MPMI Vol. 8, No. 5, 1995, pp. 733–746.

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

Hsiou-Chen Huang,[1] Rong-Hwa Lin,[1] Chun-Jung Chang,[1] Alan Collmer,[2] and Wen-Ling Deng[1]

[1]Agricultural Biotechnology Laboratories, National Chung-Hsing University, Taichung, Taiwan 40227 R.O.C.; [2]Department of Plant Pathology, Cornell University, Ithaca, NY 14853 U.S.A.

Received 6 February 1995. Accepted 18 May 1995.

***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 *Sma*I-*Sst*I 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*, *yscS/spa9/spaQ*, *yscT/spa29/spaR*, and *yscU/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 *Eco*RV-*Eco*RI 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. Tn*phoA* 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.**

Corresponding author: Hsiou-Chen Huang;
E-mail 711hchuang@vax9k.nchu.edu.tw

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 1994).

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, Tn*5-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 *Eco*RV-*Eco*RI fragment containing part of the *hrpZ* (*hrpC*, *hrpD*, and *hrpE*) and *hrpH* (*hrpG* and *hrpF*) operons and the 3.7-kb *Sma*I-*Sst*I 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 *Sma*I-*Sst*I fragment.

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

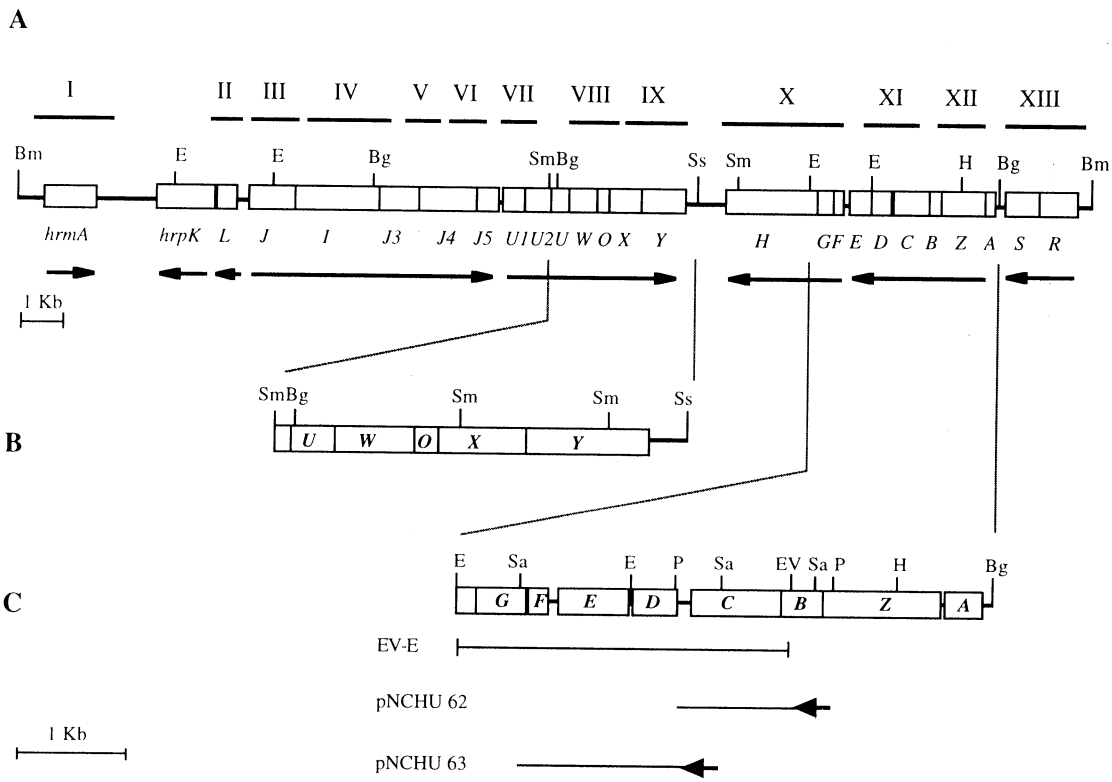


Fig. 1. The location of the *hrpZ*, *hrpH*, and *hrpU* operons in relation to the entire pHIR11 *hrp* cluster and the regions subcloned for DNA sequencing and T7 polymerase-dependent expression of *hrpC*, *hrpD*, and *hrpE*. **A,** The complementation groups were assigned as described previously (Huang 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 *Sma*I-*Sst*I fragment containing the *hrpU, hrpW, hrpO, hrpX*, and *hrpY* genes was sequenced. **C,** The nucleotide sequence of the 2.9-kb *Eco*RV-*Eco*RII 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, *Bgl*II; Bm, *Bam*HI; E, *Eco*RI; EV, *Eco*RV H, *Hin*dIII; P, *Pst*I; Sa, *Sal*I, Sm, *Sma*I; Ss, *Sst*I.

groups VIII and IX defined by Tn*phoA* mutagenesis (Huang et al. 1991). Five ORFs were found to extend from the left-hand *Bgl*II site to the right-hand *Sst*I site (Fig. 1B). The putative translational initiation site of ORF1 was to the left of the *Bgl*II site, downstream of *hrpU2*, and in the previously reported nucleotide sequence of the adjoining 4.3-kb *Bgl*II fragment (Lidell and Hutcheson 1994). Including the relevant portion of the 4.3-kb *Bgl*II fragment, the nucleotide sequence of the 3.7-kb *Sma*I-*Sst*I 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).

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 *Hin*dIII-*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.

