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Erwinia amylovora Secretes Harpin via a Type III Pathway and Contains a Homolog of *yopN* of *Yersinia* spp.

ADAM J. BOGDANOVE, ZHONG-MIN WEI, LIPING ZHAO, AND STEVEN V. BEER*

Department of Plant Pathology, Cornell University, Ithaca, New York 14853

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Type III secretion functions in flagellar biosynthesis and in export of virulence factors from several animal pathogens, and for plant pathogens, it has been shown to be involved in the export of elicitors of the hypersensitive reaction. Typified by the Yop delivery system of *Yersinia* spp., type III secretion is *sec* independent and requires multiple components. Sequence analysis of an 11.5-kb region of the *hrp* gene cluster of *Erwinia amylovora* containing *hrpI*, a previously characterized type III gene, revealed a group of eight more type III genes corresponding to the *virB* or *lcrB* (*yscN*-to-*yscU*) locus of *Yersinia* spp. A homolog of another Yop secretion gene, *yscD*, was found between *hrpI* and this group downstream. Immediately upstream of *hrpI*, a homolog of *yopN* was discovered. *yopN* is a putative sensor involved in host-cell-contact-triggered expression and transfer of protein, e.g., YopE, to the host cytoplasm. In-frame deletion mutagenesis of one of the type III genes, designated *hrcT*, was nonpolar and resulted in a Hrp$^-$ strain that produced but did not secrete harpin, an elicitor of the hypersensitive reaction that is also required for pathogenesis. Cladistic analysis of the HrpI (herein renamed HrcV) or LcrD protein family revealed two distinct groups for plant pathogens. The *Yersinia* protein grouped more closely with the plant pathogen homologs than with homologs from other animal pathogens; flagellar biosynthesis proteins grouped distinctly. A possible evolutionary history of type III secretion is presented, and the potential significance of the similarity between the harpin and Yop export systems is discussed, particularly with respect to a potential role for the YopN homolog in pathogenesis of plants.

Erwinia amylovora causes fire blight, an often devastating disease of apple, pear, and other rosaceous plants. In nonhost plants, such as tobacco plants, *E. amylovora* elicits the rapid, localized necrosis known as the hypersensitive reaction (HR). The HR is an active process (34) that is correlated with plant defense responses. Several genes required by plant-pathogenic bacteria both for elicitation of the HR and for pathogenicity have been characterized. These genes are designated *hrp* for hypersensitive response and pathogenicity (52). Large clusters of *hrp* genes have been cloned from *E. amylovora*, *Pseudomonas solanacearum*, *Xanthomonas campestris*, and *Pseudomonas syringae*. In large part, these clusters were discovered to be physically and functionally conserved (7, 50, 74, 84). The *hrp* gene cluster of *E. amylovora* was cloned from strain Ea321 in the cosmid pCPP430 and found to confer on *Escherichia coli* and other nonplant pathogens and saprophytes the ability to elicit an HR in tobacco plants (10) and to enable bacterial multiplication and induction of electrolyte leakage and necrosis in apple leaf segments (88). Harpin, an extracellular elicitor of the HR and a pathogenicity determinant, was identified as a product of the *hrpN* gene located within this cluster (82). Complementation analysis of transposon insertion mutants, assays for β-glucuronidase reporter gene fusion activity, and partial DNA sequencing have revealed eight putative transcription units within a 25-kb region of the cluster. One is *hrpN*, at least two regulate the synthesis of harpin, and the remaining five are needed for secretion of harpin (80) (see Fig. 1).

One of the secretion genes is *hrpI* (80), which lies within complementation group VI and encodes HrpI, a member of a large family of proteins involved or implicated in protein translocation. Members of this family include the following: LcrD of *Yersinia* spp.; MxiA of *Shigella flexneri*; InvA of *Salmonella typhimurium*; FlbF of *Caulobacter crescentus*; FlhA of *E. coli*, *S. typhimurium*, and *Bacillus subtilis*; and HrpO, HrpC2, and HrpI, respectively, of *P. solanacearum*, *X. campestris* pv. vesicatoria, and *P. syringae* pv. syringae (see Table 3 footnotes for references). LcrD is required for the secretion of the *Yersinia* virulence proteins called Yops (62, 77).

Secretion of Yops occurs via a pathway classified as type III (66), which is distinct from the hemolysin secretion system of *E. coli* (type I), and the pullulanase secretion system of *Klebsiella oxytoca* and the Out system of pectolytic erwiniae (both type II). Secretion of proteins through a type III pathway is *sec* independent and requires a large number of genes, possibly as many as 23 in *Yersinia* spp. (for a review, see reference 25).

Extensive similarity exists between a group of eight *Yersinia* secretion genes (*yscN* to *yscU*) and genes required for secretion of Ipa virulence determinants of *S. flexneri*, genes required for the invasive phenotype of *S. typhimurium*, and curiously, several genes required for the assembly of bacterial flagella (11). One or both counterparts of two additional components of the type III secretion system of *Yersinia* spp., YscC and YscJ, exist in *S. typhimurium* (InvG = YscC) (44), in *S. flexneri* (MxiD = YscC; MxiJ = YscJ) (2, 3), and among the flagellar biosynthesis proteins of *E. coli* and *B. subtilis* (FliF = YscJ) (43, 90). Homologs of *yscC* and *yscJ* in the *hrp* gene clusters of *P. solanacearum* (*hrpA* and *hrpI*, respectively) (31) and *X. campestris* pv. campestris (*hrpA1* and *hrpB3*, respectively) (23) were also reported. A homolog of *yscC* in *P. syringae* pv. syringae, *hrpH*, was shown to be required for the secretion of the HR elicitor HrpZ from *P. syringae* pv. syringae (35, 37). Thus, type III secretion appears to be common to an array of plant and animal pathogens and to function also in the assembly of flagella in both gram-negative and -positive bacteria.

