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

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The *hrp/hrmA* gene cluster of *Pseudomonas syringae* pv. *syringae* Pss61 has been shown to form a minimum genetic unit sufficient to enable nonpathogenic bacteria, such as *Escherichia coli*, to elicit the hypersensitive response associated with disease resistance. The biochemical functions of most of these genes have not been established. The nucleotide sequence of a 4.3-kb *Sst*I-*Bgl*II fragment carrying *hrp* apparent translational units V, VI, and VII revealed one partial open reading frame (ORF) and five complete ORFs producing 35,126-, 48,866-, 17,308-, 20,482-, and 26,364-Da gene products (*hrp*J3, J4, J5, U1, U2, respectively). The production of these proteins was confirmed by using T7 RNA polymerase-directed expression. The partial ORF was found to be identical to the C terminus of HrpJ2. The absence of apparent transcriptional terminators and promoters between *hrp*I (*hrp*J2), *hrp*J3, *hrp*J4, and *hrp*J5 together with the observation that the HrpL-dependent *hrp*J promoter directs expression of *hrp*J3–J5 indicates that these genes form a single operon controlled by the HrpL-dependent *hrp*J promoter. A second HrpL-dependent promoter consensus sequence was also identified upstream of *hrp*U1 and demonstrated to function as a HrpL-dependent promoter, thus indicating that *hrp*U1, *hrp*U2, and additional downstream genes may be part of a second operon. The deduced product of *hrp*J3 exhibits similarity to FliG of *Salmonella typhimurium*, a cytoplasmic protein that regulates flagellar rotation and biogenesis. HrpJ4 shares extensive similarity with the FliI family of ATPase-like proteins and retains the known functional domains conserved among this family of proteins. HrpJ5 has properties similar to the *S. typhimurium* FliJ. Neither HrpU1 nor HrpU2 exhibit significant similarity to known proteins. Secretion of $Harpin_{Pss}$ by *E. coli* MC4100 transformants carrying pHIR11::Tn*phoA* derivatives was blocked in *hrp*J4, J5, and U2 mutants. In view of the previously reported similarity of HrpJ2 to the LcrD superfamily that includes FlhA, these results predict that the gene products of the *hrp*J and *hrp*U operons form an inner membrane complex for translocation of proteins similar to that used by the flagellar biogenesis system of *S. typhimurium*.

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When phytopathogenic bacteria invade the tissues of a higher plant that is not their host, a rapid, localized defense response is initiated that prevents further colonization of the tissue (Keen 1992). Plants thus appear to be able to recognize by an unknown mechanism the presence of a potential pathogen and initiate a defense response that contributes to disease resistance. A laboratory manifestation of this defense response is thought to be the hypersensitive response (HR), a rapid tissue collapse and necrosis observed after an inoculum sufficient to cause 50% or more of the plant cells to respond is infiltrated into the tissue.

The ability of bacteria to elicit the HR in non-host plant species appears to be controlled in part by *hrp* genes that have been cloned from phytopathogenic pseudomonads, xanthomonads, and erwiniae (Beer *et al.* 1991; Bonas *et al.* 1991; Boucher *et al.* 1987; Huang *et al.* 1988; Hutcheson *et al.* 1993; Lindgren *et al.* 1986). The *hrp* genes were originally identified by the pleiotropic phenotype of prototrophic mutants that were nonpathogenic in previously susceptible tissue, failed to elicit the HR in other plants, and exhibited a reduced ability to multiply in any plant host (Anderson and Mills 1985; Lindgren *et al.* 1986). Introduction of a cosmid carrying the *hrp/hrm*A gene cluster isolated from *P. syringae* pv. *syringae* Pss61 into *P. fluorescens* or *Escherichia coli* enables these nonpathogenic bacteria to elicit the HR in the leaves of tobacco and other plants (Huang *et al.* 1988; Heu *et al.* 1993). A cloned *Erwinia amylovora* Ea321 *hrp* cluster has recently been shown to exhibit similar properties (Beer *et al.* 1991). These observations indicate that these *hrp* clusters form minimum genetic units for the elicitation of the HR. Regions of colinearity exist between the *E. amylovora* Ea321 and *P. s.* pv. *syringae* Pss61 clusters (Laby and Beer 1992). A secreted 44-kDa protein, called $Harpin_{Ea}$, has recently been shown to be produced by the *E. amylovora hrp*N locus and is proposed to function as an elicitor of the HR (Wei *et al.* 1992). A protein with analogous activity, $Harpin_{Pss}$, is produced by the Pss61 *hrp*Z2 locus (He *et al.* 1993; Xiao and Hutcheson 1994). These proteins appear to be heat-stable,

glycine-rich proteins that lack features typical of Sec-secreted proteins.

Relatively little is known about the function of other Hrp gene products in *P. syringae* strains. The Pss61 HrpH is a member of the *Yersinia* YscC family of proteins (Huang *et al.* 1992). YscC is an outer membrane protein associated with secretion of "Yop" virulence proteins (Michiels *et al.* 1991). The Pss61 HrpI product is a member of the *Yersinia* LcrD super-family of proteins (Huang *et al.* 1993). Proteins in this group have properties of inner membrane proteins and are associated with Sec-independent protein translocation (Galan *et al.* 1992). The HrpS product of *P. syringae* strains is an unusual member of the NtrC family of regulatory proteins (Grimm and Panopoulos 1989; Xiao *et al.* 1994), and HrpL appears to be an alternate sigma factor (Xiao *et al.* 1994; Xiao and Hutcheson 1994). The *hrp/hrm*A gene cluster thus is predicted to encode an apparently dedicated regulatory system, a postulated protein translocation system, and a secreted proteinaceous plant response elicitor (Huang *et al.* 1993; Hutcheson *et al.* 1994). Homologs to HrpH and HrpI have been identified in the *hrp* clusters of *X. campestris* pv. *vesicatoria* (Fenselau *et al.* 1992) and *P. solanacearum* (Gough *et al.* 1992), suggesting *hrp* clusters may have a common function in all phytopathogenic bacteria.

In an attempt to further characterize functional components of the Pss61 *hrp/hrm*A gene cluster, the nucleotide sequence of the 4.3-kb *Bgl*II fragment carrying apparent translational units V, VI, and VII was obtained. Here we report the nucleotide sequence for *hrp*J3, *hrp*J4, *hrp*J5, *hrp*U1, and *hrp*U2, confirmation of the deduced gene products by using a T7 RNA polymerase expression system, and evidence suggesting that the *hrp*J and *hrp*U operons form an inner membrane complex for $Harpin_{Pss}$ secretion similar to the flagellar biosynthesis complex of *S. typhimurium.*

RESULTS

Nucleotide sequence of the 4.3-kb *Bgl*II fragment.