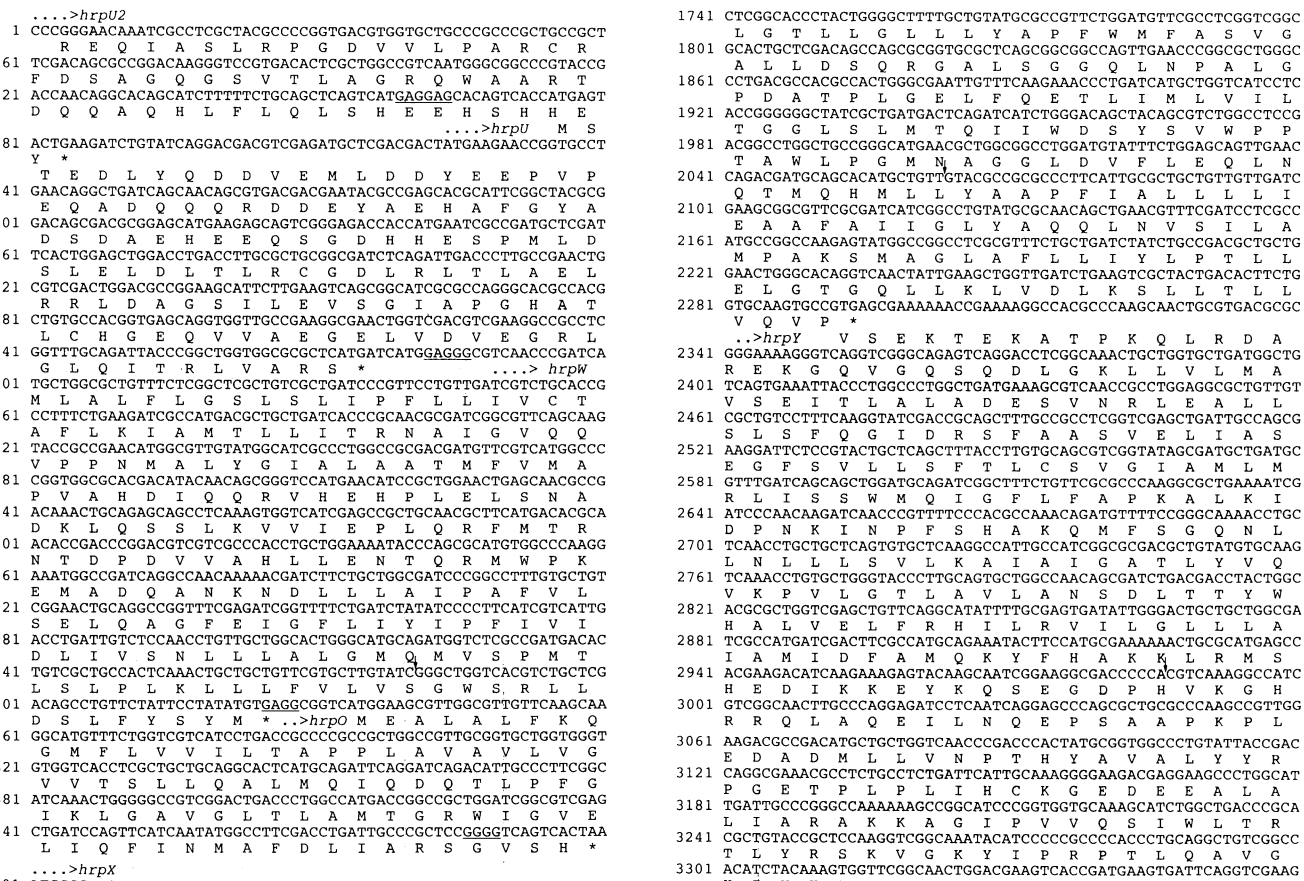


Fig. 2. Nucleotide sequence of the 3,672-bp *Sma*I-*Sst*I fragment containing the *hrpU* operon. Potential ribosome binding sites preceding the *hrpU*, *hrpW*, *hrpO*, and *hrpY* genes are underlined; vertical arrows denote Tn*phoA* insertions in *hrpW*, *hrpX*, and *hrpY* genes.

were defined by Tn*phoA* mutagenesis (Huang et al. 1991). Seven ORFs were identified within the nucleotide sequence extending from the right-hand *Eco*RV site to left-hand *Eco*RI 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 homolog, 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 *Pst*I 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 *Sal*I 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.