* Corresponding author. Phone: (607) 255-7870. Fax: (607) 255-4471. Electronic mail address: svb1@cornell.edu.

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
E. amylovora		
Ea321	Rp^r derivative of wild-type strain CNPB136	10
Ea321Δ*hrcT*	Derivative of Ea321 with 444-bp deletion in *hrcT*	This work
E. coli		
DH5	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* Nx^r	Life Technologies, Inc., Grand Island, N.Y.
DH5α	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* F^- *lacZ*ΔM15 Nx^r	Life Technologies, Inc.
SM10λpir	λ*pir* lysogen of SM10 *thi thr leu tonA lacY supE recA*::RP4-2-Tc::mu Km^r	58
Plasmids		
pBSKS+	Cloning vector, pBluescript KS+, Ap^r	Stratagene, La Jolla, Calif.
pKNG101	Suicide vector (*oriR6K*) containing *sacB mobRK2* Sm^r	45
pCPP430	Ea321 *hrp* gene cluster, Sp^r Sm^r	10
pCPP1180	pCPP1014::Δ*Rsr*II-*Sna*BI (444-bp in-frame deletion in *hrcT*)	This work
pCPP1194	4.75-kb *Xba*I-*Sal*I fragment (Δ*hrcT*) of pCPP1180 in pKNG101	This work
pCPP430 subclones in pBluescript KS+		
pCPP1031	First (leftmost) 3.8-kb *Eco*RI fragment of pCPP430	80
pCPP1149	Second 3.8-kb *Eco*RI fragment of pCPP430	This work
pCPP1014	5.2-kb *Eco*RI fragment of pCPP430	This work
pCPP1204	0.8-kb *Eco*RI-*Hin*dIII fragment of pCPP1014	This work
pCPP1106	2.4-kb *Hin*dIII fragment of pCPP1014	This work
pCPP1205	2.0-kb *Hin*dIII-*Eco*RI fragment of pCPP1014	This work
pCPP1154	3.2-kb *Eco*RI-*Bam*HI fragment of pCPP1014	This work
pCPP1139	1.8-kb *Bam*HI-*Eco*RI fragment of pCPP1014	This work

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Nx^r, nalidixic acid resistance; Sm^r, streptomycin resistance; Sp^r, spectinomycin resistance.

Recently published sequence data revealed several more conserved type III genes in the *hrp* gene cluster of *P. solanacearum* (75). This cluster governs the secretion of the HR elicitor PopA and presumably is involved in the secretion of one or more pathogenicity factors. Completion of the remaining sequences of the *hrp* gene cluster of *P. syringae* recently confirmed that this cluster also encodes a type III pathway, necessary for secretion of the *hrpZ* harpin from this bacterium. In preliminary reports, we showed that the *hrp* gene cluster of *E. amylovora* also contains, in addition to *hrpI*, several conserved type III genes (16, 17). Here, we present a complete analysis of an 11.5-kb region of the *E. amylovora hrp* gene cluster containing the genes, as well as results of nonpolar mutagenesis confirming that the *E. amylovora* harpin, an HR elicitor and pathogenicity determinant, is secreted via the type III pathway that the genes encode. We also report a particularly high degree of similarity between this portion of the *hrp* gene cluster of *E. amylovora* and the virulence loci of *Yersinia* spp., including *yopN*, which encodes a putative extracellular sensor required for the contact-dependent expression and transfer of YopE (and possibly other Yops) into mammalian cells (26, 65). Finally, we present a possible phylogeny of type III secretion based on cladistic analysis of members of the HrpI or LcrD family of proteins.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. *E. coli* DH5 and *E. coli* DH5α were used as hosts for pCPP430 and subclones of it that were used for sequencing.

Recombinant DNA techniques. Preparation of plasmid DNA, restriction enzyme digestions, ligation, and transformation of *E. coli* were performed essentially by the method of Sambrook et al. (67). Transformation of *E. amylovora* was done by electroporation by the methods of Bauer (8) with the Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). Isolation of total DNA from *E. amylovora* strains for Southern blot analysis was done as described previously (50).