The 4.3-kb *Bgl*II fragment internal to the Pss61 *hrp/hrm*A gene cluster was cloned into pLAFR3 (see Table 1) and shown to be capable of complementing mutations in *hrp* apparent translational units V, VI, and VII (Y. Lu and S. Hutcheson, unpublished results). The nucleotide sequence extending from the left-hand *Sst*I site to the right-hand *Bgl*II site was obtained for both strands. Six open reading frames were identified (Fig. 1). ORFs are predicted to extend from nucleotides 1–194, 210–1202, 1199–2548, 2551–2997, 2998–3576, and 3573–4289 (Fig. 2). The direction of transcription for these ORFs is in agreement with the orientation of *hrp*::Tn*5-gus*A1 insertions previously characterized in this region that are regulated by carbon and nitrogen source (Xiao *et al.* 1992).

The nucleotide sequence of the region encoding ORF1 was identical to that of the 3′ end of *hrp*I (Huang *et al.* 1993). Two possible translation initiation sites were identified for ORF2, each consisting of a potential ribosome binding site (AAGG or GGAGA) and an initiation codon 6–7 bp downstream (see Fig. 2). ORF2 is predicted to encode a polypep-

Table 1. Strains and plasmids used in this study

Bacterium or plasmid	Relevant characteristics	Source or reference
Escherichia coli		
MC4100	F′ *ara*D139 Δ (*arg*F-*lac*ZYA) U169 *rps*L150 *rel*A1 *flb*-5301 *pts*F25 *deo*C1	Casadaban 1976
BL21 (DE3)	B strain; F- *omp*T $r_b^-m_b^-$ *hsdS gal* (λDE3 c*Its*857 *int*l *Sam7 nin5* *lac*UV5-T7 gene 1)	Studier *et al.* 1990
DH5α	*end*A1 *hsd*R17 (r_k^- m_k^-) *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 Δ (*arg*F-*lac*ZYA) U169 φ80d*lac*ZDM15	BRL
Pseudomonas syringae pv. *syringae*		
Pss61	Nx^r, HR^+	Baker *et al.* 1987
Pss61-2081	Pss61 *hrp*J4::Tn*pho*A mutant	Huang *et al.* 1991
Pss61-2082	Pss61 *hrp*J4::Tn*pho*A mutant	Huang *et al.* 1991
Plasmids		
pLAFR3	IncP-1, Tc^r, *lac*Z′	Staskawicz *et al.* 1987
pHIR11	31-kb *P. syringae* pv. *syringae* Pss61 fragment containing the *hrp/hrm*A cluster cloned into pLAFR3	Huang *et al.* 1988
pHIR11-2074	pHIR11 derivative carrying *hrp*L::Tn*pho*A mutation	Huang *et al.* 1991
pHIR11-2081	pHIR11 derivative carrying *hrp*J4::Tn*pho*A mutation	Huang *et al.* 1991
pHIR11-2082	pHIR11 derivative carrying *hrp*J4::Tn*pho*A mutation	Huang *et al.* 1991
pHIR11-2083	pHIR11 derivative carrying *hrp*J5::Tn*pho*A mutation	Huang *et al.* 1991
pHIR11-2084	pHIR11 derivative carrying *hrp*U2::Tn*pho*A mutation	Huang *et al.* 1991
pHIR11-2092	pHIR11 derivative carrying *hrp*Z2::Tn*pho*A mutation	Huang *et al.* 1991
pHIR11-2096	pHIR11::Tn*pho*A derivative carrying wild-type *hrp*	Huang *et al.* 1991
pHIR11-5114	pHIR11 derivative carrying *hrp*J3::Tn*5-gus*A1 mutation	Xiao *et al.* 1992
pBluescriptII SK^+	ColE1, Ap^r mcs-*lac*Z	Stratagene
pMLBglII	4.3-kb *Bgl*II fragment derived from pHIR11 cloned into pBluescriptII SK^+	This report
pMLJ4-1L	1.5-kb *Hin*cII fragment carrying *hrp*J4 ligated into pLAFR3	This report
pVEX11	pET3a derivative, *bla*	Heu and Hutcheson 1993
pMLEX1	4.3-kb *Bgl*II fragment cloned in the forward orientation into pVEX11	This report
pMLEX2	4.3-kb *Bgl*II fragment cloned in the reverse orientation into pVEX11	This report
pRG970	IncP, Sp^r promoter-less *lac*Z and gusA in opposite orientation	Van den Eede *et al.* 1992
pMLPU-1R	0.79-kb *Hin*cII fragment ligated into pRG970 to create a transcriptional fusion between the *hrp*U promoter and *lac*Z	This report
pYXL2B	1-kb *Ssp*I-*Hin*cII fragment carrying the *hrp*L ORF cloned into pBluescript II SK^+ behind the *lac* promoter	Xiao *et al.* 1994

tide of 329 or 323 residues with a predicted molecular mass of 35,841 or 35,126, respectively, and pI of 5.1. ORF3 appears to begin at a GUG codon that overlaps the last translatable codon of *hrp*J3. A potential ribosome binding site (GAGG) is located 5 nt upstream of the apparent initiation codon. The ORF3 gene product is 449 aa long and has a predicted molecular mass of 48,866 and pI of 8.9. The ORF4 gene product is predicted to contain 148 aa and have a molecular mass of 17,308 with a pI of 6.7. A putative ribosome binding site (GGAG) is apparent 5 nt upstream of the deduced initiation codon. ORF5 appears to produce a relatively proline-rich protein of 190 aa with predicted molecular mass of 20,482 and pI of 6.5. A potential ribosome binding site (AAGAAGG) is located 5 nt upstream of the apparent initiation codon (GUG). The apparent initiation codon of ORF6 overlaps the last translatable codon of ORF5 and is preceded by a potential ribosome binding site (GAAGA). The ORF6 product is predicted to be 241 aa long with a molecular mass of 26,364 and pI of 6.8. The deduced ORF6 gene product contains two possible transmembrane domains (residues 52–83 and 97–120) containing amino acid residues with predominantly hydrophobic or neutral side chains (see Heijne 1987). ORF1–6 were henceforth designated *hrp*J2, *hrp*J3, *hrp*J4, *hrp*J5, *hrp*U1, and *hrp*U2, respectively. A potential translational initiation site overlapping the terminal nine codons of ORF6 suggests the presence of at least one additional ORF downstream (see below).

T7-RNA polymerase-directed expression of *hrp*J and *hrp*U operons.