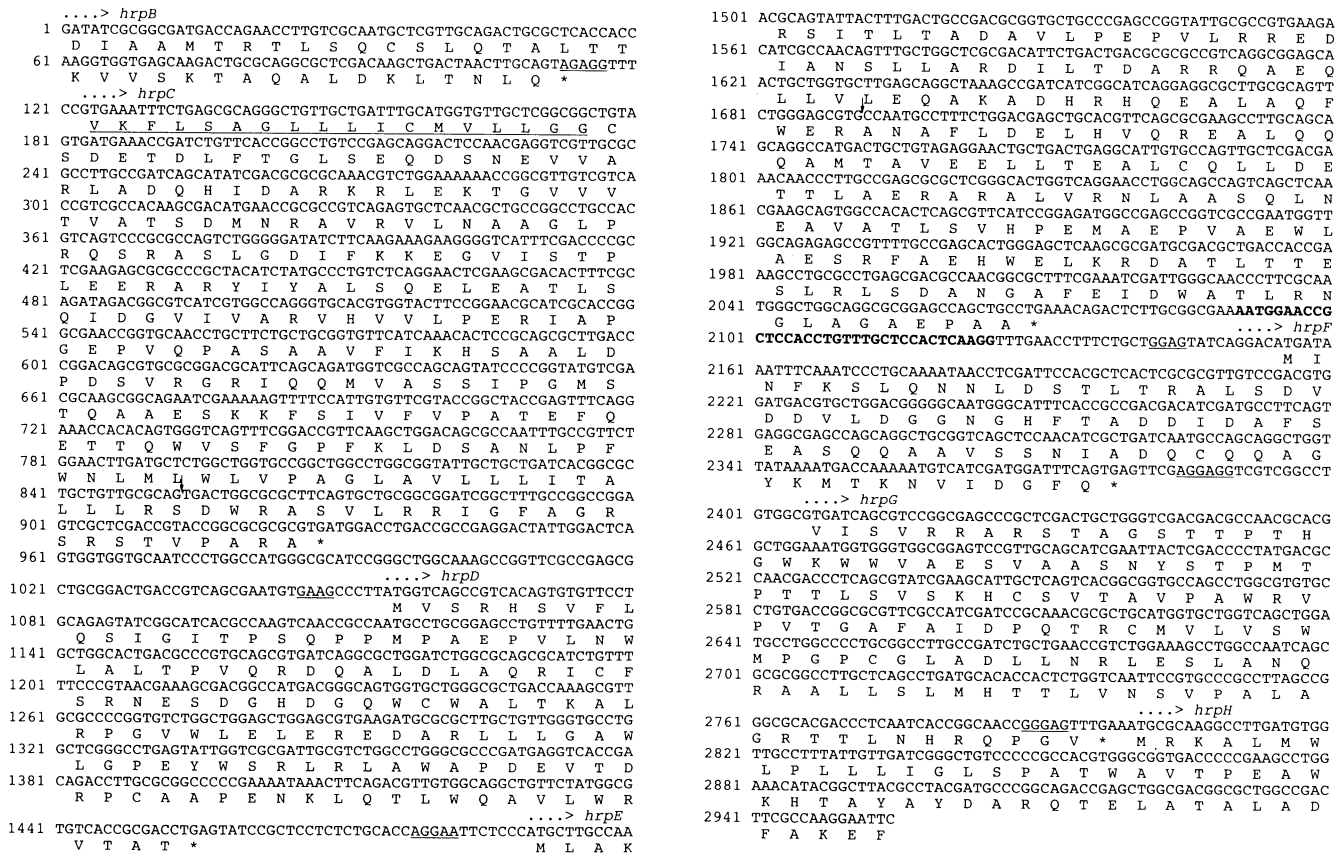


Fig 3. Nucleotide sequence of the 2,955-bp *Eco*RV-*Eco*RI fragment containing the *hrpC, hrpD, hrpE, hrpF,* and *hrpG* genes. Potential ribosome binding sites preceding the *hrpC, hrpD, hrpE, hrpF,* and *hrpG* genes are underlined. The underlined amino acids denote the predicted signal peptide of HrpC. Vertical arrows denote Tn*phoA* 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 Tn*phoA* 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 Tn*phoA* 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 Tn*phoA* 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 *ysc* 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-*

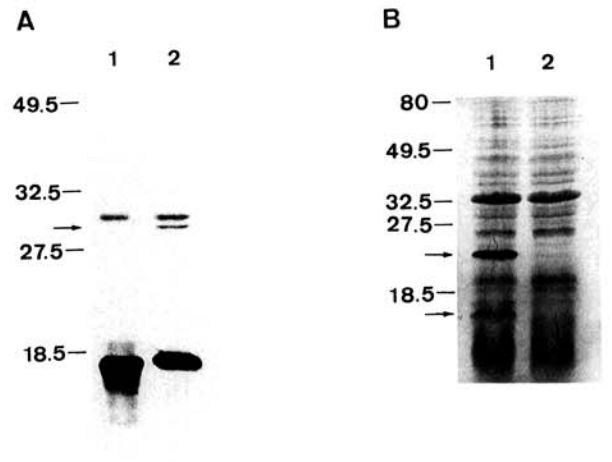


Fig. 4. Autoradiograph of the protein products of *hrpC* (**A**) and *hrpD* and *hrpE* (**B**) following T7 RNA polymerase-dependent expression in *E. coli* BL21(DE3) and SDS polyacrylamide gel electrophoresis. L-[^{35}S]methionine labeling and electrophoresis were performed as described in the text. 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