Nucleotide sequencing and analysis. The nucleotide sequence of the 11.5-kb region of the *E. amylovora hrp* gene cluster was determined by using subclones (Table 1) of pCPP430, the cosmid containing the complete cluster of *hrp* genes (10). For large subclones, either nested deletions were made in both directions with the Erase-a-base System (Promega, Madison, Wis.) or custom primers were synthesized and used in a stepwise fashion. Nucleotide sequencing was performed either manually by the method of Sanger et al. (68) or semiautomatically by the Cornell Biotechnology Sequencing Facility on a Sequenator (Applied Biosystems, Perkin-Elmer, Inc., Norwalk, Conn.). Sequence assembly and analysis were performed by using the programs of the GCG software package, version 7.1 (Genetics Computer Group, Inc., Madison, Wis.). Database searches were performed with the BLAST algorithm (5). Paired protein sequence alignments were done with BESTFIT. Statistical assessment of BESTFIT alignments was done by using the /ran=100 subcommand which repeats the alignment 100 times, each time randomizing one of the paired sequences prior to alignment. An alignment was considered significant and indicative of homology if the quality score using default parameters was at least five times the standard deviation greater than the mean quality score of the 100 randomized alignments (22). For open reading frame (ORF) 3, protein sequence was generated starting from the second potential translational start site (see table 2).

Construction of a *hrcT* in-frame deletion mutant. pCPP1014 was digested with *Rsr*II and *Sna*BI, which cut at unique sites 444 bp apart within *hrcT* (Fig. 1). After treatment with Klenow fragment to fill in the *Rsr*II overhang, the DNA was religated and then digested again with *Sna*BI to linearize any restored (full-length) plasmid. The DNA was then used to transform *E. coli* DH5α. Transformants were screened for the deletion with a restriction digest, and an appropriate clone was isolated. The insert of this clone was excised with *Xba*I and *Sal*I and recloned into the suicide vector pKNG101 (45), cut with the same enzymes, to yield pCPP1194. *E. coli* SM10λpir was used as a host strain (pKNG101 bears *oriR6K* requiring the product of the *pir* gene for replication). *E. coli* SM10λpir(pCPP1194) was mated with the rifampin (RIF)-resistant *E. amylovora* Ea321 on a Luria agar plate overnight at 28°C. Cells were streaked onto Luria agar containing RIF and streptomycin to select for transconjugants in which the plasmid had integrated into the chromosome by a single crossover. Unless stated otherwise, *E. amylovora* was always cultured at 28°C. Single colonies were isolated and inoculated to Luria broth with RIF and streptomycin, grown overnight, and then washed once and used to inoculate fresh Luria broth containing RIF only. Cells were grown to an optical density at 620 nm of 0.5 and then washed and resuspended in an *hrp* gene-inducing minimal medium (40); after 4 h, cells were plated at a $1:10^4$ dilution on Luria agar containing RIF and 5% sucrose to select for excision and loss of the plasmid by a second recombination event (pKNG101 contains the *B. subtilis sacB* gene, which is lethal in enterobacteria in the presence of 5% sucrose). Individual colonies were isolated, screened for sensitivity to streptomycin, and analyzed by Southern blotting with the 5.2-kb insert of pCPP1014 as a probe.

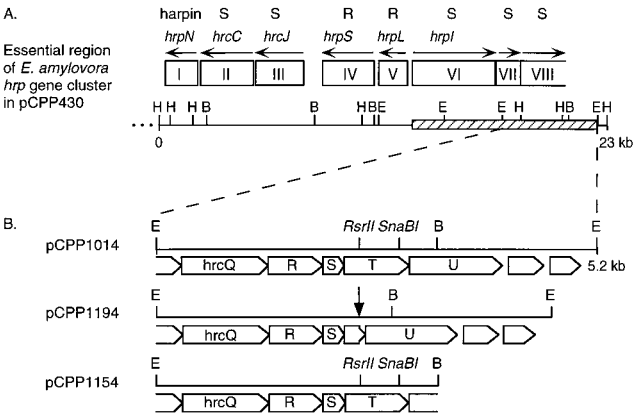

FIG. 1. The 11.5-kb region of the *hrp* gene cluster of *E. amylovora* for which nucleotide sequence was determined and plasmids used in constructing the *hrcT* deletion mutant. (A) The essential region of the *hrp* gene cluster of *E. amylovora* in cosmid clone pCPP430. Shown are complementation groups I to VIII, the function and transcriptional direction of each group, as determined by mutational analysis and reporter gene fusions (80), the locations of known genes (47, 80, 81, 83), and a restriction map for *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). At the top of the figure, S denotes a role in secretion of harpin and R denotes a role in regulation of *hrpN* and other *hrp* genes. The sequenced region is indicated with a hatched box. (B) Plasmids used to construct the *hrcT* deletion mutant. pCPP1014 was digested with *Rsr*II and *Sna*BI, filled in, and religated. The resulting fragment, lacking 444 bp in *hrcT*, was cloned into the suicide vector pKNG101 to yield pCPP1194. The vertical arrow marks the site of the deletion. pCPP1194 was used to introduce the in-frame deletion mutation into wild-type strain Ea321 by double homologous recombination. pCPP1154 contains a subclone encoding *hrcT* and several upstream (but not downstream) genes and was transferred into the mutant strain for complementation analysis (see text for details).

HR and pathogenicity assays. Tobacco leaf panels (*Nicotiana tabacum* L. 'xanthi') were infiltrated with bacterial cell suspensions as described previously (9, 82). HR was scored after 24 h. Immature Bartlett pears (*Pyrus communis* L.) were surface disinfested, trimmed to remove the calyx and stem ends, and cut longitudinally in thirds. A small tangential slice was removed from each third, and each third was inoculated by application to the exposed mesocarp of 20 μl of a 10^7-CFU/ml suspension of bacteria in 5 mM KPO_4 buffer, pH 6.8. Pears were photographed to record the amount of ooze and necrosis after incubation for 3 days at 28°C and high relative humidity.