To confirm the production of the deduced gene products, the 4.3-kb *Bgl*II fragment was cloned into pVEX11 to create pMLEX1 (forward orientation) and pMLEX2 (reverse orientation) and transformed into *E. coli* BL21(DE3). Newly synthesized proteins in lysates of BL21(DE3) transformants carrying pVEX11, pMLEX1, or pMLEX2 were visualized by autoradiography following polyacrylamide gel electrophoresis (Fig. 3). BL21(DE3)(pMLEX1) expressed unique proteins of 17, 20, 25, 35, and 44 ± 3 kDa. Although products of individual genes were not established, these observations agree well with the molecular masses predicted for HrpJ5, HrpU1, HrpU2, HrpJ3, and HrpJ4, respectively, and were not observed in lysates prepared from bacteria carrying the pVEX11 vector alone or pMLEX2. Because of the production of additional unexplained proteins in the strain carrying pMLEX1, production of HrpJ5, HrpU1, and HrpU2 was verified by cloning into pVEX11 a 2.9-kb *Not*I–*Bgl*II fragment containing a truncated *hrp*J4 locus sufficient to encode a 35-kDa protein and the complete *hrp*J5, *hrp*U1, and *hrp*U2 loci. Proteins of 18, 21, 25, and 35 kDa were detected after SDS polyacrylamide gel electrophoresis.

Relationship between *hrp*J4 and previously identified apparent translational units.

To determine the relationship between the apparent translational units defined previously (Huang *et al.* 1991) and *hrp*J4, a 1.5-kb *Hinc*II fragment carrying *hrp*J4 was cloned into pLAFR3 to create pMLJ4-1L. Transformation of pMLJ4-1L into Pss61-2081 or Pss61-2082, representing apparent translational unit V, restored their ability to elicit the HR in tobacco leaves but failed to complement Pss61 mutants carrying insertions in apparent translational units VI and VII (data not shown). By using this complementation analysis as a reference, apparent translational unit VI corresponds to *hrp*J5 and apparent translation unit VII is equivalent to *hrp*U2. Apparent translational units III and IV have been previously established to correspond to *hrp*J1 (*hrp*J) and *hrp*J2 (*hrp*I) (Huang *et al.* 1993). Several Tn*5-gus*A1 insertions have been identified (e.g., 5101, 5114) that map to *hrp*J3 (Xiao *et al.* 1992).

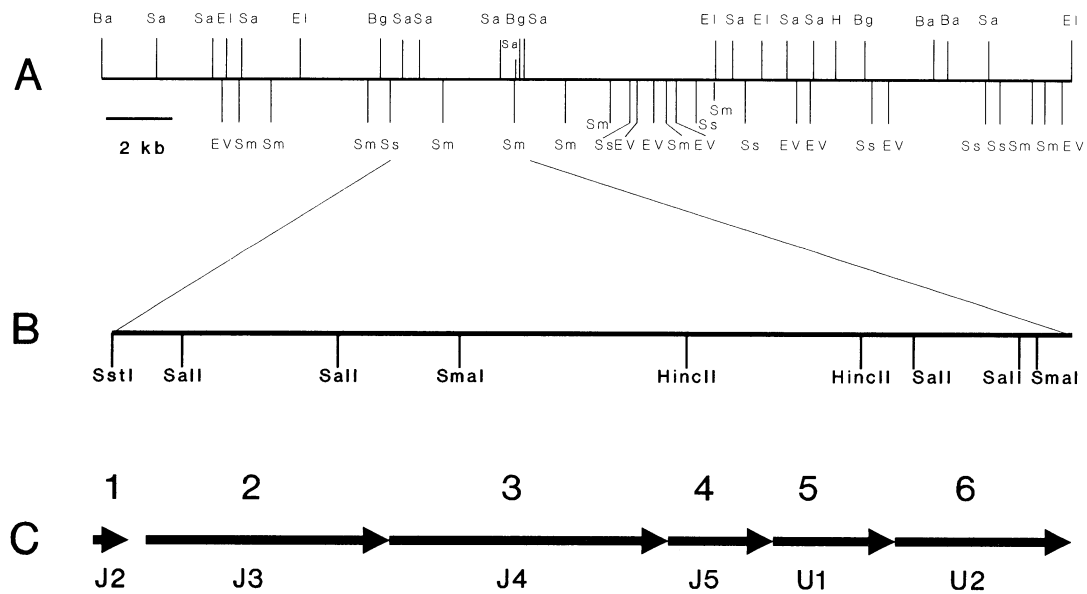

Fig. 1. Physical map of the 4.3-kb *Bgl*II fragment internal to the *Pseudomonas syringae* pv. *syringae* 61 *hrp/hrm*A gene cluster carrying apparent translational units V, VI, and VII. **A,** Restriction map of the *P. s.* pv. *syringae hrp/hrm*A gene cluster (taken from Xiao *et al.* 1992). **B,** *Sst*I-*Bgl*II fragment for which the nucleotide sequence for both strands was obtained. Restriction sites used to generate subclones are shown. **C,** Location and genetic designation for the open reading frames identified within the fragment.

Transcriptional organization of the region.

Xiao *et al.* (1992) had previously detected two apparent transcriptional units in this region based on complementation analyses in merodiploids carrying pHIR11::Tn*5gus*A1 derivatives (apparent polar mutagen) and chromosomal *hrp*::Tn*pho*A insertions (nonpolar mutations). One apparent operon consisted of *hrp*J1-J2 and the other, *hrp*J3-U2. A survey of the nucleotide sequence of the region upstream of *hrp*J3; however, failed to detect intervening transcriptional terminators or σ^{54}, σ^{70}, or HrpL-dependent promoter consensus sequences. The intergenic regions between *hrp*J2, J3, J4, and J5 were less than 15 bp. The 372-bp *Sst*I-*Sal*I fragment carrying the 200-bp region upstream of *hrp*J3 lacked promoter activity in Pss61 when cloned into pRG970 (data not shown). Attempts to detect the predicted *hrp*J transcript by Northern analysis or identify the transcriptional initiation point by primer extension were unsuccessful.

Previous experiments had identified a HrpL-dependent promoter located 29 nt upstream of *hrp*J1 (Xiao *et al.* 1994; Xiao and Hutcheson 1994). In an attempt to determine whether the *hrp*J promoter controls the expression of *hrp*J3, a 3.5-kb *Dra*I–*Sal*I fragment that includes the region extending