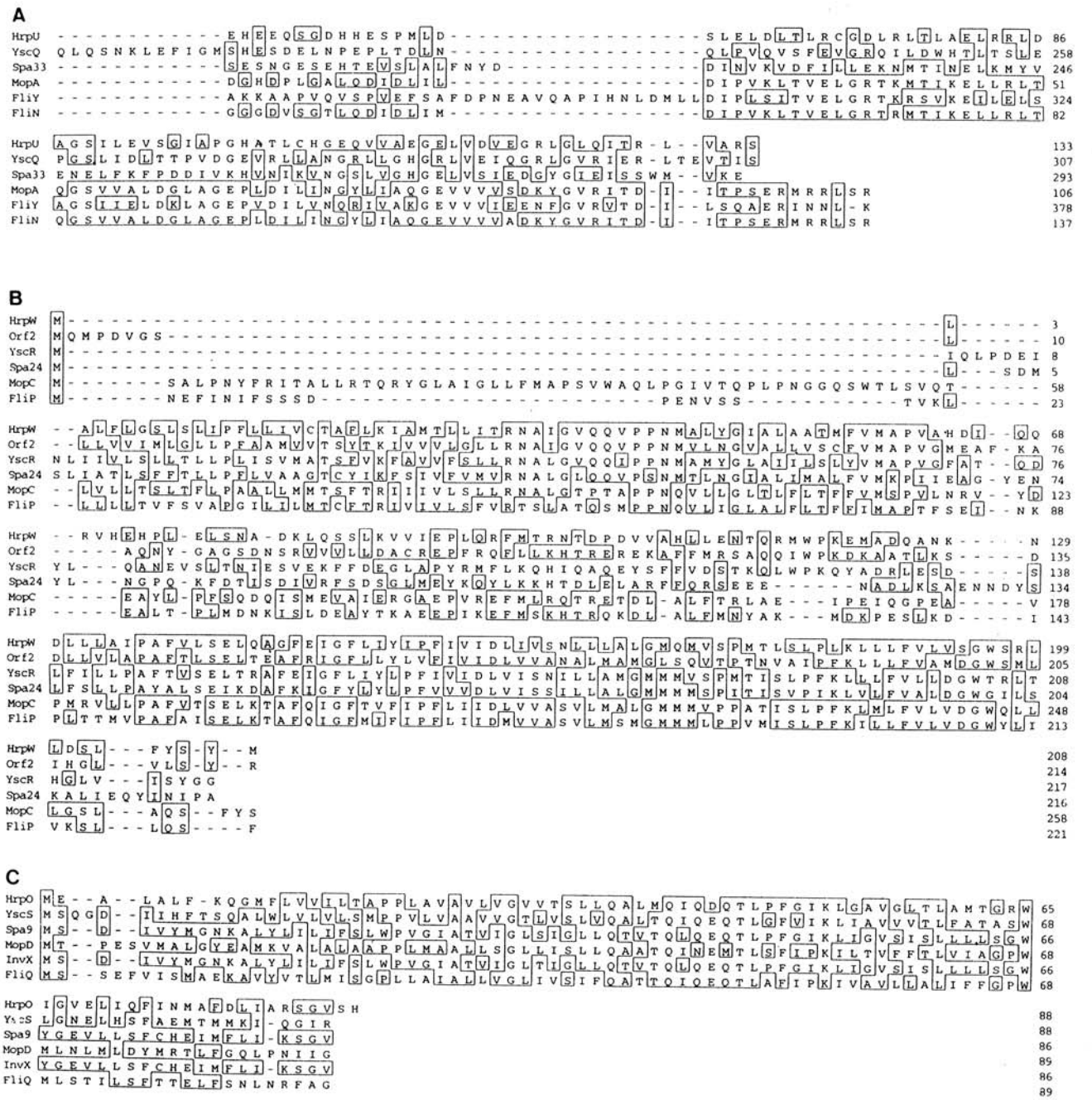
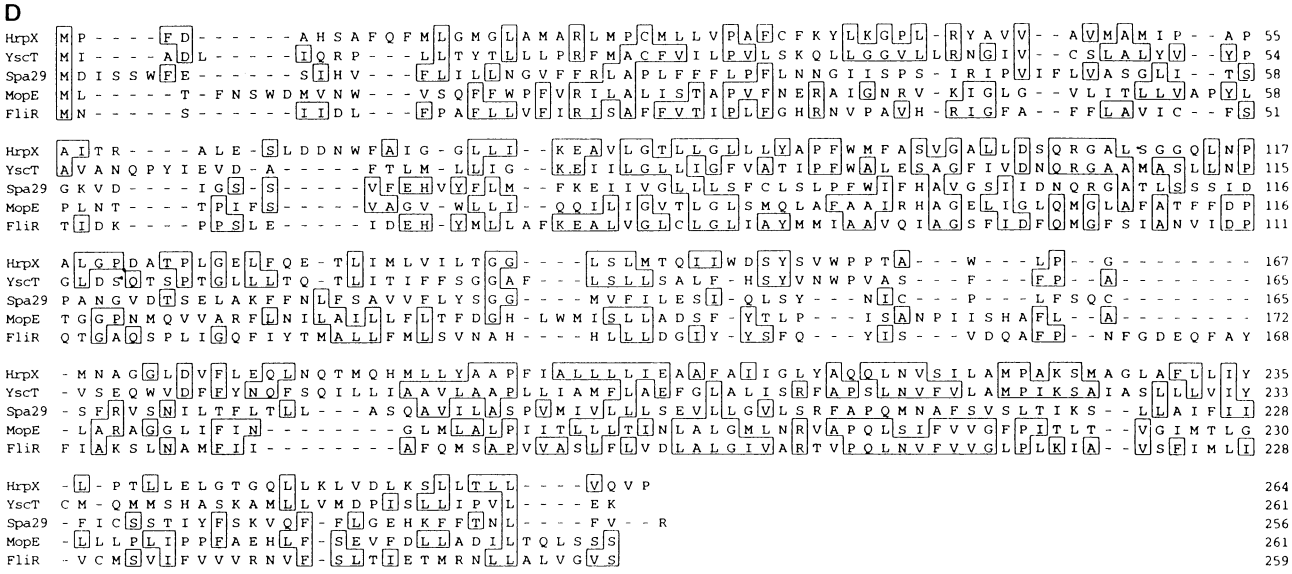


Fig 5. Alignment of *Pseudomonas syringae* pv. *syringae hrpU* operon proteins with potential homologs from other bacteria using the Cluster method in DNA Star. YscQ, YscR, YscS, YscT, and YscU are from *Y. pseudotuberculosis* (see text for all references). ORF2 is from *Xanthomonas campestris* pv. *glycines*. 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 Tn*phoA* 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. solanacearum hrp* cluster.