Detection of extracellular and cell-associated harpin. Strains were grown to an optical density at 620 nm of 0.95 in an *hrp* gene-inducing minimal medium (40) at 22°C. To detect extracellular harpin, 20 μl of each culture was spotted onto an Immobilon-P membrane (Millipore Corp., Bedford, Mass.) and immunostained with antiharpin antiserum (in Tris-buffered saline without detergent) as previously described (80). To detect cell-associated harpin, cells were harvested and resuspended in one-half volume of 5 mM KPO_4 buffer, pH 6.8, and 15 μl of each suspension was treated to lyse the cells. These whole-cell lysates were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting (immunoblotting), and immunostaining with antiharpin antiserum as described previously (80).

Phylogenetic analysis. Multiple alignments of each gene family were made by using the PILEUP Program of the GCG package, and the HrpI (herein renamed HrcV) multiple alignment was chosen for input into the PAUP computer program (Phylogenetic Analysis Using Parsimony; D. L. Swafford, Smithsonian Institution, Washington, D.C.) to construct a phylogenetic tree. The bootstrap method with a heuristic search for 100 replicates was used. Trees were also constructed in the same way for the highly conserved N-terminal half of the HrcV multiple alignment and for an alignment of HrcS and homologs.

Nucleotide sequence accession number. The nucleotide sequence upstream and downstream of *hrpI* has been combined with the previously published *hrpI* sequence and deposited in GenBank under accession number L25828.

RESULTS

Nucleotide sequence analysis of the 11.5-kb region comprising complementation groups VI, VII, and VIII. The DNA sequence of the entire 11.5-kb region encompassing complementation groups VI, VII, and VIII of the *E. amylovora hrp* gene cluster (Fig. 1) was determined. It contains 11 adjacent or overlapping ORFs, including the previously described *hrpI* gene (80), followed 64 bp downstream by two small ORFs, spaced 58 bp apart. The translational direction of the ORFs is in agreement with that predicted previously with β-glucuronidase reporter gene fusions (83). Each ORF is preceded by an appropriately spaced potential ribosome binding site. ORF 3 has two potential translational start sites. ORF 11 begins with the unusual start codon UUG. The predicted physical characteristics of the proteins encoded by ORFs 1 to 13 and the potential ribosome binding sites and start codons are presented in Table 2. T7 promoter-driven specific expression of a DNA fragment containing what are now known to be ORFs 2 to 5 resulted in the specific production of four proteins (80) with apparent molecular masses (66, 25, 38, and 21 kDa) roughly corresponding to those predicted for the proteins encoded by these ORFs (Table 2). Sequences beginning 62 bp upstream of ORF 1 and 59 bp upstream of ORF 12 were found to be similar (strictly conserved bases are shown below underlined) to the putative *avr* and *hrp* promoter consensus sequence of *P. syringae*, GGAACc(N)$_{16}$NcCACNNA (N is any base, and lowercase letters denote ≥75% conservation in *P. syringae*; 42, 53, 71, 86). Whether these sequences are in fact *hrp*-specific promoters in *E. amylovora* will be addressed later. Nowhere in the sequence could be detected any palindromic sequences typical of a *rho*-independent terminator by using the terminator search program of the GCG software package. The

TABLE 2. Predicted characteristics of the encoded proteins and potential ribosome binding sites and start codons for ORFs 1 to 13

ORF	Name of encoded protein	MM[a] (kDa)	pI[a]	No. of TMRs[b]	Sequence with potential RBS(s) and start codon(s)[c]
1	HrpJ	39.8	9.9	0	GAAGGTTCGCCAGG*ATG*
2	HrcV (HrpI)	77.6	5.4	6–8	AGGACGGCGCTGTAA*ATG*
3	HrpQ	33.9	5.1	1	AGAACTTCAGGAAA*ATG*ACC*ATG*
4	HrcN	49.1	6.3	0	GGAAAACATCAGGGTGAA*ATG*
5	HrpO	17.7	6.9	0	GGAGGTCAGCGCCC*ATG*
6	HrpP	17.9	5.7	0	GGAGGAGGCGTG*ATG*
7	HrcQ	36.4	4.7	0	GGAGAAGCGCC*ATG*
8	HrcR	24.1	6.1	4	AGGAGGTTGG*ATG*
9	HrcS	9.2	6.6	2	GAGGTAAGA*ATG*
10	HrcT	29.0	6.5	5–6	AGGGCATAGT*ATG*
11	HrcU	40.6	7.2	4	GGGACACTAGC*TTG*
12		16.0	7.0	0	GAGGAAACCGGCCG*ATG*
13		14.4	8.9	0	AGGAACAAAC*ATG*

[a] Molecular mass (MM) and isoelectric point (pI) were calculated by using the PEPSTATS program of the GCG software package.
[b] The number of transmembrane regions (TMRs) was predicted for each protein on the basis of a hydropathy plot by the method of Kyte and Doolittle (49).
[c] Sequences similar to the *E. coli* ribosome binding site (RBS) consensus sequence are underlined. Start codons are italicized.

