTTCTGGAGCAGTTGAAC
	TAWLPGM N _i AGGLD V F L E Q L N
2041	CAGACGATGCAGCACATGCTGTTGTACGCCGCGCCCTTCATTGCGCTGCTGTTGTTGATC
	Q T M Q H M L L Y A A P F I A L L L I
2101	GAAGCGGCGTTCGCGATCATCGGCCTGTATGCGCAACAGCTGAACGTTTCGATCCTCGCC
0161	E A A F A I I G L Y A Q Q L N V S I L A
2161	ATGCCGGCCAAGAGTATGGCCGGCCTCGCGTTTCTGCTGATCTATCT
2221	M P A K S M A G L A F L L I Y L P T L L
2221	GAACTGGGCACAGGTCAACTATTGAAGCTGGTTGATCTGAAGTCGCTACTGACACTTCTG
2201	E L G T G Q L L K L V D L K S L L T L L
2281	GTGCAAGTGCCGTGAGCGAAAAAACCGAAAAAGGCCACGCCCAAGCAACTGCGTGACGCGC
	' * ' *
2241	>hrpY V S E K T E K A T P K Q L R D A GGGAAAAGGGTCAGGTCGGCAGACTCGGCAAACTGCTGGTGCTGATGGCTG
2341	
2401	R E K G Q V G Q S Q D L G K L L V L M A TCAGTGAAATTACCCTGGCCTGGCTGATGAAAGCGTCAACCGCCTGGAGGGGCGTTTGT
2401	V S E I T L A L A D E S V N R L E A L L
2461	CGCTGTCCTTTCAAGGTATCGACCGCAGCTTTGCCGCCCCCGGTCGAGCTGATTGCCAGCG
2401	S L S F O G I D R S F A A S V E L I A S
2521	AAGGATTCTCCGTACTGCTCAGCTTTACCTTGTGCAGCGTCGGTATAGCGATGCTGATGC
2321	E G F S V L L S F T L C S V G I A M L M
2581	
2301	R L I S S W M O I G F L F A P K A L K I
2641	ATCCCAACAAGATCAACCCGTTTTCCCACGCCAAACAGATGTTTTCCGGGCAAAACCTGC
	D P N K I N P F S H A K Q M F S G Q N L
2701	TCAACCTGCTGCTCAGTGTGCTCAAGGCCATTGCCATCGGCGCGACGCTGTATGTGCAAG
	LNLLLSVLKAIAIGATLYVQ
2761	TCAAACCTGTGCTGGGTACCCTTGCAGTGCTGGCCAACAGCGATCTGACGACCTACTGGC
	V K P V L G T L A V L A N S D L T T Y W
2821	ACGCGCTGGTCGAGCTGTTCAGGCATATTTTGCGAGTGATATTGGGACTGCTGCTGCCGA
	H A L V E L F R H I L R V I L G L L L A
2881	TCGCCATGATCGACTTCGCCATGCAGAAATACTTCCATGCGAAAAAACTGCGCATGAGCC
	I A M I D F A M Q K Y F H A K K L R M S
2941	ACGAAGACATCAAGAAAGAGTACAAGCAATCGGAAGGCGACCCCCACGTCAAAGGCCATC
	H E D I K K E Y K Q S E G D P H V K G H
3001	GTCGGCAACTTGCCCAGGAGATCCTCAATCAGGAGCCCAGCGCTGCGCCCCAAGCCGTTGG
	RRQLAQEILNQEPSAAPKPL
3061	AAGACGCCGACATGCTGGTCAACCCGACCCACTATGCGGTGGCCCTGTATTACCGAC
	EDADMLLVNPTHYAVALYYR
3121	CAGGCGAAACGCCTCTGCTCTGATTCATTGCAAAGGGGAAGACGAGGAAGCCCTGGCAT
	P G E T P L P L I H C K G E D E E A L A
3181	TGATTGCCCGGGCCAAAAAAGCCGGCATCCCGGTGGTGCAAAGCATCTGGCTGACCCGCA
	LIARAKKAGIPVVQSIWLTR
3241	
	T L Y R S K V G K Y I P R P T L Q A V G
3301	
	HIYKVVRQLDEVTDEVIQVE
3361	TCGAGCTGTGATGGCTGTTTAGCCATACGCGTACCCTTCCAGCCAAGTGACCTCAACCGA
	VEL*
3421	CGTTATCTCGTGGTCGTCGAAGCCTGCATGAATCACAGACAG
3481	CGGCTGCATTGCCCATTGGCGGCCTGGATGTCGGGGCTGCTCCGATGCCATGGCGCTTGA
3541	
3601	GGTCGTCCCATCCTGATGGCAGTGCTCGCTGCCGGGGTGACAGCTGTGCAGTGTCGAAAA

Fig. 2. Nucleotide sequence of the 3,672-bp SmaI-SstI fragment containing the hrpU operon. Potential ribosome binding sites preceding the hrpU, hrpW, hrpO, and hrpY genes are underlined; vertical arrows denote TnphoA insertions in hrpW, hrpX, and hrpY genes.

3661 CCGACCGAGCTC

were defined by TnphoA mutagenesis (Huang et al. 1991). Seven ORFs were identified within the nucleotide sequence extending from the right-hand EcoRV site to left-hand EcoRI site (Fig. 1C). ORF1, from nucleotide 1 to 111, was the C-terminal part of the hrpB gene product (Preston et al. 1995). ORF7, from nucleotide 2800 to 2956, corresponded to the N-terminal part of the HrpH protein (Huang et al. 1992). ORFs 2, 3, 4, 5, and 6 were predicted to encompass nucleotides 123-926, 1055-1453, 1490-2068, 2155-2376, and 2406-2795, and were designated hrpC, hrpD, hrpE, hrpF, and hrpG, respectively (Fig. 3). Because of the short intergenic regions and lack of apparent promoter sequences between them, hrpB, hrpC, hrpD, and hrpE appear to belong to the hrpZ operon (Preston et al. 1995). Since a putative hrp/avr promoter sequence was noted between hrpE and hrpF (Xiao and Hutcheson 1994; Fig. 3), hrpF-H may form a separate transcriptional unit.

The putative ribosome binding sites, molecular weight, and pI for these genes and their products are listed in Table 1. For the *hrpC* product, a putative amino-terminal signal peptide (18 amino residues) was identified by several characteristics of signal peptide sequences (Fig. 3): a positively charged N-terminus followed by a stretch of uncharged, mostly hydrophobic residues ending in Gly (von Heijne 1985). Furthermore, HrpC is a potential lipoprotein, since a cysteine residue follows the signal peptide cleavage site, and its ho-

molog, MixJ, has been shown to be a lipoprotein (Allaoui et al. 1992).

T7 RNA polymerase-dependent expression of *hrpC*, *hrpD*, and *hrpE* in *E. coli*.