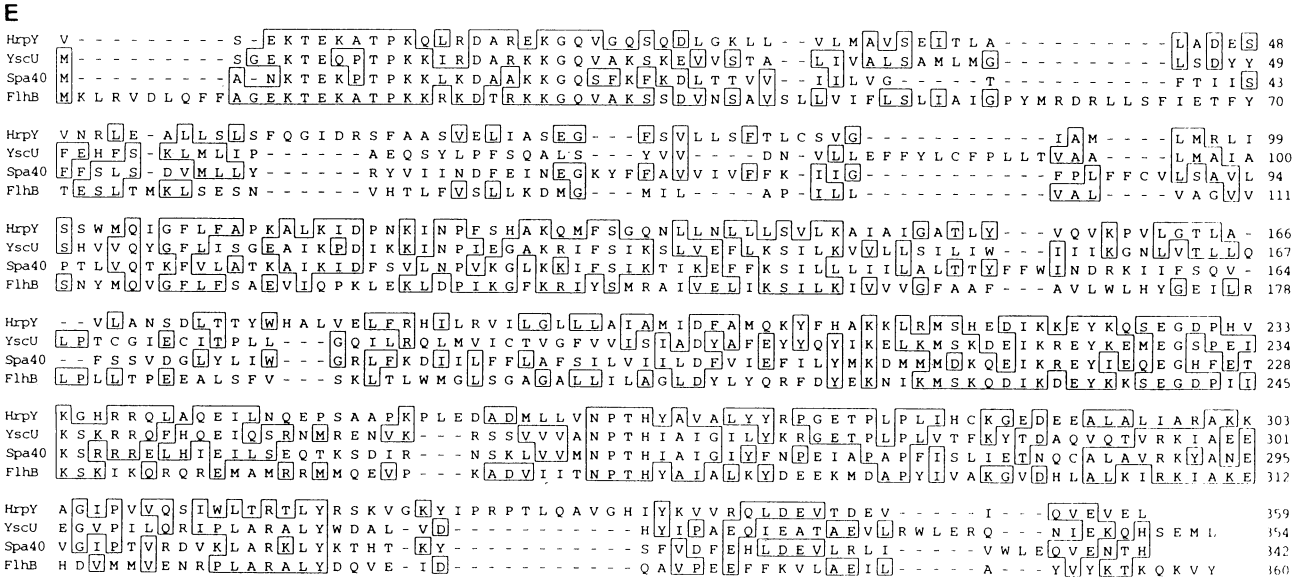


Fig. 5. *(continued from preceding page)*

For example, the *P. solanacearum hrp* homologs of *yscT* and *yscU* are not arranged colinearly with those of *yscQ, 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* NolT. **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/similarity[a] of homologs of Hrp proteins

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

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

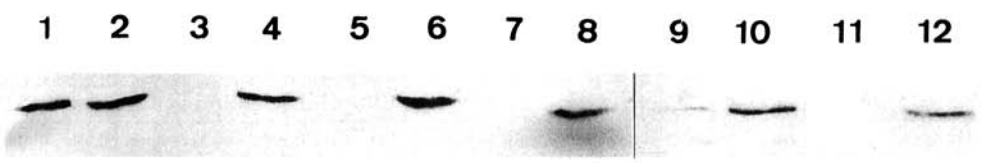


Fig 7. Cellular location of HrpZ in *hrpC, hrpE, hrpW, hrpX,* and *hrpY* mutants as detected by Western blots probed with anti-HrpZ antibodies. Lanes: 1, supernatant of wild type *Pseudomonas syringae* pv. *syringae*; 2, cell fraction of *P. s.* pv. *syringae*; 3, supernatant of *hrpW* mutant, 61-12; 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 $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 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 ($CaCl_2$ 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 *Sma*I 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 *Eco*RI-*Hin*dIII and 1.4-kb

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

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.[c]; secretion likely[d] (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			$Harpin_{Pss}$, 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.

*Eco*RI-*Eco*RI 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 Tn*phoA* insertions in the *hrpC, hrpE, hrpW, hrpX*, and *hrpY* mutations were confirmed by determining the flanking DNA sequences using primers homologous to Tn*phoA*. 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 *Pst*I 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 OD_{600} 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 μg/ml) was then added, and incubation was continued for a further 10 min. L-[^{35}S]methionine (10 μCi) 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 μl of polyacrylamide gel sample buffer, and heated to 100°C for 2 min. A 10-μl 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 *Sal*I 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.