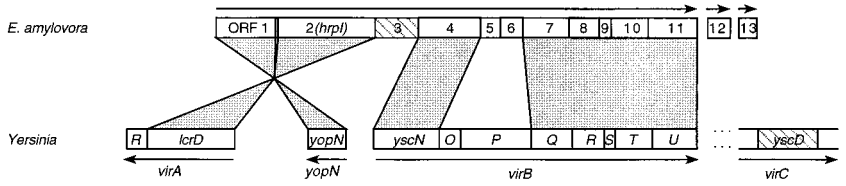

FIG. 2. Genetic organization of the sequenced 11.5-kb region of the *E. amylovora hrp* gene cluster and similarity to the *virA*, *yopN*, *virB*, and *virC* loci of *Yersinia* spp. (for a review, see reference 20). Shaded blocks indicate regions of significant similarity between the two sets of genes. ORF 3 and *yscD* (hatched boxes) are also significantly similar.

above results suggest that ORFs 1 to 11 are in a single operon and that ORFs 12 and 13 may be transcribed independently.

Similarity to virulence loci of *Yersinia* spp. Previous results indicated homology of HrpI (ORF 2) and members, including LcrD of *Yersinia pestis*, of a large family of integral membrane proteins involved or implicated in protein secretion (80). Comparison of the predicted amino acid sequences of the remaining ORFs to sequences in the major databases revealed that many of the ORFs downstream of *hrpI* are similar to genes in the *virB* or *lcrB* (*yscN*-to-*yscU*) locus of *Yersinia* spp. involved in secretion of the virulence proteins called Yops (11, 24). The spatial organization of these ORFs was perfectly conserved (Fig. 2). ORF 1, immediately upstream of *hrpI*, shows significant similarity to the *Yersinia* gene *yopN* (*lcrE*) (Fig. 3A) that lies between and adjacent to the *virA* (*lcrD* and *lcrR*) locus and the *virB* locus on the virulence plasmids of *Yersinia* spp. *yopN* and *virA* are transcribed in the same direction, opposite that of *yscN* to *yscU*. ORF 3, immediately downstream of *hrpI*, shows significant similarity to *yscD* (Fig. 3B), the fourth gene in the *Yersinia virC* locus, located downstream of the *virB* locus. It appears that the entire region encompassing ORFs 1 to 11 is conserved, to a greater or lesser degree for any given ORF and with some rearrangement, between *Yersinia* spp. and *E. amylovora* (Fig. 2).

ORFs homologous with genes in the *Yersinia virC* locus also showed, as the *ysc* genes do (see Fig. 1 of reference 11), similarity to, and in many cases colinearity with, *spa* genes of *S. flexneri* (70, 76) and *S. typhimurium* (32), *mop* genes of *Erwinia carotovora* (60), and flagellar genes of *B. subtilis* (1, 12, 13, 19), *C. crescentus* (64, 89), *E. coli* (55, 56), and *S. typhimurium* (46, 59, 78), as well as recently published *hrp* genes in *P. solanacearum*. ORF 3 is similar to *hrpW* of *P. solanacearum* (75) and to *hrpJ3* of *P. syringae* pv. syringae (51), but no homologs of this gene in *S. typhimurium* or *S. flexneri* have been reported. ORFs 1 to 11 are particularly highly similar to and are colinear with genes in the *hrpJ* (51) and *hrpU* (38, 51) operons in the *P. syringae* pv. syringae *hrp* gene cluster, except that the upstream two-thirds of ORF 7 is similar to *hrpU2* and the downstream one-third of ORF 7 is similar to *hrpU*; *hrpU2* and *hrpU* have been reported to follow *hrpU1*.

In accordance with a proposal for standardizing *hrp* gene nomenclature, ORFs homologous with broadly conserved type III genes were given the new designation *hrc* (see Discussion) followed by the capital letter of the corresponding *ysc* gene, and *hrpI* was renamed *hrcV*. The *yopN* homolog was named *hrpJ*, after the *P. syringae* homolog. ORFs 12 and 13, which showed no significant similarity to any known genes in the database and were not characterized further, were not named. The names of the proteins encoded by ORFs 1 to 11 are presented in Table 2, and the findings of the database searches are presented in detail in Table 3.

Nonpolar mutagenesis of *hrcT*. An in-frame deletion was made in *hrcT* contained in a subclone of the *hrp* gene cluster (Fig. 1). This unmarked mutation was introduced into strain Ea321 using the *sacB* gene on the suicide vector pKNG101 for positive selection of double recombination events (45). Four of ten sucrose-resistant strains tested showed the deletion (data not shown). One of these strains was chosen for phenotypic characterization and complementation analysis and was designated Ea321Δ*hrcT*. In contrast to the wild-type strain, Ea321Δ*hrcT* was Hrp$^-$ (Fig. 4A and B) and failed to secrete detectable levels of harpin when grown in *hrp* gene-inducing