To visualize the hrpC gene product, a 1.4-kb PstI fragment carrying hrpC was cloned into the expression vector pET21a in both orientations with respect to the T7 promoter. The hrpC product was visualized by using the T7 RNA polymerase-dependent system and a construct (pNCHU62) in which the predicted transcriptional direction of hrpC was the same as that of the T7 promoter in the vector (Fig. 1C). The apparent molecular weight of the HrpC polypeptide (29.5 kDa), as determined by SDS-polyacrylamide gel electrophoresis, agreed with that predicted from the nucleotide sequence (Fig. 4A, lane 2). To identify the hrpD and hrpE gene products, a 1.7-kb SalI fragment was cloned into pBluescript KS to construct pNCHU63 and pNCHU64 (representing two orientations of the insert) (Fig. 1C). Again, the construct (pNCHU64), in which the predicted transcriptional direction of the hrpD and hrpE genes was under control of the T7 promoter, produced two polypeptides, 16.5 and 22.5 kDa, respectively, that were visualized (Fig. 4B, lane 1). These molecular weights are also in agreement with those predicted by the nucleotide sequences.

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1 GATATCGCGGCGATGACCAGAACCTTGTCGCAATGCTCGTTGCAGACTGCGCTCACCACC
   D I A A M T R T L S Q C S L Q T A L T T 61 AAGGTGGTGAGCAAGACTGCGCAGGCGCTCGACAAGCTGACTACTTGCAGTAGAGGTTT
         K V V S K T A Q A L D K L T N ....> hrpC
  121 CCGTGAAATTTCTGAGCGCAGGGCTGTTGCTGATTTGCATGGTGTTGCTCGGCGGCTGTA
  V K F L S A G L L L I C M V L L G G C

181 GTGATGAAACCGATCTGTTCACCGGCCTGTCCGAGGACTCCAACGAGGTCGTTGGGC
  241 GCCTTGCCGATCAGCATATCGACGGCGGCGATGAACGTCTGGAAAAAACGGGGGTTGTTCGTCA
R L A D Q H I D A R K R L E K T G V V V
301 CCGTCGCCACAAGCGACATGAACCGCGGCGTCAGAGTGCTCAACGCTGCCGGCCTGCCAC
  TCGAAGAGCGCCCGCTACATCTATGCCCTGTCTCAGGAACTCGAAGCGACACTTTCGC
  Q I D G V I V A R V H V V L P E R I A P 541 GCGAACCGGTGCAACCTGCTTCTGCTGCGGTGTTCATCAAACACTCCGCAGCGCTTGACC
       G E P V Q P A S A A V F I K H S A A L D CGGACAGCGTGCGCGGACGCATTCAGCAGATGTCGCCAGCAGTATCCCCGGTATGTCGA
      P D S V R G R I Q Q M V A S S I P G M S CGCAAGCGGCAGAATCCAAAAGTTTTCCATTGTGTTCGTACCGGGTACCGAGTTTCAGG T Q A A E S K K F S I V F V P A T E F Q AAACCACACAGTGGGTCAGTTTCGGACCGTTCAAGCTGGACAGCGCCAATTTGCCGTTCT
  661
       E T T Q W V S F G P F K L D S A N L P F GGAACTTGATGCTCTGGCTGGCCGGCTGGCCTGGCGGTATTGCTGATCACGGCGC
       W N L M L W L V P A G L A V L L L I T A
TGCTGTTGCGCAGTGACTGCGCGCTTCAGTGCTGCGGCGGATCGCTTTGCCGCCGGA
T T T R S D W R A S V L R R I G F A G R
       L L L R S D W R A S V L R R I G F A G R GTCGCTCGACCGTACCGGCGCGCGCGTGATGGACCTGACCGCGAGGACTATTGGACTCA
      S R S T V P A R A \star GTGGTGGTGCAATCCCTGGCCATGGGCGCATCCGGGCTGGCAAAGCCGGTTCGCCGAGCG
1021 CTGCGGACTGACCGTCAGCGAATGT<u>GAAG</u>CCCTTATGGTCAGCCGTCACAGTGTGTTCCT

M V S R H S V F L

1081 GCAGAGTATCGGCATCACCGCCAAGTCAACCGCCAATGCCTGCGGAGCCTGTTTTGAACTG
L A L T P V Q R D Q A L D L A Q R I C F 1201 TTCCCGTAACGAAAGCGACGGCCATGACGGGCAGTGGTGCTGGGCGCTGACCAAAGCGTT
S R N E S D G H D G Q W C W A L T K A L 1261 GCGCCCCGGTGTCTGGCTGGAGCTGGAGCGTGAAGATGCGCGCTTGCTGTTGGGTGCCTG
1321 GCTCGGGCCTGAGTATTGGTCGCGATTGCGTCTGGCCTGGGCGCCCGATGAGGTCACCGA
1321 GCTCGGGCCTGAGTATTGGTCGCGATTGCGTCGGCCCCCCAAAATAAACTTCAGACGTTGTGCGAGGCTGTTCTATGGCG