Tn*phoA* 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::Tn*phoA* (Manoil and Beckwith 1985) as described (Huang et al. 1991). Mutated (Kan^R) plasmids were extracted and directly restriction mapped. Those with Tn*phoA* 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(*arg*F-*lac*ZYA)U169 ϕ80d *lac*ZΔM15	Life Sciences Technologies (Gaithersburg, MD)
HB101	F′ *hsd20 recA13 thr leu thi pro* Sm^r	Maniatis et al. 1982
MC4100	F$^-$ Δ(*lac*)U169 *araD*136 *relA rpsL thi*	Oliver and Beckwith 1981
BL21(DE3)	B strain, F-*omp*T $r_b^-m_b^-$ *hsd*S *gal* (λDE3 c*Its*857 *int*1 Sam7 *nin*5 *lac*UV5-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*::Tn*phoA* mutation	This study
61-19	Strain 61 derivative carrying *hrpE*::Tn*phoA* mutation	This study
61-2087	Strain 61 derivative carrying *hrpX*::Tn*phoA* mutation	Huang et al. 1991
61-2088	Strain 61 derivative carrying *hrpY*::Tn*phoA* mutation	Huang et al. 1991
61-2091	strain 61 derivative carrying *hrpC*::Tn*phoA* mutation	Huang et al. 1991
Plasmids		
pRK2013	IncP Tra RK2+ Δ*repRK2 repE1*$^+$ Kan^r	Ditta et al. 1980
pBluescript KS or SK	ColE1, Ap^r, mcs-*lacZ*	Stratagene
pET21a	Ap^r, *lacI*	Novagene
pGP1-2	Kan^r, *cI857*, 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 *Eco*RI fragment from pHIR11 subcloned in pLARF3	S. W. Hutcheson
pCPP2145	10.6-kb *Bgl*II from pHIR11 subcloned in pCPP30	Huang *et at.* 1992
pCPP30	IncP *lacZ*′; Tc^r	D.W. Bauer
pSH14-12	pSH14 derivative carrying *hrpW*::Tn*phoA* mutation	This study
pCPP2145-19	pCPP2145 derivative carrying *hrpE*::Tn*phoA* mutation	This study
pNCHU61	1.4-kb *Pst*I fragment from pCPP2145 subcloned in pET21a; opposite orientation of T7 promoter	This study
pNCHU62	1.4-kb *Pst*I fragment from pCPP2145 subcloned in pET21a; same orientation as T7 promoter	This study
pNCHU63	1.7-kb *Sal*I fragment from pCPP2145 subcloned in pBluescript KS; opposite orientation of T7 promoter	This study
pNCHU64	1.7-kb *Sal*I 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. Tn*phoA* 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 OD_{600} 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 µl of 10 mM Tris buffer, and boiled after adding an equal volume of 2× 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*.

ACKNOWLEDGMENTS

We are very grateful to Z.-S. Chang for technical assistance. This work was supported by NSC grant NSC82-0418-B-005-117 and NSC83-0203B-005-004.

LITERATURE CITED

Allaoui, A., Sansonetti, P. J., and Parsot, C. 1992. MixJ, a lipoprotein involved in secretion of *Shigella* Ipa invasins, is homologous to YscJ, a secretion factor of the *Yersinia* Yop proteins. J. Bacteriol. 174:7661-7669.

Allaoui, A., Woestyn, S., Sluiters C., and Cornelis, G. R. 1994. YscU, a *Yersinia enterocolitica* inner membrane protein involved in Yop secretion. J. Bacteriol. 176:4534-4542.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Arlat, M., Gough, C. L., Barber, C. E., Boucher, C., and Daniels, M. J. 1991. *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 4:593-601.

Arlat, M., Van Gijsegem, F., Huet, J. C., Pernollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induced a hypersensitive-like response on specific Petunia genotypes, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. EMBO J. 13:543-553.

Baker, C. J., Atkinson, M. M., and Collmer, A. 1987. Concurrent loss in Tn*5* mutants of *Pseudomonas syringae* pv. *syringae* of the ability to induce the hypersensitive response and host plasma membrane K^+/H^+ exchange in tobacco. Phytopathology 77:1268-1272.

Beer, S. V., Bauer, D. W., Jiang, X. H., Laby, R. J., Sneath, B. J., Wei, Z.-M., Wilcox, D. A., and Zumoff, C. H. 1991. The *hrp* gene cluster of *Erwinia amylovora*. Pages 53-60 in: Advances in Molecular Genetics of Plant-Microbe Interactions. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht.

Bergman, T., Erickson, K., Galyov, E., Persson, C., and Wolf-Watz, H. 1994. The *lcrB* (*yscN/U*) gene cluster of *Yersinia pseudotuberculosis* is involved in Yop secretion and shows high homology to the *spa* gene clusters of *Shigella flexneri* and *Salmonella typhimurium*. J. Bacteriol. 176:2619-2626.

Bischoff, D. S., Weinreich, M. D., and Ordal, G. W. 1992. Nucleotide sequences of *Bacillus subtilis* flagellar biosynthetic genes *flip* and *fliQ* and identification of a novel flagellar gene, fliZ. J. Bacteriol. 174:4017-4025.

Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. Pages 79-98 in: Current Topics in Microbiology and Immunology, Vol. 192: Bacterial Pathogenesis of Plants and Animals—Molecular and Cellular Mechanisms. J. L. Dangl, ed. Springer-Verlag, Berlin.

Bonas, U., Schulte, R., Fenselau, S., Minsavage, G. V., Staskawicz, B. J., and Stall, R. E. 1991. Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interact. 4:81-88.

Boucher, C. A., Van Gijsegem, F., Barberis, P. A., Arlat, M., and Zischek, C. 1987. *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. J. Bacteriol.169:5626-5632.

Brown, I., Mansfield, J., Irlam, I., Conrads-Strauch, J., and Bonas, U. 1993. Ultrastructure of interactions between *Xanthomonas campestris* pv. *vesicatoria* and pepper, including immunocytochemical localization of extracellular polysaccharides and the AvrBs3 protein. Mol. Plant-Microbe Interact. 6:376-386.