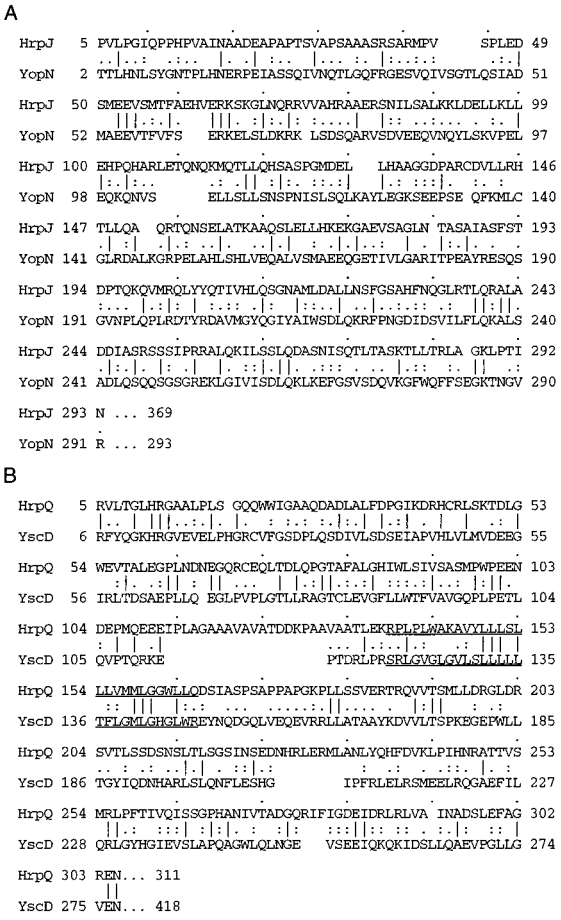

FIG. 3. BESTFIT alignments (21) of predicted amino acid sequences of HrpJ and YopN (26) (A) and HrpQ and YscD (57) (B). The HrpJ-YopN alignment was generated by using default parameters. For the HrpQ-YscD alignment, the gap penalty and gap length weight were increased to 5 and 0.13, respectively, to obtain better alignment of a dominant hydrophobic stretch (underlined) containing a putative transmembrane region. Between the sequences, vertical lines indicate identical residues, colons indicate highly similar residues, and periods indicate moderately similar residues.

TABLE 3. Similarities of deduced *E. amylovora* proteins to proteins involved in plant or animal pathogenesis, flagellar biosynthesis, and proton translocation

Category and species	Similarity of protein to *E. amylovora* protein[a]										
	HrpJ (369 aa)	HrcV (698 aa)	HrpQ (311 aa)	HrcN (455 aa)	HrpO (144 aa)	HrpP (158 aa)	HrcQ (339 aa)	HrcR (218 aa)	HrcS (87 aa)	HrcT (266 aa)	HrcU (361 aa)
Plant pathogenicity proteins											
P. syringae	HrpJ[51] (26/15.8)	HrpI[39] (64/79.7)	HrpJ3[51] (35/27.0)	HrpJ4[51] (62/160.6)	HrpJ5[51] (27/7.7)	HrpU1[51] (26/6.0)	HrpU2[51] (29/14.8[b])	HrpW[38] (73/49.8)	HrpO[38] (64/23.5)	HrpX[38] (48/35.5)	HrpY[38] (52/54.5)
P. solanacearum		HrpO[30] (38/68.9)	HrpW[75] (23/6.1)	HrpE[75] (49/37.3)			HrpQ[75] (23/4.6)	HrpT[75] (35/37.2)	HrpU[75] (32/10.8)	HrpC[75] (28/12.3)	HrpN[75] (30/16.7)
X. campestris		HrpC2[23] (39/71.4)		HrpB6[23] (48/61.0)				Pro2[41] (38/19.6)			
Animal pathogenicity proteins											
Yersinia spp.	YopN[26,77] (21/5.8[c])	LcrD[62,77] (43/63.6)	YscD[33,57] (26/5.5)	YscN[11,85] (51/56.0)			YscQ[11,24] (24/10.6)	YscR[11,24] (42/28.5)	YscS[11,24] (51/17.6)	YscT[11] (34/22.9)	YscU[4,11] (31/31.2)
S. typhimurium		InvA[28] (39/84.1)		SpaL[32] (47/71.0[d])			SpaO[32] (22/4.5)	SpaP[32] (36/27.9)	SpaQ[32] (42/14.9)	SpaR[32] (25/23.3)	SpaS[32] (30/19.8)
S. flexneri		MxiA[6] (39/52.7)		Spa47[70,76] (43/34.3)			Spa33[70,76] (19/7.8)	Spa24[70,76] (36/21.0)	Spa9[70] (39/12.1)	Spa29[70] (26/20.4)	Spa40[70] (27/23.4)
E. coli									InvX[36] (39/19.4)		
Flagellar biosynthesis proteins											
B. subtilis		FlhA[18] (34/58.4)		FliI[1] (42/58.4)			FliY[12] (24/6.1)	FliP[13] (35/17.6)	FliQ[13] (32/10.7)	FliR[19] (23/10.5)	FlhB[19] (30/22.5)
C. crescentus		FlbF[63] (32/32.6)					FliN[64] (25/8.7[e])		FliQ[89] (33/8.3)	FliR[89] (22/12.8)	
E. coli, S. typhimurium		FlhA[59] (36/42.5)		FliI[78] (42/34.3)			FliN[56,46] (30/10.1[e])	FliP[55] (37/20.6)	FliQ[55] (36/10.9)	FliR[55] (24/6.9)	FlhB[59] (33/30.1)
E. carotovora							MopA[60] (33/10.1[f])	MopC[60] (41/23.9)	MopD[60] (33/10.1)	MopE[60] (25/10.1)	
Proton translocation proteins											
E. coli				AtpB[79] (29/27.5)							
B. subtilis				AtpB[69] (27/18.2)							