R P C A A P E N K L Q T L W Q A V L W R ....> hrpE
1441 TGTCACCGCGACCTGAGTATCCGCTCCTCTCTGCACCAGGAATTCTCCCATGCTTGCCAA
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1501 ACGCAGTATTACTTTGACTGCCGACGCGGTGCTGCCCGAGCCGGTATTGCGCCGTGAAGA
I A N S L L A R D I L T D A R R Q A E Q
1621 ACTGCTGGTGCTTGAGCAGGCTAAAGCCGATCATCGGCATCAGGAGGCGCTTGCGCAGTT
L L V L E Q A K A D H R H Q E A L A Q F 1681 CTGGGAGGCTGCCAATGCCTTTCTGGACGAGCTTCAGCGCGAAGCCTTGCAGCA
1741 GCAGGCCATGACTGATGAGGAACTGCTGACTGAGGCATTGTGCCAGTTGCTCGACGA
Q A M T A V E E L L T E A L C Q L L D E
1801 AACAACCCTTGCCGAGCGCCTCGGGCACTGGTCAGGCAGCCAGTCAGCTCAA
1 T L A E R A R A L V R N L A A S Q L N
1861 CGAAGCAGTGGCCACACTCAGCGTTCATCCGGAGATGGCCGAGCGGTCGCCGAATGGTT
E A V A T L S V H P E M A E P V A D W T
 1921 GGCAGAGAGCCGTTTTGCCGAGCACTGGGAGCTCAAGCGCGATGCGACGCTGACCACCGA
A E S R F A E H W E L K R D A T L T T E
1981 AAGCCTGCGCCTGAGCGACGCCAACGGCGCTTTCGAAATCGATTGGGCAACCCTTCGCAA
S L R L S D A N G A F E I D W A T L R N 2041 TGGGCTGGCAGGCGCGGAGCCAGCTGCTGAAACAGACTCTTGCGGCGAAAATGGAACCG
G L A G A E P A A * ....> hrpf
2101 CTCCACCTGTTTGCTCCACTCAAGGTTTGAACCTTTCTGCTGGAGTATCAGGACATGATA
2161 AATTTCAAATCCCTGCAAAATAACCTCGATTCCACGCTCACTCGCGCGTTGTCCGACGTG
D D V L D G G N G H F T A D D I D A F S
2281 GAGGCGAGCCAGCAGCTGCGTCAACATCGCTGATCAATGCCAGCAGGCTGGT
L A S Q Q A A V S S N I A D Q C Q Q A G 2341 TATAAAATGACCAAAAATGTCATCGATGGATTTCAGTGAGTTCGAGGAGGTCGTCGGCCT Y K M T K N V I D G F Q *
2401 GTGGCGTGATCAGCGTCCGGCGAGCCCGCTCGACTGCTGGGTCGACGACGCCAACGCACG V I S V R R A R S T A G S T T P T H
2521 CAACGACCCTCAGCGTATCGAAGCATTGCTCAGTCACGGCGGTGCCAGCCTGGCGTGTGC
P T T L S V S K H C S V T A V P A W R V CTGTGACCGGCGCGTTCGCCATCGATCGCCAACCGCTGCATGGTGCTGGTCAGCTGGA
P V T G A F A I D P Q T R C M V L V S W 2641 TGCCTGGCCCTTGCGGCCTTGCCGATCTGCTGAACCGTCTGGAAAGCCTGGCCAATCAGC
2761 GGCGCACGACCCTCAATCACCGGCAACCGGGAGTTTGAAATGCGCAAGGCCTTGATGTGG
G R T T L N H R Q P G V * M R K A L M W
2821 TTGCCTTTAATTGTTGATCGGCTGTCCCCCGCCACGTGGGGGTGACCCCCGAAGCCTG
L P L L L I G L S P A T W A V T P E A W
K H T A Y A
2941 TTCGCCAAGGAATTC
```

Fig 3. Nucleotide sequence of the 2,955-bp EcoRV-EcoRI fragment containing the hrpC, hrpD, hrpE, hrpF, and hrpG genes. Potential ribosome binding sites preceding the hrpC, hrpD, hrpE, hrpF, and hrpG genes are underlined. The underlined amino acids denote the predicted signal peptide of HrpC. Vertical arrows denote TnphoA insertion sites in hrpC and hrpE mutants. The bold nucleotides are predicted promoter sequences of the hrpH operon.

Similarities of products of the hrpU and hrpZ operons to proteins required for the secretion of virulence factors by animal pathogens and for assembly of the bacterial flagellum.

The amino acid sequences of all deduced polypeptides encoded by hrp genes presented in this study were compared with sequences in GenBank by the BLAST program (Altschul et al. 1990; Pearson and Lipman 1988). Alignments were determined with the Cluster method of the DNAStar program (DNAStar, Inc.) and are presented in Figures 5 and 6. The similarities of these proteins with those previously reported are summarized in Table 2 and yield several general observations. (i) The genes in the hrpU operon encoding the HrpU, HrpW, HrpO, HrpX, HrpY proteins are arranged colinearly with similar genes in the lcrB(virB) locus of Yersinia spp. (Allaoui et al. 1994; Bergman et al. 1994; Fields et al. 1994) and (with slightly less similarity) with related spa genes in region 5 of Shigella flexneri (Sasakawa et al. 1993; Venkatesan et al. 1992) and Salmonella typhimurium (Groisman and Ochman 1993). Additionally, HrpW and HrpO are similar to ORF2 of X. c. pv. glycines (Hwang et al. 1992) and InvX of animal pathogenic E. coli (Hsia et al. 1993). The corresponding loci in these animal pathogens are involved with the secretion of virulence proteins by the type III pathway. (ii) The genes in the hrpZ operon similarly correspond to a different block of genes in the virC operon of Yersinia spp. (Michiels et al. 1991; Rimpilainen et al. 1992). (iii) The similarity of the five hrpU genes can also be extended to genes involved with flagellum biogenesis in E. coli (Malakooti et al. 1989, 1994), S. typhimurium (Kihara et al. 1989), Caulobacter crescentus (Ramakrishnan, et al. 1994; Zhuang and Shapiro 1995) and B. subtilis (Bischoff et al. 1992) and with motility and virulence in Erwinia carotovora (Mulholland et al. 1993). (iv) The hrpC gene is similar in its predicted product to previously reported genes from plant-associated bacteria, P. solanacearum hrpI (Gough et al. 1992), X. campestris hrpB3 (Fenselau et al. 1992), and Rhizobium fredii nolT (Meinhardt et al. 1993). (v) Among the 10 putative ORFs analyzed, only HrpF and HrpG showed no similarity to previously characterized proteins.

Involvement of Hrp proteins in the secretion of the HrpZ protein.

The location of TnphoA mutations in the hrpC, hrpE, hrpW, hrpX, and hrpY genes was confirmed by directly sequencing the insertion sites (Figs. 2 and 3). These mutants, 61-2091 (hrpC), 61-19 (hrpE), 61-12 (hrpW), 61-2087 (hrpX), and 61-2088 (hrpY) were not able to cause the HR in tobacco leaves (data not shown). The TnphoA insertions in hrpE and hrpW were isolated and marker-exchanged into the P. syringae pv. syringae genome as part of this work. The construction of the others was previously described (Huang et al. 1991). None of the TnphoA insertions produced fusions with alkaline phosphatase activity. To determine the function of these genes in harpin secretion, mutant cultures were grown in Hrp-inducing minimal medium, and the distribution of HrpZ between extracellular and cell-bound fractions was determined with Western blots probed with anti-HrpZ antiserum. HrpZ was secreted by the wild-type cells and still synthesized by all of the mutants, but in each mutant the protein accumulated in the cell-bound fraction (Fig. 7). The results indicated that the mutations affected the secretion of HrpZ.

DISCUSSION