Devereaux, J., Haeberli, P., and Smithies, O. 1984. A comprehensive set of sequence analysis programs for the VAX. Gene 12:387-395.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium melioti*. Proc. Natl. Acad. Sci. USA 77:7347-7351.

Doolittle, R. F. 1986. Of Urfs and Orfs: A Primer on How to Analyze Derived Amino Acid Sequences. University Science Books, Mill Valley, CA. 103 pp.

Dreyfus, G., Williams, A. W., Kawagishi, I., and Macnab, R. M. 1993. Genetic and biochemical analysis of *Salmonella typhimurium* FliI, a flagellar protein related to the catalytic subunit of the F_0F_1 ATPase

and to virulence proteins of mammalian and plant pathogen. J. Bacteriol. 175:3131-3138.

Fellay, R., Rahme, L. G., Mindrinos, M. N., Frederick, R. D., Pisi, A., and Panopoulos, N. J. 1991. Genes and signals controlling the *Pseudomonas syringae* pv. *phaseolicola*-plant interaction. Pages 45-52 in: Advances in Molecular Genetics of Plant-Microbe Interactions. H. Hennecke and D. P. S. Verma, ed. Kluwer Academic Publishers, Dordrecht.

Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. Mol. Plant-Microbe Interact. 5:390-396.

Fields, K. A., Plano, G. V., and Straley, S. 1994. A low-Ca^{2+} response (LCR) secretion (*ysc*) locus lies within the *lcrB* region of the LCR plasmid in *Yersinia pestis*. J. Bacteriol. 176:569-579.

Genin, S., Gough, C. L., Zischek, C., and Boucher, C. A. 1992. Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. Mol. Microbiol. 6:3065-3076.

Groisman, E. A., and Ochman, H. 1993. Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. EMBO J. 12:3779-3787.

Gough, C. L., Genin, S., Zischek, C., and Boucher, C. A. 1992. *hrp* genes of *Pseudomonas solanacearum* are homologous to pathogenicity determinants of animal pathogenic bacteria and are conserved among plant pathogenic bacteria. Mol. Plant-Microbe Interact. 5:384-389.

Grimm, C., and Panopoulos, N. J. 1989. The predicted protein product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several prokaryotic regulatory proteins. J. Bacteriol. 171:5031-5038.

He, S. Y., Huang, H.-C; and Collmer, A. 1993. Pseudomonas syringae pv. syringae HarpinPss: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. Cell 73:1255-1266.

Heu, S., and Hutcheson, S. W. 1993. Nucleotide sequence and properties of the *hrmA* locus associated with the *Pseudomonas syringae* pv. *syringae hrp* gene cluster. Mol. Plant-Microbe Interact. 6:553-564.

Hsia, R.-C., Small, P. L. C., and Bavoil, P. M. 1993. Characterization of virulence genes of enteroinvasive *Escherichia coli* by Tn*phoA* mutagenesis: Identification of *invX*, a gene required for entry into HEp-2 cells. J. Bacteriol. 175:4817-4823.

Huang, H.-C., Xiao, Y., Lin, R.-H.; Lu, Y., Hutcheson, S. W., and Collmer, A. 1993. Characterization of the *Pseudomonas syringae* pv. *syringae hrpJ* and *hrpI* genes: Homology of HrpI to a superfamily of proteins associated with protein translocation. Mol. Plant-Microbe Interact. 6:515-520.

Huang, H.-C., He, S. Y., Bauer, D. W., and Collmer, A. 1992. The *Pseudomonas syringae* pv. *syringae* 61 *hrpH* product: An envelope protein required for elicitation of the hypersensitive response in plants. J. Bacteriol. 174:6878-6885.

Huang, H.-C., Hutcheson, S. W., and Collmer, A. 1991. Characterization of the *hrp* cluster from *Pseudomonas syringae* pv. *syringae* 61 and Tn*phoA* tagging of genes encoding exported or membrane-spanning Hrp proteins. Mol. Plant-Microbe Interact. 4:469-476.

Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W., and Collmer, A. 1988. Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco. J. Bacteriol. 170:4748-4756.

Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377.

Hwang, I., Lim, S. M., and Shaw, P. D. 1992. Cloning and characterization of pathogenicity genes from *Xanthomonas campestris* pv. *glycines*. J. Bacteriol. 174:1923-1931.

Kazmierczak, B. I., Mielke, D. L., Russel, M., and Model, P. 1994. pIV, a filamentous phage protein that mediates phage export across the bacterial cell envelope, forms a multimer. J. Mol. Biol. 238:187-198.

Kihara, M., Homma, M., Kutsukake, K., and Macnab, R. M. 1989. Flagellar switch of *Salmonella typhimurium*: Gene sequences and deduced protein sequence. J. Bacteriol. 171:3247-3257.

King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Med. 22:301-307.

Klememt, Z. 1982. Hypersensitivity, Pages 49-177 in: Phytopathogenic Prokaryotes, Vol. 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.

Lidell, M. C., and Hutcheson, S. W. 1994. Characterization of the *hrpJ* and *hrpU* operons of *Pseudomonas syringae* pv. *syringae* Pss61: Similarity with components of enteric bacteria involved in flagellar biogenesis and demonstration of their role in $Harpin_{Pss}$ secretion. Mol. Plant-Microbe Interact. 7:488-497.

Lindgren, P. B., Peet, R. C., and Panopoulos, N. J. 1986. Gene cluster of *Pseudomonas syringae* pv. "phaseolicola" controls pathogenicity of bean plants and hypersensitivity on nonhost plants. J. Bacteriol. 168:512-522.