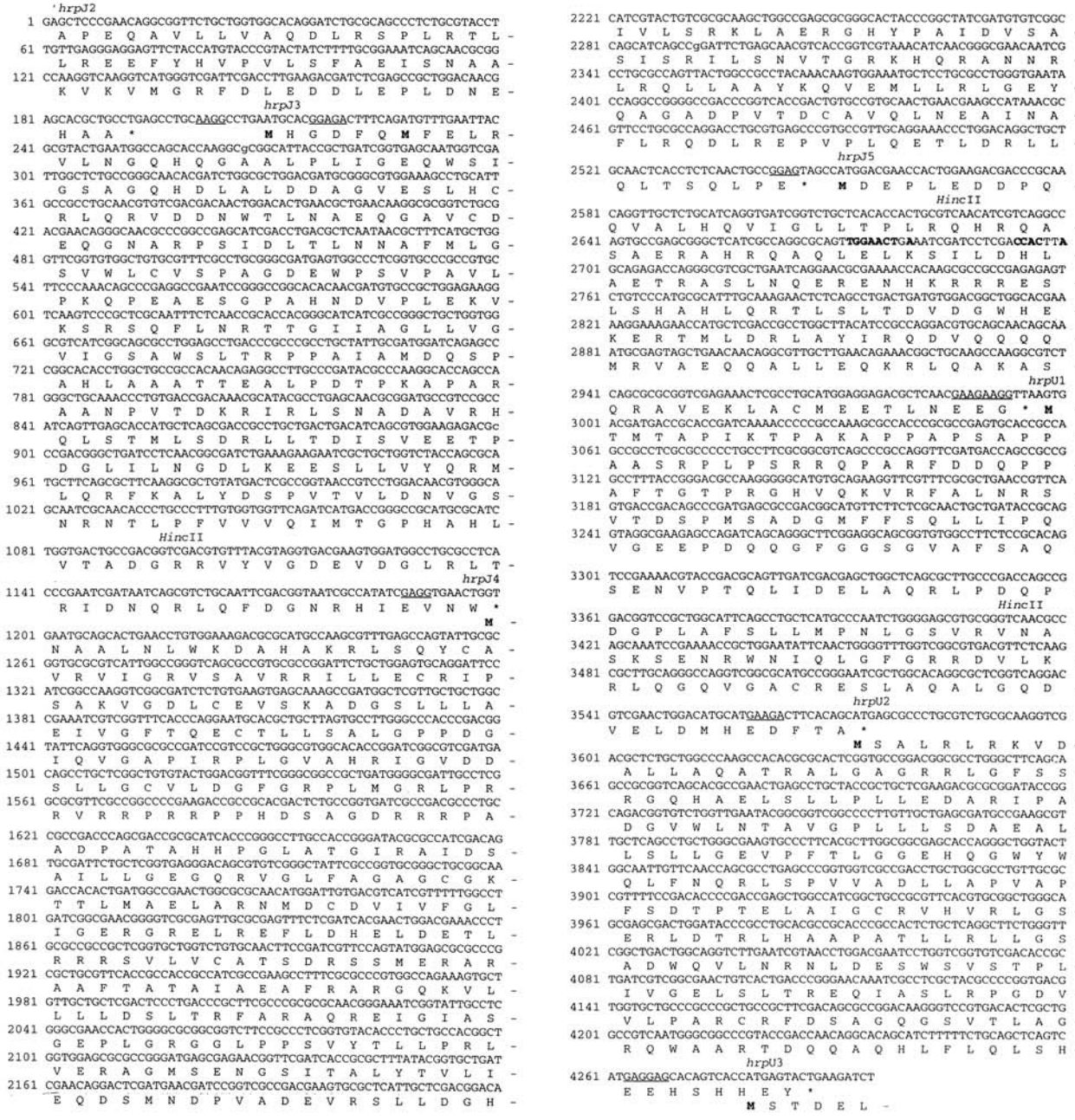

Fig. 2. Nucleotide sequence of the 4.3-kb *Bgl*II fragment internal to the *P. s.* pv. *syringae* 61 *hrp*/*hrm*A gene cluster carrying apparent complementation groups V, VI and VII. Sequencing strategy is described in the text. Synthetic oligonucleotide primers were used to bridge the sequence between adjacent restriction fragments. Deduced amino acid sequence of ORFs shown below. A HrpL-dependent promoter consensus sequence is shown in bold. Potential ribosome binding sites are underlined and termination codons marked with an asterisk.

from 0.15 kb upstream of *hrp*J1 to 0.15 kb internal to *hrp*J3 was cloned into pRG970 to create a transcriptional fusion with *lac*Z. The resulting fragment was promoter-active in *E. coli* MC4100 (pHIR11-2096) but not in MC4100 (pHIR11-2074) transformants carrying a *hrp*L::Tn*pho*A insertion (Fig. 4). In contrast, the 3.0-kb *Eco*R1–*Sal*I fragment lacking the *hrp*J promoter failed to exhibit promoter activity in either MC4100 derivative when tested in a similar manner. These observations indicate that *hrp*J3 expression is directed by *hrp*J promoter as predicted by the sequence data. Since the previous analysis of the region had predicted that *hrp*J3–J5 are transcriptionally linked, these data support the hypothesis deduced from the sequence data that *hrp*J1–J5 form a single operon.

A HrpL-dependent promoter consensus sequence (Xiao and Hutcheson 1994) was also identified upstream of *hrp*U1 (Fig. 2). To determine if the region upstream of *hrp*U1 is promoter active, a 0.79-kb *Hinc*II fragment that includes 46 bp upstream of the HrpL promoter consensus sequence was cloned into pRG970 to construct pMLPU-1R. After transformation into MC4100 (pHIR11-2096), this fragment was promoter active when cloned in the forward orientation as described above (Fig. 4). This suggests that *hrp*U1 and *hrp*U2 may be part of a separate operon, but because of the short apparent intergenic region between *hrp*J5 and *hrp*U2 this observation does not exclude the possibility that the *hrp*J promoter may also direct expression of the apparent *hrp*U operon.

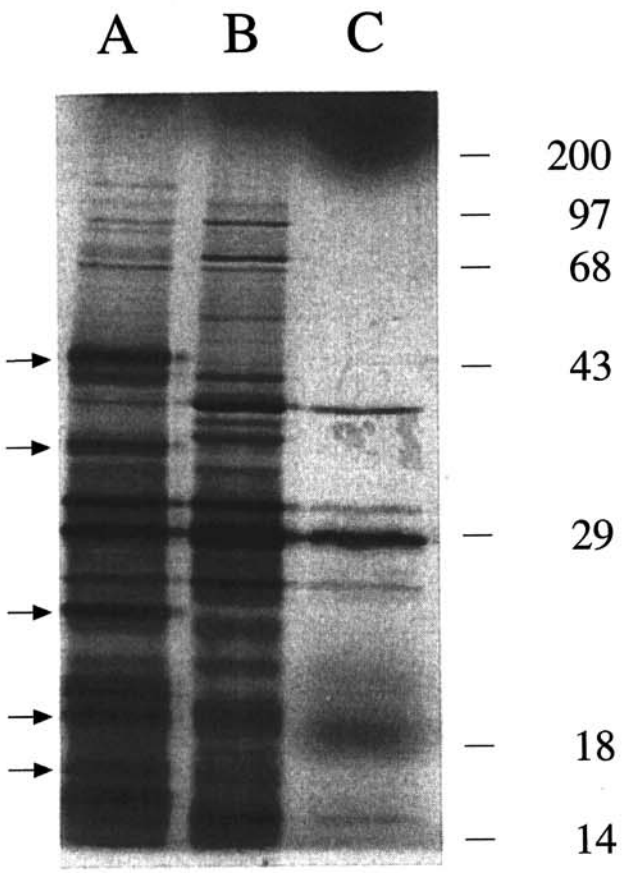

Fig. 3. T7 RNA polymerase-directed expression of the *hrp*J and *hrp*U operons. Proteins of *E. coli* BL21 (DE3) carrying pMLEX1 (A), pMLEX 2 (B), or the vector pVEX11 (C) were labeled with [^{35}S]methionine after induction of T7 RNA polymerase production as described in the text. Whole cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis and an autoradiograph obtained. Positions of molecular weight standards are indicated on the right.