P. s. pv. syringae contains a 25-kb hrp/hrmA cluster, cloned in cosmid pHIR11, that is sufficient for elicitation of the HR in tobacco leaves (Huang et al. 1991). The 10 ORFs reported here, in combination with previous and accompanying reports, complete the nucleotide sequence of the pHIR11 hrp/hrmA cluster, which is apparently organized into eight transcriptional units and potentially encodes 26 polypeptides (including HrmA). The biochemical functions of these polypeptides are expected to be involved in the regulation (e.g., HrpR, HrpS, and HrpL) (Xiao et al. 1994) and secretion (e.g., HrpH) of extracellular proteins (e.g., HrpZ) that are biologically active in plants (He et al. 1993). The 10 ORFs reported here reveal extensive homologies with proteins associated with the type III protein secretion pathway in Yersinia (Allaoui et al. 1994; Bergman et al. 1994; Fields et al. 1994), Shigella (Sasakawa et al. 1993; Venkatesan et al. 1992), and Salmonella spp. (Groisman and Ochman 1993) and with proteins required for biogenesis of the bacterial flagellum (Bischoff et al. 1992; Dreyfus et al. 1993; Kihara et al. 1989; Malakooti et al. 1989, 1994; Ramakrishnan, et al. 1994; Vogler et al. 1991; Zhuang et al., unpublished). Particularly striking is the presence in the hrp cluster of nine genes in two operons involved with HrpZ secretion (hrpB, hrpC, hrpD, and hrpE in the hrpZ operon; hrpU, hrpW, hrpO, hrpX, and hrpY in the hrpU operon), that are colinear with two blocks of vsc genes in Yersinia spp. The genes in the hrpU operon are also colinear with spa genes of Shigella flexneri and Salmonella typhimurium.

The complete inventory of pHIR11 hrp genes shown in Table 3 reveals a large subset that is conserved with type III pathway and flagellar biogenesis genes, including 12 that appear related to Yersinia ysc genes. The relationship of the some of these hrp and ysc genes is relatively weak. The relatively high divergence of HrpB and HrpD within the P. syrin-

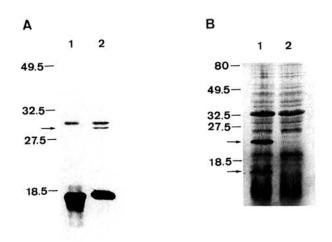


Fig. 4. Autoradiograph of the protein products of *hrpC* (**A**) and *hrpD* and *hrpE* (**B**) following T7 RNA polymerase-dependent expression in *E. coli* BL21(DE3) and SDS polyacrylamide gel electrophoresis. L-[3⁵S]methionine labeling and electrophoresis were performed as described in the text. The samples in each lane were derived from cells carrying the following plasmids. **A,** Lane 1, pNCHU61; lane 2, pNCHU62. **B,** Lane 1, pNCHU63; lane 2, pNCHU64.

gae pathovars suggests that the sequences of these proteins are less conserved than that of HrpC, for example, which has more certain homology with YscJ (Preston et al. 1995). Thus, the similarities of HrpB with YscI and HrpD with YscK were initially revealed by BLAST searches with P. s. pv. tomato Hrp sequences, which generally show stronger similarity with Yersinia sequences, and are documented in the context of comparisons involving multiple P. syringae pathovars

(Preston et al. 1995). The notion of a common ancestry for these genes is supported by multiple pathovar comparisons, the general similarity of the predicted products, and the colinearity of the encoding genes (Preston et al. 1995). The products of eight of the 12 ysc-related hrp genes also show significant similarity to proteins involved in flagellar biogenesis (Table 3). Additional sequences of homologous hrp genes from other plant pathogens and data on the structure

	Α																																																						
	HrpU YscQ Spa33 MopA FliY FliN	Q L	Q 5	S N	K L	E -	F I	G - - -	- E	HEGK	E S H K	SNDAD	D E E P L	G S G V	D N E A Q	H I P I H C L C	H E P	L V I	T S D E D	M L L F	L N A L I L S A	F - F	- N - D	- Y - P	D .	 E A	- - - v	- - - 0		 P I	- - H	- - - N	LI	 O M	L L	- (PPP	г 	D II O V K I S I K I	J T T	F F V V	R E I E E	LC	RERRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRRR	L Q K T T	R I N K R	L T M T M T R S M T	L V I	H I		R F K M L F L E	L		36 258 246 51 324 82
	HrpU YscQ Spa33 MopA FliY FliN	A G P G E N Q G A G	S I E I S V S I	L I F V	E V K F A L E L	P D C	G I T P G L K L	V I A A	P C D C V K G E G E	H H P P	V V L	RIP	I L	H V I V I	G N N N N	E G	R L S L Y L R I	I A T A	G G A A	E H O K O	G E E E E E E	L L V V	V V V V	D E S V V	V I	E G E D E D E E	R R K N K	L L Y Y F	G ! G ! G !	L Q / R / R / R	I	E S T T	RSDD	L	T	E .	V A V T V K I T	RIE	S A S	E F	R M	R N R	R	L S L S	R										133 307 293 106 378 137
	B HrpW Orf2 YscR Spa24 MopC FliP	M M M	м р 	D 1	 / G 	S	 5 A		 - N I N	- - - Y I	- - F	 R I	т 5 S	- - - A D	- - L	 L R	T	- - - Q	- - R	 Y (. L	- - A	- - I	- - G !		 . F	- - - M	- -	 P S	v	- - - W	- - A	Q L	- - P	- - - G E	· · · · · · · · · · · · · · · · ·		- - Q s	- - - P	 L P	- - N	- - G	- · ·	 2 s	- - W	- - T	L 3	 5 V	-[-[-[L - L -	- L -	P D	E I	- 3 - 1 - 8 - 9 - 2	3 10 3 5 5 5 8 23
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Fig 5. Alignment of *Pseudomonas syringae* pv. syringae hrpU operon proteins with potential homologs from other bacteria using the Cluster method in DNA Star. YscQ, YscR, YscS, YscT, and YscU are from *Y. pseudotuberculosis* (see text for all references). ORF2 is from *Xanthomonas campestris* pv. glycines. Spa33, Spa24, Spa9, Spa29, and Spa40 proteins are from *S. flexneri*. MopA, MopC, MopD, and MopE are from *E. carotovora* subsp. atroseptica (Mulholland et al. 1993). InvX and FliN are from *E. coli*. FliY, FliP, FliQ, FliR and FlhB are from *B. subtilis*. Identical amino acids (open boxes) in the predicted sequences of various hrpU operon proteins and homologs are in shown in the following panels: HrpU (A), HrpW (B), HrpO (C), HrpX (D), and HrpY (E). (continued next page)

and function of the Hrp proteins may reveal a common ancestry for even more of the *hrp*, *ysc*, and flagellar genes, and they may confirm the significance of intriguing but tentative similarities that have already been discerned (Table 3).

Our current evidence for the role of many of the *P. s.* pv. *syringae* Hrp proteins in HrpZ secretion is fragmentary and sometimes presumptive (Table 3). Some TnphoA mutations in *P. s.* pv. *syringae* apparently permit expression of downstream genes (Huang et al. 1991), but complementation analyses with appropriate subclones are required to prove the necessity of each target gene in secretion. Only with *hrpH*, *hrpE*, and *hrpY* do we have evidence that an irrefutably nonpolar mutation directly affects HrpZ secretion, as determined by the

visualization of HrpZ localization (Huang et al. 1992; He et al. 1993; Fig. 7). Nevertheless, indirect evidence argues that several other *hrp* genes are also involved in the secretion of HrpZ and possibly other proteins (Table 3 and references therein).

In a recent report, Van Gijsegem et al. (1995) present the remaining unpublished sequences for the *P. solanacearum hrp* cluster and an inventory of homologies with Ysc and flagellar biogenesis proteins that is identical to that found for *P. s.* pv. syringae 61, except for YscI and YscK. Interestingly, examination of these two *hrp* clusters confirms earlier indications that the *P. syringae hrp* cluster is more closely related to the *Yersinia ysc* genes than is the *P. solanacear m hrp* cluster.