[a] Similarity of deduced *E. amylovora* protein (length of protein in amino acid residues [aa]) to proteins involved in plant or animal pathogenesis, flagellar biosynthesis, and proton translocation. The numbers in parentheses are the percent identity to the *E. amylovora* sequence based on a BESTFIT alignment (21) by using default parameters, and for the quality score of that alignment, the number of standard deviations above the mean of 100 alignments for which one of the paired sequences had been randomized prior to alignment (22). Except where noted, each alignment spans the full length of the *E. amylovora* protein. References for sequence data taken from previous studies are given as superscripts. When sequences of the same gene from more than one species are available, data are presented for the sequence that yields the highest percent identity (underlined reference).

[b] Aligns to the N-terminal two-thirds of HrcQ. The protein encoded by the reported neighboring gene, *hrpU* (38), aligns to the remainder of HrcQ (38% identity, 25.7 standard deviations).

[c] The whole sequence aligns to the N-terminal 80% of HrpJ.

[d] The known sequence aligns to the C-terminal three-fourths of HrcN.

[e] The whole sequence aligns to the C-terminal third of HrcQ.

[f] The known sequence aligns to the C-terminal fourth of HrcQ.

minimal medium (Fig. 4C). Harpin was present in equivalent amounts in lysates of wild-type and mutant cells (Fig. 4D). A cloned fragment (in pCPP1154) containing *hrcQ*, *-R*, *-S*, and *-T*, but not *hrcU*, complemented the deletion in *hrcT* (Fig. 4), confirming that the mutation is nonpolar. Thus, *hrcT* is required for the secretion of harpin, for the HR, and for pathogenicity.

Phylogenetic analysis. Given the prevalence of type III secretion in a diverse array of bacterial systems, it was of interest to explore the phylogeny of the type III genes based on an analysis of sequence relationships. The HrcV multiple alignment (Fig. 5) was chosen for this purpose, because it shows a particularly high degree of conservation in its N terminus and because sequence is available for homologs in each of the bacterial systems represented in Table 3. The phylogenetic tree generated using a bootstrap analysis with heuristic search for 100 replicates is presented in Fig. 6. Similar trees were obtained (not shown) when the same analysis was undertaken using only the highly conserved N-terminal portion of the HrcV multiple alignment or the HrcS multiple alignment, which by virtue of its short length and high degree of conservation is also relatively unambiguous.

DISCUSSION

DNA sequencing and analysis of an 11.5-kb region of the *E. amylovora hrp* gene cluster revealed 11 adjacent or overlapping ORFs including the previously characterized *hrpI* gene. Eight of the ten new ORFs constitute a conserved group of type III secretion genes. The eight genes are colinear with their coun-

terparts in the animal pathogens and are most similar to *yscN* to *yscU* of *Yersinia* spp. The genes were named (in order of transcription) *hrcN*, *hrpO*, *hrpP*, *hrcQ*, *hrcR*, *hrcS*, *hrcT*, and *hrcU*. The other two new ORFs also have homologs in *Yersinia* spp. One of these two ORFs, located between *hrcN* and *hrpI*, is a homolog of *yscD* that we have named *hrpQ*. Surprisingly, the other, located upstream of *hrpI*, is a homolog of *yopN*, which encodes an extracellular protein in *Yersinia* spp. The *yopN* homolog has been named *hrpJ*.

We designated any ORF with significant similarity to a broadly conserved type III gene *hrc* (rather than *hrp*) followed by the capital letter designation of the corresponding *ysc* gene in accordance with a forthcoming proposal for standardizing *hrp* gene nomenclature (15). The *hrc* designation is intended to indicate that the ORF is associated with the HR-eliciting ability of the bacterium (i.e., it lies within the *hrp* gene cluster or is required for the Hrp phenotype) and that it is conserved among pathogens both of plants and animals (*hr* for HR and *c* for conserved). Similarity was considered significant if a BESTFIT alignment (21) of predicted amino acid sequences yielded a quality score by using default parameters of at least five times the standard deviation above the mean quality score of 100 alignments for each of which one of the sequences had been randomized prior to alignment (22). *hrpI* has been renamed *hrcV* to indicate that it is also a conserved type III secretion gene. (The *Yersinia* homolog is *lcrD*, functionally a *ysc* gene, but "*V*" was chosen instead of "*D*" in order to avoid confusion of *hrcV* as a *yscD* homolog. "*V*" currently is not used in the *ysc* series.)

hrpJ to *hrcU* appear to constitute a large operon. There is no typical terminator sequence within the tightly packed group of genes, and *hrpJ* is preceded by a putative *hrp* promoter sequence. Nevertheless, the DNA spans previously defined complementation groups VI, VII, and VIII. Therefore, although no recognizable promoter sequences were found within the putative operon, it likely contains two or more internal promoters. β-Glucuronidase gene fusions in complementation groups VII and VIII showed slightly different levels of activity (83), suggesting differential expression of genes. The unusual start codon of *hrcU* may play a role in differential expression at the translational level. Further biochemical analysis is required to define precisely the transcriptional organization of this region.