Similarities of HrpJ products to proteins associated with flagellar biosynthesis.

A survey of the databases revealed numerous similarities between HrpJ products and proteins associated with flagellar biogenesis. In addition to the similarities reported previously with other members of the LcrD family of proteins, HrpJ2 also exhibits similarity with the *E. coli* FlhA (% Similarity (S)/Identity (I) = 61/37) which functions in flagellar biosynthesis (MacNab 1992). HrpJ4 was found to exhibit substantial similarity to HrpB6 from *Xanthomonas campestris* (%S/I = 66/47)(Fenselau *et al.* 1992), FliI of *S. typhimurium* (%S/I = 62/42)(Vogler *et al.* 1991), FliI of *Bacillus subtilis* (%S/I = 61/43)(Albertini *et al.* 1991), Spa47 of *Shigella flexneri* (%S/I = 58/39)(Venkatesan *et al.* 1992), and the β subunit of the F_0F_1 ATP synthase from *E. coli* (%S/I = 55/33)(Saraste *et al.* 1981). All five proteins are of similar length and the similarity extends throughout the sequence (Fig. 5). Strongest similarity is retained in the mononucleotide binding (Walker *et al.* 1982) and the Mg^{2+} binding domains (Yoshida *et al.* 1982), and in the ATPase signature sequence, a putative active site domain (Futai *et al.* 1989). Conservation of these domains among the five proteins indicates these proteins share similar functions, probably as cytoplasmic ATPases.

Weaker similarities were detected between the deduced HrpJ3 product and the FliG proteins of *S. typhimurium* (%S/I = 45/20)(Kihara *et al.* 1989) and *B. subtilis* (%S/I = 46/20) (Albertini *et al.* 1991)(Fig. 6) and between the HrpJ5 product and FliJ of both *S. typhimurium* and *B. subtilis* (%S/I = 52/29)(Fig. 7)(Vogler *et al.* 1991). Both proteins are associated with flagellar biogenesis (MacNab 1992). Although the similarity is not as strong as that described above, the HrpJ3 and FliG products and the HrpJ5 and FliJ products

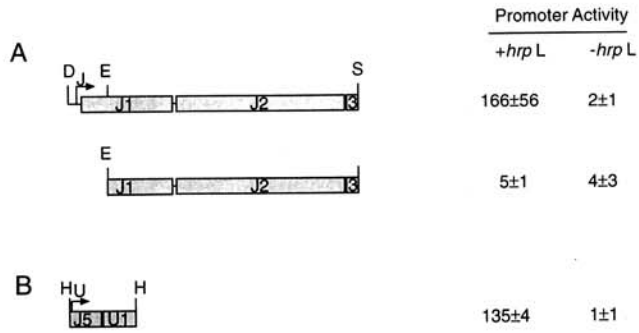

Fig. 4. Activity of the *hrp*J and *hrp*U promoters in *E. coli* MC4100 transformants carrying the *Pseudomonas syringae* Pss61 *hrp*/*hrm*A gene cluster. **A,** Promoter activity of *hrp*J fragments. The indicated 3.5-kb *Dra*I–*Sal*I or 3.0-kb *Eco*R1–*Sal*I were cloned into pRG970 to create transcriptional fusions with *lac*Z and transformed into MC4100 (pHIR11-2096) [+*hrp*L] and MC4100 (pHIR11-2074) [–*hrp*L]. β-Galactosidase activity indicative of promoter activity was determined in Miller units after induction in M63 medium for 6 hr as described in the Materials and Methods. In parallel experiments, both MC4100 derivatives carrying pRG970 exhibited 2±1 units of activity. Restriction sites: D, *Dra*I; E, *Eco*R1; S, *Sal*I. Bent arrow, *hrp*J promoter. Shaded boxes, ORFs identified from the sequence data (Huang *et al.* 1993; this report). **B,** Activity of the *hrp*U promoter. The apparent *hrp*U promoter was cloned into pRG970 as *Hinc*II fragment and its activity measured in MC4100 transformants as described above. H, HincII.

are of the same approximate size and the similarity extends over the length of the proteins. Of those residues that are conserved between the *S. typhimurium* and *B. subtilis* FliG and FliJ products, more than 50% are conserved in the corresponding *hrp*J gene product. These similarities indicate that homologs to three of the six gene products of the *S. typhimurium fli*FGHIJK operon are retained in the *P. syringae* *hrp*J operon and their arrangement within their respective operons appears similar.

Neither HrpU1 nor HrpU2 exhibited any significant similarity to protein sequences stored in the current databases that could give an indication of function.