```
D
         MP----FD-----AHSAFQFMLGMGLAMARLMPCMLLVPAFCFKYLKGPL-RYAVV--AVMAMIP--AP 55
MI----ADDL-----IQRP---LLTYTLLLPRFMACFVILPVLSKQLLGGVLLRNGIV--CSLALYV--YP 54
MDISSWFE-----SIHV---FLILLNGVFFRLAPLFFFLPFLNNGIISPS-IRIPVIFLVASGLI--TS 58
ML----T-FNSWDMVNW---VSQFFWPFVRILALISTAPVFNERAIGNRV-KIGLG--VLITLLVAPYL 58
HrpX
Spa29
MonE
         M N - - - -
                                     ----IIDL---FPAFLLVFIRISAFFVTIPLFGHRNVPAVH-RIGFA--FFLAVIC--FS 51
FliR
         AITR----ALE-SLDDNWFAIG-GLLI--KEAVLGTLLGLLLYAPFWMFASVGALLDSQRGALSGGQLNPAVANQPYIEVD-A----FTLM-LLIG--KEIILGLLIGFVATIPFWALESAGFIVDNQRGAAMASLLNPGKVD----IGS-S-----VFEHVYFLM--FKEIIVGLLLSFCLSLPFWIFHAVGSIIDNQRGAAMASLLNPPLNT----TPIFS-----VAGV-WLLI--QQILIGVTLGLSMQLAFAAIRHAGELIGLQMGLAFATFFDPTIDK----PPSLE----IDEH-YMLLAFKEALVGLCLGLIAYMMIAAVQIAGSFIDFOMGFSIANVIDP
HrpX
 YscT
FliR
        YscT
 Spa29
FliR
         - M N A GGLDVFL EOLN Q T M Q H M L L YA A PFIALL L I E AAFAII G L YAQO L N V S I L A M PAK S M A G L A F L L I Y 235
- V S E Q W V D F F Y N O F S Q I L L I A A V L A A P L L I A M F L A E F G L A L I S R F A P S L N V F V L A M P I K S A I A S L L L V I Y 233
- S F R V S N I L T F L T L L - - - A S Q A V I L A S P V M I V L L L S E V L L G V L S R F A P Q M N A F S V S L T I K S - - L L A I F I I 228
- L A R A G G L I F I N - - - - - G L M L A L P I I T L L L I T N L L A C M L N R V A P Q L S I F V V G F P I T L T - V G I M T L G 230
F I A K S L N A M P I I - - - - - - - - A F Q M S A P V V A S L F L V D L A L G I V A R T V P Q L N V F V V G L P L K I A - V S F I M L I 228
YscT
 Spa29
FliR
        -L-PTLLELGTGQLLKLVDLKSLLTLL----VQVPCM-QMMSHASKAMLLVMDPISLLIPVL----EK
-FICSSTIYFSKVQF-FLGEHKFFTNL----FV--
-LLLPLIPPFAEHLF-SEVFDLLADILTQLSSS
-VCMSVIFVVVRNVF-SLTIETMRNLLALVGVS
 YscT
                                                                                                                                                                                                                            261
 Spa29
                                                                                                                                                                                                                            256
                                                                                                                                                                                                                            259
F
             HrpY
 Sna40
         MKLRVDLQFFAGEKTEKATPKKRKDTRKKGOVAKSSDVNSAVSLLVIFLSLTAIGPYMRDRLLSFIETFY
                           ALLSLSFQGIDRSFAASVELIASEG---FSVLLSFTLCSVG-----
 SSWMOIGFLFAPKALKIDPNKINPFSHAKOMFSGONLLNLLLSVLKAIAIGATLY- - VQVKPVLGTLA - 166
SHVVQYGFLISGEAIKPDIKKINPIEGAKRIFSIKSLVEFLKSILKVYVLLSILIW- - - IIIIKGNLVTLLQ 167
PTLVQTKFVLATKAIKIDFSVLNPVKGLKKIFSIKTIKEFFKSILLLIILALTTYFFWINDRKIIFSQV - 164
SNYMQVGFLFSAEVIQPKLEKLDPIKGFKRIYSMRAIVELIKSILKIVVVGFAAF- - AVLWLHYGEILR 178
HrpY
 Yscu
 Spa40
FlhB
HTPY --VLANSDLTTYWHALVELFRHILRVILGLLLAIAMIDFAMOKYFHAKKLRMSHEDIKKEYKQSEGDPHV 233
YSCU LPTCGIECITPLL---GOILROLMVICTVGFVVIISIADVAFEVYQYIKELKMSKDEIKREYKEMEGSPEI 234
SPA40 --FSSVDGLYLIW---GRLFKDIILFFLAFSILVIILDFVIEFILYMKDMMMDKQEIKREYIEQEGHFET 228
F1hB LPLLTPEEALSFV---SKLTLWMGLSGAGALLILAGLDYLYQRFDYEKNIKMSKODIKDEYKKSEGDPII 245
         KGHRROLAQEILNQEPSAAPKPLEDADMLLVNPTHYAVALYYRPGETPLPLIHCKGEDEEALALIARAKK
KSKRROFHOEIQSRNMRENVK---RSSVVVANPTHIAIGILYKRGETPLPLVTFKYTDAQVQTVRKIAEE
KSRRRELHIEILSEQTKSDIR---NSKLVVMNPTHIAIGIYFNPEIAPAPFISLIETNQCALAVRKYANE
KSKIKQRQREMAMRRMMQEVP---KADVIITNPTHYAIALKYDEEKMDAPYIVAKGVDHLALKIRKIAKE
HrpY
 Spa40
HTPY AGIPVVOSIWLTRTLYRSKVGKYIPRPTLQAVGHIYKVVROLDEVTDEV----II--QVEVEL
YSCU EGVPILQRIPLARALYWDAL-VD-------HYIPAEQIEATAEVLRWLERQ---NIEKQHSEML
Spa40 VGIPTVRDVKLARKLYKTHT-KY------SFVDFEHLDEVLRLI----VWLEQVENTH
FINB HDVMMVENRPLARALYDQVE-ID------QAVPEEFFKVLAEIL----A---YVYKTKQKVY
                                                                                                                                                                                                                             359
                                                                                                                                                                                                                             354
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Fig. 5. (continued from preceding page)