Macnab, R. M. 1992. Genetics and biogenesis of bacterial flagella. Annu. Rev. Genet. 26:131-158.

Malakooti, J., Komeda, Y., and Matsumura, P. 1989. DNA sequence analysis, gene product identification, and localization of flagellar motor components of *Escherichia coli*. J. Bacteriol. 171:2728-2734.

Malakooti, J., Ely, B., and Matsumura, P. 1994. Molecular characterization, nucleotide sequence, and expression of the *fliO, fliP, fliQ,* and *fliR* genes of *Escherichia coli*. J. Bacteriol. 176:189-197.

Manoil, C., and Beckwith, J. 1985. Tn*phoA*: A transposon probe for protein export signals. Proc. Natl. Acad. Sci. U.S.A. 82:8129-8133.

Meinhardt, L. W., Krishnan, H. B., Balatti, P. A., and Pueppke, S. G. 1993. Molecular cloning and characterization of a *sym* plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. Mol. Microbiol. 9:17-29.

Michiels, T., Vanooteghem, J.-C., de Rouvroit, C. L., China, B., Gustin, A., Boudry, P., and Cornelis, G. R. 1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. J. Bacteriol. 173:4994-5009.

Mulholland, V., Hinton, J. C. D., Sidebotham, J., Toth, I. K., Hyman, L. J., Perombelon, M. C. M., Reeves, P. J., and Salmond G. P. C. 1993. A pleiotropic reduced virulence (Rvi^-) mutant of *Erwinia carotovora* subspecies *atroseptica* is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. Mol. Microbiol. 9: 343-356.

Pearson, W. R., and Lipman, D. J. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. U.S.A. 85:2444-2448.

Plano, G. V., Barve, S. S., and Straley, S. C. 1991. LcrD, a membrane-bound regulator of the *Yersinia pestis* low-calcium response. J. Bacteriol. 173:7293-7303.

Preston, G., Huang, H.-C., He, S. Y., and Collmer, A. 1995. 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 irrespective of bacterial host range. Mol. Plant-Microbe Interact . 8:717-732.

Pugsley, A. P. 1993. The complete general protein secretory pathway in gram-negative bacteria. Microbiol. Rev. 57:50-108.

Rimpilainen, M., Forsberg, A., and Wolf-atz, H. 1992. A novel protein, LcrQ, involved in the low-calcium response of *Yersinia pseudotuberculosis* shows extensive homology to YopH. J. Bacteriol. 174:3355-3363.

Russel, M. 1993. Protein-protein interactions during filamentous phage assembly. J. Mol. Biol. 231:689-697.

Russel, M. 1994. Phage assembly: A paradigm for bacterial virulence factors export? Science 265:612-614.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Sasakawa, C., Komatsu, K., Tobe, T., Suzuki, T., and Yoshikawa, M. 1993. Eight genes in region 5 that form an operon are essential for invasion of epithelial cells by *Shigella flexneri* 2a. J. Bacteriol. 175:2334-2346.

Sory, M.-P., and Cornelis, G. R. 1994. Translocation of a hybrid YopE-adenylate cyclase from *Yersinia enterocolitica* into HeLa cells. Mol. Microbiol. 14:583-594.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185:60-89.

Tabor, S., and Richardson, C. C. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82:1074-1078.

Van Gijsegem, F., Genin, S., and Boucher, C. 1993. Conservation of secretion pathways for pathogenicity determinants of plant and animal bacteria. Trends Microbiol. 1:175-180.

Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. 1995. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. Mol. Microbiol. 15:1095-1114.

Venkatesan, M., Buysse, J. M., and Oaks, E. V. 1992. Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the *spa* locus. J. Bacteriol. 174:1990-2001.

Vogler, A. P., Homma, M., Irikura, V. M., and Macnab, R. M. 1991. *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of FliI to F_0F_1, vacuolar, and archaebacterial ATPase subunits. J. Bacteriol. 173:3564-3572.

Von Heijne, G. 1985. Structure and thermodynamic aspects of the transfer of proteins into and across membranes. Pages 151-179 in: Current Topics in Membranes and Transport. Vol. 24. Membrane Protein Biosynthesis and Turnover. P. A. Knauf and J. S. Cook, eds. Academic Press, Orlando, Canada.

Wattiau, P., and Cornelis, G. R. 1993. SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE. Mol. Microbiol. 8:123-131.

Wei, Z.-M., and Beer, S. V. 1993. HrpI of *Erwinia amylovora* functions in secretion of Harpin and is a member of a new protein family. J. Bacteriol. 175:7958-7967.

Wei, Z.-M., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science 257:85-88.

Woestyn, S., Allaoui, A., Wattiau, P., and Cornelis, G. R. 1994. YscN, the putative energizer of the *Yersinia* Yop secretion machinery. J. Bacteriol. 176:1561-1569.

Young, S. A., White, F. F., Hopkins, C. M., and Leach, J. E. 1994. AVRXa10 protein is in the cytoplasm of *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. 7:799-804.

Xiao, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 1994. Identification of a putative alternate sigma factor and characterization of multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrmA* genes. J. Bacteriol. 176:1025-1036.

Xiao, Y., and Hutcheson, S. W. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. J. Bacteriol. 176:3089-3091.

Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. J. Bacteriol. 174:1734-1741.

Zhuang, W. Y., and Shapiro, L. 1995. Caulobacter FliQ and FliR proteins, required for flagellar biogenesis and cell division, belong to a family of virulence factor export proteins. J. Bacteriol. 177:343-356.