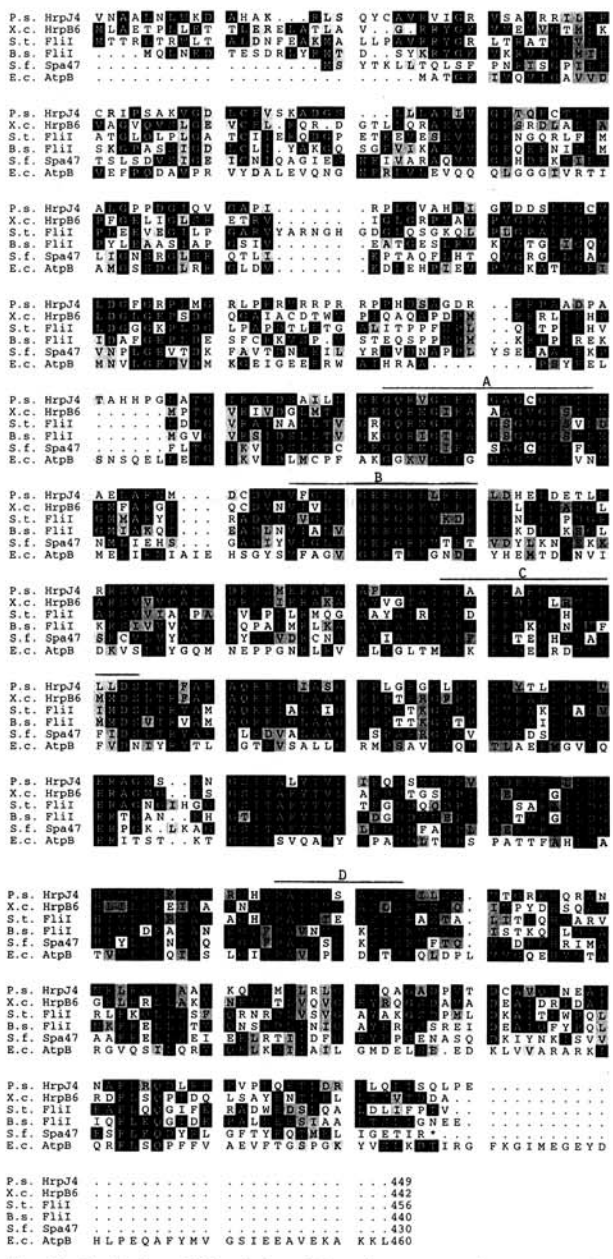

Fig. 5. Similarity of the deduced *Pseudomonas syringae* pv. *syringae* Pss61 HrpJ4 product with a super-family of protein subunits associated with ATPases. Sequences were aligned by using the algorithm PILEUP (Devereux *et al.* 1984). Identical residues are enclosed in black boxes. Conservative substitutions are indicated by the shaded boxes. Conserved domains are overlined: A and C, mononucleotide binding motifs (Walker *et al.* 1982); B, Mg^{++} binding motif (Yoshida *et al.* 1982); and D, ATPase signature sequence (Futai *et al.* 1989). Abbreviations: P.s., *P. syringae*; X.c., *X. campestris*; S.t. *S. typhimurium*; B.s., *B. subtilis;* S.f., *Shigella flexneri*; E.c., *E. coli.*

Ability of *P. syringae hrp* mutants to regenerate flagella.

The aforementioned similarities of HrpJ2 with FlhA, HrpJ3 with FliG, HrpJ4 with FliI, and HrpJ5 with FliJ could suggest that the *hrp* genes function in flagellar biogenesis. The phenotype of several *S. typhimurium* FliI mutations is most evident during flagellar regeneration (Vogler *et al.* 1991). Mutants lack the ability to regenerate flagella after treatment to remove the flagella. To determine if *P. syringae hrp*J4 mutants are capable of regenerating flagella, Pss61 and Pss61-2082 were grown in minimal salts medium for 6 hr and flagella removed by serial passage through a syringe needle. Greater than 45% of the bacteria in untreated preparations were motile, while none of the treated population was motile immediately after shearing. The wild-type strain exhibited increased motility beginning 10 min after treatment and ~45% of the population was motile after 40 min. Motility of *P. s.* pv. *syringae* Pss61-2082 recovered at the same rate as the wild-type strain (data not shown).

Fig. 6. Similarity of the deduced *Pseudomonas syringae* pv. *syringae* Pss61 HrpJ3 product with the FliG products of *S. typhimurium* and *B. subtilis*. Sequences were aligned as described in Figure 4.

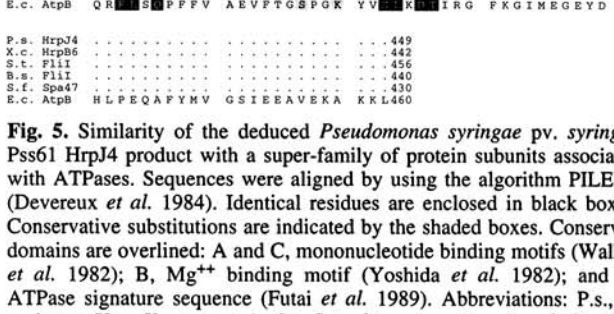

Fig. 7. Similarity of the deduced *Pseudomonas syringae* pv. *syringae* Pss61 HrpJ5 product with FliJ of enteric bacteria and *Bacillus* strains. See Figure 4 for conditions and abbreviations.

Role of HrpJ4, HrpJ5, and HrpU2 in the secretion of Harpin$_{Pss}$.

The similarities reported here coupled with those reported previously (Huang *et al.* 1992; Huang *et al.* 1993) suggest that the products of the *P. syringae hrp* cluster function in Harpin$_{Pss}$ secretion (He *et al.* 1993). To determine if HrpJ4, HrpJ5, and HrpU2 also function in Harpin$_{Pss}$ secretion, MC4100 (pYXL2B) transformants carrying pHIR11: :Tn*pho*A derivatives with *hrp*J4, *hrp*J5, *hrp*U2, or *hrp*Z2 mutations were screened for their ability to secrete HrpZ. Tn*pho*A insertions have previously been established to be nonpolar mutations (Huang *et al.* 1991, 1992, 1993, Xiao *et al.* 1992). The plasmid pYXL2B was found to be necessary to enhance production of harpin$_{Pss}$ for these experiments. The MC4100(pYXL2B)(pHIR11-2096) cells carrying the complete *hrp/hrm*A cluster were able to elicit the HR in tobacco leaves without lytic treatment (Table 2). In contrast, transformants containing pHIR11 derivatives carrying *hrp*J4, *hrp*J5, or *hrp*U2 mutations only produced the HR when lysed *in planta* by the procedures of He *et al.* 1993 (He *et al.* 1993). Approximately 80% of the lysates screened induced a response. In comparison, less than 33% of the MC4100 (pYXL2B) or MC4100(pYXL2B)(pHIR11-2092) lysates tested elicited a necrotic response similar to the HR. Basis for this residual activity of *E. coli* strains has not been established.

DISCUSSION

DNA sequence analyses of *hrp* genes in several plant pathogenic bacteria have recently revealed significant similarity between the predicted products of several *hrp* loci and determinants controlling protein translocation in enteric bacteria (Fenselau *et al.* 1992; Gough *et al.* 1992; Huang *et al.* 1992; Huang *et al.* 1993; Wei and Beer 1994). The previous analyses of the *P. s.* pv. *syringae* Pss61 *hrp/hrm*A gene cluster have indicated that HrpH is an outer membrane protein similar to the *Yersinia* YscC product necessary for Yop protein secretion (Huang *et al.* 1992) and that HrpJ2 is a member of the LcrD super-family of inner membrane proteins associated with protein translocation and virulence (Huang *et al.* 1993). The sequence analysis of the 4.3-kb *Sst*I-*Bgl*II fragment carrying complementation groups V, VI, and VII identified five additional genes, three of which have properties that suggest they could also be associated with protein translocation. These results, then, provide further evidence that the *P. s.* pv. *syringae* 61 *hrp/hrm*A gene cluster forms a protein secretion system for Harpin$_{Pss}$ (see He *et al.* 1993; Huang *et al.* 1993; Hutcheson *et al.* 1994).