For example, the P. solanacearum hrp homologs of yscT and yscU are not arranged colinearly with those of yscO, yscR, and yscS, as they are in P. s. pv. syringae 61. It is also noteworthy that completely different positive activator proteins control the expression of the hrp genes in the two systems: HrpB of P. solanacearum is homologous to the AraC family (Genin et al. 1992), but the HrpR and HrpS proteins of P. syringae belong to the NtrC family involved in two component regulatory systems (Fellay et al. 1991; Grimm and Panopoulos 1989; Xiao et al. 1994), and no homolog has been found in P. solanacearum for P. syringae HrpL, a putative alternative sigma factor in the AlgU family (Xiao et al. 1994). Our combined results reveal that gram-negative bacteria possess conserved sets of proteins involved with flagellar biogenesis, the polar delivery of antihost proteins to mammalian cells (by Yersinia and other animal pathogens), and the secretion of glycine-rich elicitor proteins (by diverse plant pathogens).

Little is known about how the type III secretion pathway functions to secrete the glycine-rich elicitor proteins of plant pathogens or the Yop and Ipa virulence factors of animal pathogens across the cell membranes, as well as the exact

numbers of proteins participating in secretion. Twenty or more Ysc proteins (encoded by the virC and virB loci) (Bergman et al. 1994; Michiels et al. 1991), or at least 15 Hrp proteins may participate in forming an export organelle that involves outer membrane, inner membrane, and cytoplasmic proteins. HrpH, one protein of this complex located in the outer membrane, possesses sequence similarity to the pIV protein of filamentous phages involved in extruding phage particles (Huang et al. 1992). Ten to 12 molecules of pIV protein probably form a disk-shaped multimeric complex in the outer membrane, whose opening could be gated by an energized pI protein as soon as it receive a signal of packaging of phage particles (Kazmierczak et al. 1994; Russel 1993). Russel (1994) has suggested that homologs of pIV may play the same role in the type II protein secretion pathway. Therefore, it is worth investigating whether the HrpH protein forms the same structure. If HrpH does that, the HrpJ4 protein could be potentially a candidate to regulate the opening of this pore since it contains an ATPase domain (Lidell and Hutcheson 1994). The roles played in this secretory machinery of HrpC and its homolog, YscJ or MixJ proteins located in the outer

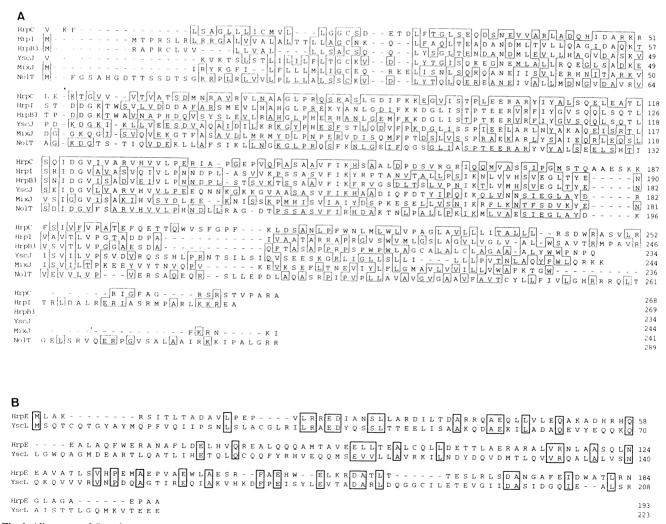


Fig 6. Alignment of *Pseudomonas syringae* pv. syringae hrpZ operon proteins with potential homologs from other bacteria using the Pileup algorithm (Devereaux et al. 1984). A, Identical amino acids (open boxes) in the predicted sequences of *Pseudomonas syringae* pv. syringae HrpC, *P. solanacearum* HrpI, *Xanthomonas campestris* pv. vesicatoria HrpB3, *Y. enterocolitica* YscJ, *S. flexneri* MixJ, and *R. fredii* NoIT. B, Alignment of identical amino acids in the predicted sequences of *P. s.* pv. syringae HrpE and *Y. enterocolitica* YscL.

membrane, are still a puzzle. They may function in stabilizing the HrpH multimer, or in mediating any communication with inner membrane proteins in this complex.

The roles played by a group of inner membrane proteins, HrpI, HrpW, HrpO, HrpX, and HrpY, in the secretion process remain elusive. SpaS, the homolog of HrpY, contains a motif that defines a group of mitochondrial proteins responsible for energy transfer (Groisman and Ochman 1993). Also, YscU, the homolog of HrpY, is thought to interact with cytoplasmic YscN, the homolog of HrpJ4 (Allaoui et al. 1994). So is it possible that HrpY interacts with HrpJ4 in energy transfer during the process? The C-terminal portion of ORF2 of X. campestris pv. glycines, a homolog of HrpW, is quite similar to that of the gamma subunit of oxaloacetate decarboxylase, which is involved in sodium ion transport (Hwang et al. 1992). A reasonable possibility is that all of these proteins assemble into a very tight channel in the inner membrane. Several Fli/Flh proteins of enteric bacteria form a complex in

the inner membrane to allow the secretion of flagellar proteins (Macnab 1992). However, some Ysc proteins, for instance LcrD and YscU, appear to have dual functions, one in secretion, another in the regulation of Yop protein production (Allaoui et al. 1994; Plano et al. 1991). The Hrp proteins which are homologs of these Ysc proteins may not have such dual functions. For example, HrpI of *E. amylovora*, a homolog of LcrD, functions only in the secretion of Harpin_{Ea} (Wei and Beer 1993).

Unlike the type II protein secretion pathway, which employs the Sec machinery to cross the inner membrane, harpin-like proteins and Yop proteins bypass the Sec pathway in their travel to the cell exterior. However, the *Yersinia* YopE protein requires a specific chaperone-like SycE protein to protect it from denaturation and subsequent proteolytic degradation before being secreted (Wattiau and Cornelis 1993), just as the Sec-mediated translocation of some envelope proteins requires SecB (Pugsley 1993). Hence, it is reasonable to predict

Table 2. Identity/similaritya of homologs of Hrp proteins

Pss 61 proteins	HrpC	HrpE	HrpU	HrpW	HrpO	HrpX	HrpY
Secretion components	80 m	14.800 - 2.000	12412 A224		2000 747-	4121 200	
Y. spp	YscJ	YscL	YscQ	YscR	YscS	YscT	YscU
	37/59	21/47	28/53	45/72	46/68	30/59	32/59
S. flexneri	MixJ		Spa33	Spa24	Spa9	Spa29	Spa40
	25/58		16/47	34/61	33/69	25/56	26/56
S. typhimurium			SpaO	SpaP	SpaQ	SpaR	SpaS
			28/53	37/63	36/63	27/59	28/57
P. solanacearum	HrpI	HrpF	HrpQ	HrpT	HrpU	HrpC	HrpN
	36/58	25/45	23/48	42/64	31/56	28/54	31/56
X. campestris	HrpB3			ORF2			
000 (1.17 J.)	35/58			43/63			
E. coli					InvX		
					33/69		
R. fredii	NolT						
	37/58						
Flagellar proteins				-		13004207	72272
B. subtilis			FliY 24/51	FliP 37/62	FliQ 32/62	FliR 20/53	FlhB 34/59
							34/39
E. coli			FliN	FliP	FliQ	FliR	
			25/56	37/62	32/59	21/53	
C. crescentus	FliF		FliN		FliQ	FliR	
	25/58		25/56		35/63	23/53	
E. carotovora			MopA	MopC	MopD	MopE	
			28/58	42/65	34/59	24/54	
S. typhimurium	FliF		FliN				
5(%)	24/56		25/56				

a The identity/similarity values are expressed in percentages as estimated by the Bestfit program of the Genetics Computer Group software.



Fig 7. Cellular location of HrpZ in hrpC, hrpE, hrpW, hrpX, and hrpY mutants as detected by Western blots probed with anti-HrpZ antibodies. Lanes: 1, supernatant of wild type Pseudomonas syringae pv. syringae; 2, cell fraction of P. s. pv. syringae; 3, supernatant of hrpW mutant, 61-12; 4, cell fraction of 61-12; 5, supernatant of hrpX mutant, 61-1087; 6, cell fraction of 61-1087; 7, supernatant of hrpY mutant, 61-1088; 8, cell fraction of 61-1088; 9, supernatant of hrpC mutant, 61-2091, 10, cell fraction of 61-2091; 11, supernatant of hrpE mutant, 61-19; 12, cell fraction of 61-19.

that among the *hrp* gene products are chaperone-like proteins that have the same function in the Hrp secretion pathway. A few cytoplasmic Hrp proteins with unknown function are potential candidates as chaperones, for example, HrpF, HrpG, HrpK, or HrpA.