Several of the newly identified Pss61 gene products were found to be similar to proteins associated with flagellar biogenesis in *S. typhimurium* and *B. subtilis*. HrpJ2, HrpJ3, HrpJ4, and HrpJ5 are similar to FlhA, FliG, FliI, and FliJ, respectively. The FlhA and FliI similarities are significant because key features are retained in the corresponding Hrp gene product. As described previously (Huang *et al.* 1993), HrpJ2 exhibits the typical features of the LcrD super-family of inner membrane proteins that includes FlhA. HrpJ4 retains the catalytic domains described for the β subunit of F_1/F_0 ATPases (see Futai *et al.* 1989; Walker *et al.* 1982; Yoshida *et al.* 1982), but is most similar to members of the protein family associated with protein translocation, such as FliI. An ATPase is likely to be necessary to provide the energy necessary for protein translocation. Directed mutagenesis of conserved residues in *S. typhimurium* FliI known to be catalytically important for ATP hydrolysis produced nonflagellated cells, indicating that protein translocation was impaired (Dreyfus *et al.* 1993; MacNab 1992). Similarly, a *hrp*J4::Tn*pho*A mutation impairs Harpin$_{Pss}$ secretion.

Although the similarity of HrpJ3 and HrpJ5 to the FliG and FliJ products of *S. typhimurium* and *B. subtilis* appears weaker, at least 50% of the apparently conserved amino acid residues in the FliG and FliJ products are retained in the HrpJ3 and HrpJ5 products and some parallels are observed in the transcriptional organization of the respective operons. FliG is part of the *fli*FGHIJK operon that is expressed early in flagellar biogenesis and is predicted to form the assembly platform and machinery necessary for flagellar assembly (MacNab 1992). FliG has been localized to the cytoplasmic face of the cell membrane where it interacts with FliF and forms the M ring of the flagellar basal body (MacNab 1992). FliG, along with FliM and FliN, is also postulated to form a switch determining the clockwise or counterclockwise rotation of the flagellar motor and mediates the chemotactic process of bacteria by interacting with CheY (Francis *et al.* 1992; Irikura *et al.* 1993; MacNab 1992). It is unlikely that HrpJ3 functions in the flagellar switch assembly of *P. syringae*. Amino acid residues which genetic analyses suggest function in the switch activity of FliG do not appear to be conserved in the HrpJ3 product (see Irikura *et al.* 1993). The function of HrpJ3 therefore remains to be established. HrpJ5 exhibits

Table 2. Response of tobacco leaves to *E coli* MC4100 cells carrying derivatives of the *Pseudomonas syringae* pv. *syringae* Pss61 *hrp/hrm*A gene cluster

		Plant response[c]	
Plasmid[a]	Mutation[b]	Intact[d]	Lysed[e]
pHIR11-2096	WT	+[f]	+[g]
pHIR11-2081	J4	−	+
pHIR11-2083	J5	−	+
pHIR11-2086	U2	−	+
pHIR11-2092	Z2	−	−
None		−	−

[a] *E. coli* MC4100 (pYXL2B) transformant carrying the indicated pHIR11 derivative.

[b] The *hrp*::Tn*pho*A mutation carried by the indicated plasmid. WT = wild type.

[c] Response of *Nicotiana tabacum* cv. Samsun leaves 48 hr after infiltration with the indicated bacterial strain. At least three leaves were infiltrated during each experiment, and the experiment was repeated four times.

[d] Cells were grown for 6 hr in M63 media supplemented with 1 mM $MgSO_4$, 10 mM mannitol, and appropriate antibiotics, harvested, and resuspended in H_2O to an OD_{600} of 0.3–0.5. The bacterial suspension was infiltrated directly without further treatment.

[e] *In situ* lysis as described by He *et al.* 1993. Cells, grown as described above, were harvested and resuspended in 50 mM glucose, 25 mM Tris-HCl (pH 8.0), and 20 mM EDTA to an OD_{600} of 0.3–0.5. After incubating 10 min at room temperature, cells were harvested by centrifugation and resuspended in 1/2 volume of 10 mM Tris-HCl (pH 8.0) containing 2 mg/ml lysozyme. Cell suspensions were immediately infiltrated into leaves.

[f] + = All suspensions tested elicited a necrotic reaction typical of the hypersensitive response; − = none of the suspensions elicited a visible plant response.

[g] + = Greater than 66% of the preparations tested produced a necrotic reaction similar to the HR; − = less than 33% of preparations elicited a necrotic response.

similarity to FliJ, but unfortunately little is known of its role in flagellar assembly or operation (MacNab 1992). A mutation in the *B. subtilis* homolog to *fliJ* affects chemotactic responses (Ying *et al.* 1991), suggesting these proteins could function in signal transduction.

The similarities observed could be suggestive of a role of *hrp* genes in flagellar biosynthesis. The *hrp* genes, however, are not required for export of flagellar components. A *P. syringae hrp*J4 mutant retained the ability to regenerate flagella (as indicated by the recovery of motility shown here), whereas *S. typhimurium* FliI mutants cannot (Vogler *et al.* 1991). Instead, we predict that *hrp*J, and possibly *hrp*U, encode an inner membrane complex for the Sec-independent secretion of $Harpin_{Pss}$. Two of the deduced gene products of the operon have properties of inner membrane proteins: HrpJ2 and HrpU2. Consistent with the hypothesis that the *P. syringae hrp* cluster functions in protein secretion is the observation that *E. coli* MC4100 carrying Pss61 *hrp* cluster is capable of eliciting the HR when physiologically intact, whereas *hrp*J4, *hrp*J5, and *hrp*U2 mutants require treatments to permeabilize or lyse the cells before they elicit the HR. Since the ability to elicit the HR has been linked to extracellular $Harpin_{Pss}$ (He *et al.* 1993), these observations indicate that $Harpin_{Pss}$ secretion is blocked in *hrp*J4, *hrp*J5, and *hrp*U2 mutants. Compartmentation of the $Harpin_{Pss}$ in the various *hrp* mutants awaits further study. The observation that transformation of MC4100 with pHIR11 confers the ability to secrete the HrpZ product indicates that the *hrp/hrm*A gene cluster encodes the major components of a separate protein secretion pathway. Further analysis will be necessary to establish whether any *E. coli* components function in this pathway.

The sequence data together with the promoter analysis suggests that the genes in this region are organized into two apparent operons, *hrp*J and *hrp*U. No obvious transcriptional terminators or obvious promoters are apparent in the short intergenic regions between *hrp*J1, J2, J3, J4, J5, U1, and U2. The nature of *P. syringae* transcriptional termination signals, however, has not been established. The presence of a potential translational initiation site at the 3′ end of *hrp*U2 suggests that the *hrp*U operon contains additional genes. The sequence of the adjacent region appears to confirm this deduction (H. C. Huang, unpublished results; Xiao and Hutcheson 1994). Interestingly, *lux-npt*I insertions into the *Bgl*II site at the fifth codon of the apparent *hrp*U3 ORF retain a Hrp^+ phenotype (Xiao *et al.* 1992). Either this putative locus is tolerant of insertions at this site or the predicted gene product is not essential for Harpin secretion.