Recently discovered properties of the type III protein secretion pathway may be important to plant-pathogen interactions. For example, Xanthomonas avr gene products have been predicted to be secreted via a hrp-dependent pathway (Fenselau et al. 1992), although so far, there is no experimental evidence that these proteins are released from the cell (Brown et al. 1993; Young et al. 1994). However, it has recently been observed that the type III pathway in Yersinia spp. permits the polarized transfer of antihost proteins directly into mammalian cells (Rosqvist et al. 1994; Sory and Cornelis 1994). The extensive similarities documented here between the type III pathway components in Yersinia spp. and P. syringae raises the possibility that the Hrp pathway may have a similar translocation capability. The completion of the nucleotide sequence of the entire functional hrp cluster of P. s. pv. syringae 61 should facilitate exploration of the mechanism by which harpins and possibly other proteins are secreted by plant pathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 4. *Escherichia coli* was grown in Luria-Bertani (LB) medium (Sambrook et al. 1989) at 37°C. *Pseudomonas*

strains were grown on King's B medium (KB) (King et al. 1954) or Hrp minimal medium (50 mM potassium phosphate buffer, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, 1.7 mM NaCl, 10 mM mannitol, and 10 mM fructose, pH 5.7) (Huynh et al. 1989) at 28°C to 30°C. The alkaline phosphatase substrate used here was 40 µg/ml 5-bromo-4-choro-3-indolyl-phosphate (XP). Antibiotics used in selective media were (in micrograms per milliliter): ampicillin, 100; kanamycin, 50; tetracycline, 20; nalidixic acid, 25.

General DNA manipulations.

Restriction enzyme digestions, agarose gel electrophoresis, plasmid extractions, DNA ligation, and bacterial transformations (CaCl₂ procedure) were performed as described by Sambrook et al. (1989). DNA fragments used in subcloning were separated by electrophoresis through a 0.7% agarose gel and then purified by electroelution from excised gel pieces in an Elutrap apparatus (Schleicher & Schuell, Keene, NH) or by using the Jetsorb Extraction kit (Genomed. Inc.).

DNA sequencing.

To determine the nucleotide sequences of the *hrpU*, *hrpH*, and *hrpZ* operons (the regions containing complementation groups VIII, IX, and XI) by the dideoxy method (Sanger et al. 1977), appropriate restriction fragments were subcloned into the sequencing vectors, pBluescript KS or SK. Three *SmaI* fragments (1.7, 1.5, and 1.5 kb, respectively), which are adjacent to each other and encompass the sequences of group VIII and group IX, were subcloned individually from pSH14 into pBluescript. The 2.3-kb *EcoRI-HindIII* and 1.4-kb

Table 3. Characteristics and homologies of Pseudomonas syringae pv. syringae 61 Hrp proteins*

Hrp proteins ^a	Yersinia proteins	Flagellar proteins	Hrp protein characteristics
HrpK			Unknown function (Xiao et al. 1994)
HrpL			Alternative sigma factor (Xiao et al. 1994)
HrpJ			Unknown function (Huang et al. 1993)
HrpI	LcrD	FlhA	6 m.d. ; secretion likely (Huang et al. 1993)
HrpJ3		(FliG)	Secretion likely (Lidell and Hutcheson 1994)
HrpJ4	YscN	FliI	ATPase domain; secretion likely (Lidell and Hutcheson 1994)
HrpJ5		(FliJ)	Secretion likely (Lidell and Hutcheson 1994)
HrpU1			Secretion likely (Lidell and Hutcheson 1994)
HrpU2			Similar to ORF1 from X. c. pv. glycines; secretion likely (Lidell and Hutcheson 1994)
HrpU	YscQ	FliY/FliN	Secretion likely; cytoplasmic (This work)
HrpW	YscR	FliP	3 m.d.; secretion likely (This work)
HrpO	YscS	FliQ	2 m.d.; secretion likely (This work)
HrpX	YscT	FliR	3 m.d.; secretion likely (This work)
HrpY	YscU	FlhB	4 m.d.; secretion (This work)
HrpH	YscC		Outer membrane; secretion (Huang et al. 1992)
HrpG			Potential ORF; function unknown
HrpF			Potential ORF; function unknown
HrpE	YscL		Secretion (This work)
HrpD	(YscK)		Secretion likely (Preston et al. 1995; this work)
HrpC	YscJ	FliF	Outer membrane; secretion likely (This work)
HrpB	YscI		Secretion likely (Preston et al. 1995)
HrpZ			Harpin _{Ber} , elicitor of HR (He et al. 1993)
HrpA			Unknown function (Preston et al. 1995)
HrpR, HrpS			NtrC family; positive activator (Xiao et al. 1994)
25	11 + (1)	8 + (2)	Total number of proteins

^a The criterion for probable homology is based on a difference between the scores for the optimized and the average of 100 random alignments being at least 5 times the standard deviation for the randomized alignments (Doolittle 1986). Proteins with similarities less than this value are presented in parentheses.

b Proteins are listed in the order of the arrangement of their genes, from left to right, in the pHIR11 hrp cluster (Fig 1).

^c Number of m.d. refers to number of transmembrane domains.

d Evidence for secretion is discussed in the text.

EcoRI-EcoRI fragments spanning the hrpH and hrpZ operons were also cloned from pCPP2145 into pBluescript. A series of nested deletions was generated with the Erase-A-Base Kit (Promega), and then the sequences of both DNA strands were determined by using the Sequenase version 2.0 sequencing kit (U.S. Biochemical Co.). The junction between fragments was confirmed by synthesis of appropriate primers, which were prepared by the Agricultural Biotechnology Labs of National Chung Hsing University. The TnphoA insertions in the hrpC, hrpE, hrpW, hrpX, and hrpY mutations were confirmed by determining the flanking DNA sequences using primers homologous to TnphoA. Data were analyzed with the Microgenie 7.0, DNA Star, and Genetics Computer Group programs (Devereaux et al. 1984), and the homology search was done with the BLAST program (Altschul et al. 1990; Pearson and Lipman 1988).

T7 RNA polymerase-dependent expression of *hrpC*, *hrpD*, and *hrpE*.

To express the hrpC gene in $E.\ coli$, a 1.4-kb PsI fragment isolated from pCPP2145 was cloned into pET21a to construct pNCHU61 and pNCHU62 (with the insert in different orientations). Then these plasmids were transformed into $E.\ coli$ MC4100 harboring pGP1-2 (Tabor and Richardson 1985). The transformants were grown in Luria-Bertani broth to an OD₆₀₀ of 0.4 at 30°C, washed with M9 minimal medium, and then suspended in the same minimal medium. Incubation was continued for 1 h at 30°C before the culture was shifted to 42°C for 20 min to induce expression of the T7 RNA polymerase gene. Rifampin (final concentration, 200 μ g/ml) was

then added, and incubation was continued for a further 10 min. L-[35 S]methionine (10 μ Ci) was then added to 1 ml of culture, which was incubated for an additional 10 min at 30°C. Labeled cells were pelleted, suspended in 80 μ l of polyacrylamide gel sample buffer, and heated to 100°C for 2 min. A 10- μ l sample then was subjected to sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis. Labeled proteins were visualized by exposing Kodak X-Omat AR film to the gel at -80°C for 16 h.