Recent genetic analyses have characterized a multicomponent regulatory cascade controlling *hrp* expression (Xiao *et al.* 1994). A key component of this regulatory cascade is HrpL, a putative alternate sigma factor, and a HrpL-dependent promoter consensus sequence has been identified (Xiao and Hutcheson 1994). A HrpL-dependent promoter consensus sequence is found upstream of *hrp*J1 and *hrp*U1 (Xiao and Hutcheson 1994). Both regions exhibit promoter activity in *E. coli* MC4100 derivatives expressing *hrp*L. Attempts to confirm the transcriptional organization of the cluster by characterization of the RNA transcripts produced by this region proved unsuccessful. Instead a *hrp*J3′-*lac*Z fusion was constructed that included the *hrp*J promoter and its expression shown to be dependent upon the *hrp*J promoter. These data coupled with previous analyses (Xiao *et al.* 1992) support the argument that *hrp*J1–J5 are transcriptionally linked and that *hrp*U1-U2 represent a second potential operon.

The *hrp* clusters of *P. solanacearum*, *X. campestris,* and *P. syringae* share at least three genes in common. HrpA from *P. solanacearum* (Gough *et al.* 1992), and HrpA1 from *X. c.* pv. *vesicatoria* (Fenselau *et al.* 1992) are similar to the *P. s.* pv. *syringae* Pss61 HrpH product. The *P. solanacearum* HrpO (Gough *et al.* 1992), and *X. c.* pv. *vesicatoria* HrpC2 (Fenselau *et al.* 1992) products have been shown to be similar to HrpJ2. The *P. solanacearum* HrpE (C. Boucher, personal communication) and the *X. campestris* HrpB6 gene products share strong similarity to the *P. syringae* HrpJ4 product. Since regions homologous to probes carrying *P. s.* pv. *syringae* 61 *hrp*J, *hrp*U, and *hrp*H have been identified in the *E. amylovora hrp* cluster (Laby and Beer 1992), it is also likely that this cluster carries homologs to HrpJ2 (LcrD family), HrpH (YscC family), and HrpJ4 (FliI family) as well. A HrpJ2 homolog has recently been identified in the *E. amylovora hrp* cluster (Wei and Beer 1994). Both *P. solanacearum hrp*I and *X. campestris hrp*B3 loci produce proteins homologous to the *Yersinia* YscJ protein (Fenselau *et al.* 1992; Gough *et al.* 1992). This predicts the *P. syringae hrp* cluster may also carry a homolog to YscJ. Although qualitative similarities between *hrp* clusters in the various plant pathogenic bacteria have been noted for the aforementioned genes, the *hrp* clusters in *P. syringae*, *X. campestris*, and *P. solanacearum* are not identical. The *P. solanacearum* and the *X. campestris hrp* clusters do not carry homologs to HrpJ1, HrpR, HrpS, HrpL, or HrmA (C. Boucher, personal communication; U. Bonas, personal communication). The transcriptional organization of the *hrp* clusters in these bacteria appears to be different.

The proteins similar to the *P. syringae* HrpJ products which have been identified thus far in other bacteria function in one of two cellular processes: pathogenicity or flagellar assembly. The possible involvement of these genes in diverse bacterial functions like pathogenicity and flagellar operation may indicate that they may be part of a general protein export pathway adapted by different bacteria for specific functions. Although the products that appear to be secreted by these genes in other bacteria are highly heterogeneous, they share a common feature in that they lack the N-terminal signal sequence typical of Sec-dependent protein export systems (Pugsley *et al.* 1990). The N-terminal sequence of $Harpin_{Pss}$ also lacks evidence of a Sec-dependent signal sequence (He *et al.* 1993). The export of these flagellar and pathogenicity proteins thus appears to represent a third distinct pathway for protein export pathway in bacteria as proposed by Salmond and Reeves 1993.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. Plasmids were propagated in *E. coli* DH5α. *E. coli* strains were grown in LB medium at 37 C (Hanahan 1983). M9 minimal medium containing glucose as carbon source (unless indicated otherwise) was used to culture *E. coli* BL21(DE3) for the T7 RNA polymerase-directed expression

of *hrp* genes (Heu and Hutcheson 1993). Media were supplemented with ampicillin at a concentration of 100 mg/ml.

Nucleotide sequence analysis.

The nucleotide sequence of the 4.3-kb *Sst*I-*Bgl*II fragment was obtained essentially as described by Huang *et al.* (1993). The DNA sequencing strategy employed pMLBglII which carries the 4.3-kb *Bgl*II fragment cloned into pBluescript II SK+ and *Sst*I, *Sma*I, and/or *Hin*cII fragments thereof subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA.). Synthetic oligonucleotide primers were used to bridge gaps in the sequence data. Sequencing reactions were carried out on double-stranded templates employing Sequenase Version 2.0 (U.S. Biochemical, Cleveland, OH) according to the manufacturer's instructions. Synthetic oligonucleotide primers were synthesized by the Protein/Nucleic Acid Laboratory, University of Maryland, College Park, MD. Both dGTP and dITP reaction mixtures were examined. Data were obtained for both strands and analyzed with the Wisconsin Genetics Computer Group sequence analysis software Version 7.2 programs (Devereux *et al.* 1984). The complete nucleotide sequence has been submitted to GenBank under accession number U07346.

T7 RNA polymerase driven expression of ORF1–6 in *E. coli* Bl21(DE3).

The plasmid vector, pVEX11 (a gift of Adhya Sankar of NCI, NIH, Bethesda, MD) is a derivative of pET-3a (Studier *et al.* 1990) modified to carry a multicloning site, Shine-Delgarno sequence, and a translational start codon. The pVEX11 expression vector was digested by *Xba*I and *Sma*I to remove its Shine-Dalgarno sequence and ATG start codon, treated with mung bean nuclease, and then religated prior to use.

The 4.3-kb *Bgl*II fragment was excised from pMLBglII and subcloned into the modified pVEX11 to form pMLEX1 in which all native translational start sequences and putative open reading frames are oriented downstream of the vector's T7 promoter. The fragment subcloned in the reverse orientation was designated pMLEX2. The pVEX11, pMLEX1, and pMLEX2 plasmids were then transformed into *E. coli* BL21(DE3) and the protein product determined as described by Huang *et al.* (1993).

Flagellar regeneration.

Regeneration of flagella was monitored as described by (Vogler *et al.* 1991). Flagella were sheared off cells by 50 serial passages through a #26 syringe needle. Motility was monitored by optical microscopy.

β-Galactosidase activity.

β-Galactosidase activity was measured in crude lysates as described by Xiao *et al.* (1994). Data was recorded in Miller units and is the mean of three replicates. Each experiment was repeated twice with similar results.

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