To express the *hrpD* and *hrpE* genes in *E. coli* cells, a 1.7-kb *SaII* fragment isolated from pCPP2145 was cloned into pBluescript KS to construct pNCHU63 and pNCHU64 (with the insert in different orientations). These plasmids were transformed into *E. coli* BL21(DE3) (Studier et al. 1990). The methods described above were then used, except that IPTG (final concentration, 0.4 mM) was used to induce the expression of the T7 RNA polymerase gene to permit selective expression of the gene of interest.

TnphoA mutagenesis of pCPP2145 and pSH14, triparental matings, and marker-exchange mutagenesis of *P. syringae*.

Plasmids pCPP2145 and pSH14, carried in *E. coli* CC118, were mutagenized with λ ::TnphoA (Manoil and Beckwith 1985) as described (Huang et al. 1991). Mutated (Kan^R) plasmids were extracted and directly restriction mapped. Those with TnphoA inserted in the region of interest were individually mobilized by triparental matings into *P. s.* pv. syringae 61 for subsequent marker-exchange mutagenesis. Triparental matings (Ditta et al. 1980) were carried out by

Table 4. Bacterial strains and plasmids used in this study

Strains or plasmids	Relevant characteristics	Source or reference
E. coli		
DH10B	endA1 hsdR17 recA1 relA Δ(argF-lacZYA)U169 φ80d lacZΔM15	Life Sciences Technologies (Gaithersburg, MD)
HB101	F' hsd20 recA13 thr leu thi pro Sm ^r	Maniatis et al. 1982
MC4100	$F^-\Delta(lac)U169$ araD136 relA rpsL thi	Oliver and Beckwith 1981
BL21(DE3)	B strain, F-ompT r _b -m _b -hsdS gal (λDE3 clts857 int1 Sam7 nin5 lacUV5-T7 gene1)	Studier et al. 1990
P. syringae pv. syringae		
61	Wild type isolated from Wheat, Nal ^r	Huang et al. 1988
61-12	Strain 61 derivative carrying hrpW::TnphoA mutation	This study
61-19	Strain 61 derivative carrying hrpE::TnphoA mutation	This study
61-2087	Strain 61 derivative carrying hrpX::TnphoA mutation	Huang et al. 1991
61-2088	Strain 61 derivative carrying hrpY::TnphoA mutation	Huang et al. 1991
61-2091	strain 61 derivative carrying hrpC::TnphoA mutation	Huang et al. 1991
Plasmids		
pRK2013	IncP Tra RK2+ ΔrepRK2 repE1* Kan'	Ditta et al. 1980
pBluescript KS or SK	ColE1, Apr, mcs-lacZ	Stratagene
pET21a	Ap ^r , lacI	Novagene
pGP1-2	Kan ^r , c1857, T7 RNA polymerase	Tabor and Richardson 1985
pHIR11	30-kb fragment of strain 61 containg hrp cluster cloned in pLAFR3	Huang et al. 1988
pSH14	13.5-kb EcoRI fragment from pHIR11 subcloned in pLARF3	S. W. Hutcheson
pCPP2145	10.6-kb BglII from pHIR11 subcloned in pCPP30	Huang et at. 1992
pCPP30	IncP $lacZ^{r}$; Tc ^r	D.W. Bauer
pSH14-12	pSH14 derivative carrying hrpW::TnphoA mutation	This study
pCPP2145-19	pCPP2145 derivative carrying hrpE::TnphoA mutation	This study
pNCHU61	1.4-kb PstI fragment from pCPP2145 subcloned in pET21a; opposite orientation of T7 promoter	This study
pNCHU62	1.4-kb PstI fragment from pCPP2145 subcloned in pET21a; same orientation as T7 promoter	This study
pNCHU63	1.7-kb SalI fragment from pCPP2145 subcloned in pBluescript KS; opposite orientation of T7 promoter	This study
pNCHU64	1.7-kb SalI fragment from pCPP2145 subcloned in pBluescript KS; same orientation as T7 promoter	This study

mixing the recipient *P. s.* pv. *syringae*, donor *E. coli* CC118 (containing mutated cosmids), and helper strain *E. coli* HB101(pRK2013) at a ratio of 10:1:1 on KB agar and incubating at 30°C. The mating mixture was then spotted on KB agar supplemented with nalidixic acid, tetracycline, and kanamycin at 30°C for 3 days. TnphoA insertions were then marker-exchanged into the *P. s.* pv. *syringae* 61 genome as described (Huang et al. 1988).

HR assay in tobacco plants.

Tobacco (*Nicotiana tabacum* L. var. Xanthi) plants were grown under greenhouse conditions and then transferred to the laboratory and maintained at room temperature for the HR assays. Bacteria were prepared by suspending 24-h cultures grown on KB plates in 10 mM phosphate buffer at a concentration of 5×10^8 . Inoculations were performed by pricking leaves with a dissecting needle and then pressing the blunt end of a tuberculin syringe against the leaf surface while supporting the leaf with a finger (Baker et al. 1987). The plants were observed within 24 h for development of the HR.

Western blotting of HrpZ protein with anti-HrpZ antibody.

The wild-type P. s. pv. syringae 61, and hrpC, hrpE, hrpW, hrpX, and hrpY mutants were first grown in 5 ml of KB broth at 30°C to an OD₆₀₀ of 0.5. Cells were collected by centrifugation, washed once in 5 ml of Hrp minimal broth, resuspended in 5 ml of the same medium, and incubated with shaking for 5 h. The cells and extracellular fractions were then separated by centrifugation. The supernatants were immediately precipitated with TCA at a final concentration of 5%, washed with acetone, dissolved in 30 ul of 10 mM Tris buffer, and boiled after adding an equal volume of 2x loading buffer (0.625 M Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol) for 1 min. The cell pellets were washed with 10 mM Tris buffer (pH 8.0), resuspended in 2 ml of the same buffer, sonicated, precipitated with 5% of TCA, washed with acetone, dissolved in 30 µl of 10 mM Tris buffer, and boiled with an equal volume of 2×loading buffer for 1 min. A 20-μl sample of each fraction was subjected to SDS-10% polyacrylamide gel electrophoresis in a 0.75 mm thick gel in a Hoefer Mighty Small apparatus. Prestained molecular size standards (Bio-Rad Laboratories, Richmond, CA) were used to calibrate protein mobilities on the blot. After separation, the protein bands were transferred to an Immobilon-P membrane (Millipore Inc., Bedford, MA) in a high-field electroblotting apparatus (American Bionetics Inc.) as described by the manufacturer's instructions. The transblotted filter was probed with an anti-HrpZ antiserum (He et al. 1993). Immunodetection of the bands was performed with a rabbit alkaline phosphatase-conjugated antibody (The Binding Site) as described by manufacturer's instruction.

Nucleotide sequence accession numbers.

The nucleotide sequences reported in this paper have been deposited in GenBank under accession numbers U25812 for the *Sma*I-*Sst*I fragment bearing *hrpU*, *O*, *W*, *X*, and *Y*, and U25813 for the *Eco*RV-*Eco*RI fragment bearing *hrpC*, *D*, *E*, *F*, and *G*.

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