On the basis of sequence analysis, HrcN and its homologs are predicted to be cytoplasmic. They show similarity to the β-subunit of the F_1F_0 ATPase of *E. coli* (79) and may function in energizing export. HrpO and HrpP do not have significant sequence similarity with the products of genes (*yscO* and *yscP* [11]; *spaM* and *spaN* [32]; *spa13* and *spa32* [70, 76]) in the corresponding positions in the clusters of the animal pathogens (which are likewise different from one another). HrpO and HrpP do have significant similarity to HrpJ5 and HrpU1, respectively, of *P. syringae* pv. syringae (51). The products of the genes in these positions in each of the groups may represent two sets of highly divergent, system-specific homologs. HrcQ and its homologs are hydrophilic, but HrcR, HrcS, HrcT, and HrcU (and homologs) are each predicted to have two or more membrane-spanning regions.

Strains carrying chromosomal insertion mutations within the region comprising this group of genes produced harpin but failed to export the protein and were Hrp^- (80). Each of the homologs of *hrcN* to *hrcU* in *S. flexneri* is required for the export of the Ipa proteins (70). In *Yersinia* spp., *yscN*, *yscQ*, *yscR*, *yscS*, and *yscU* have each been shown to be required for Yop secretion (4, 11, 24, 85). Results of Tn*phoA* mutagenesis of *hrpY*, apparently the last gene in the *P. syringae hrpU* operon, strongly suggest a requirement for this homolog of *hrcU* in the secretion of *P. syringae* pv. syringae harpin. We constructed an in-frame deletion mutation of *hrcT*. The mutant strain failed to secrete harpin and was Hrp^-. A fragment containing *hrcT* and genes upstream (but not downstream) complemented the mutation for harpin secretion and restored the Hrp^+ (wild-type) phenotype, indicating that the mutation is nonpolar. Our sequence analysis and mutagenesis results, combined with the previous characterization of *hrpI* (now *hrcV*), confirm that the *hrp* gene cluster of *E. amylovora* encodes a type III pathway that, as a route for export of harpin and possibly other factors, is an indispensable component of the pathogenic and HR-eliciting ability of this bacterium.

The homology of HrpJ with YopN is intriguing. YopN is a surface protein that plays a key role in the cell-contact-triggered expression and polarized transfer of YopE. Specifically, YopN is thought to be involved in sensing contact with the target cell and transmitting the contact signal to intracellular regulatory factors (26, 65, 87). Rosqvist et al. (65) presented evidence suggesting that secretion of YopE is localized to the zone of interaction between *Yersinia* and target cells. These investigators hypothesized that YopN is involved in directing assembly of the secretion apparatus to the zone of interaction

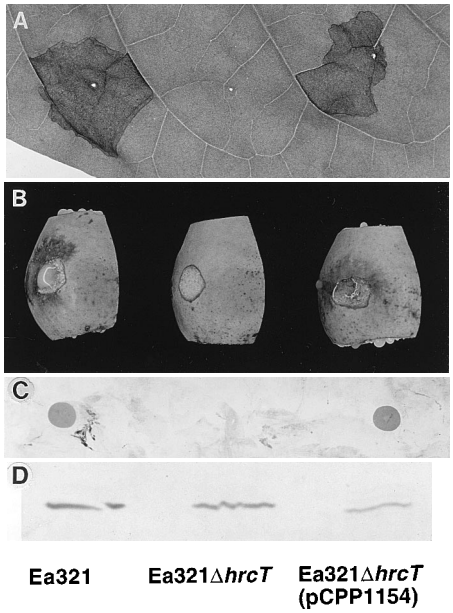

FIG. 4. Phenotype and complementation analysis of the *hrcT* deletion mutant strain. Shown in each panel are the results for the wild-type strain Ea321, the mutant strain Ea321Δ*hrcT*, and the mutant strain containing pCPP1154. (A) HR assay. Tobacco leaf shown 24 h after infiltration with suspensions of each strain. Bacteria were grown to an optical density at 620 nm of 0.8 in Luria broth, harvested by centrifugation, and resuspended in 5 mM KPO_4 buffer (pH 6.8) for infiltration. (B) Pathogenicity assay. Immature Bartlett pears were surface disinfested, trimmed to remove the calyx and stem ends, and cut longitudinally in thirds. A small tangential slice was removed from each third, and each third was inoculated with one of the three strains by application to the exposed mesocarp of 20 μl of a 10^7-CFU/ml suspension of bacteria in 5 mM KPO_4 buffer, pH 6.8. Shown are the thirds from one pear typical of six replicate pears after incubation for 3 days at 28°C and high relative humidity. (C) Whole culture immunoblot. Strains were grown in an *hrp* gene-inducing minimal medium (40), and 20-μl portions of each culture were spotted onto Immobilon-P membranes (Millipore Corp.) and immunostained with antiharpin antiserum as previously described (80). (D) Western blot. Cells were grown as described above for panel C, harvested, and resuspended in one-half volume of 5 mM KPO_4 buffer, pH 6.8. Portions (15 μl) of each suspension were treated to lyse the cells and subjected to SDS-PAGE, Western blotting, and immunostaining with antiharpin antiserum as previously described (80).

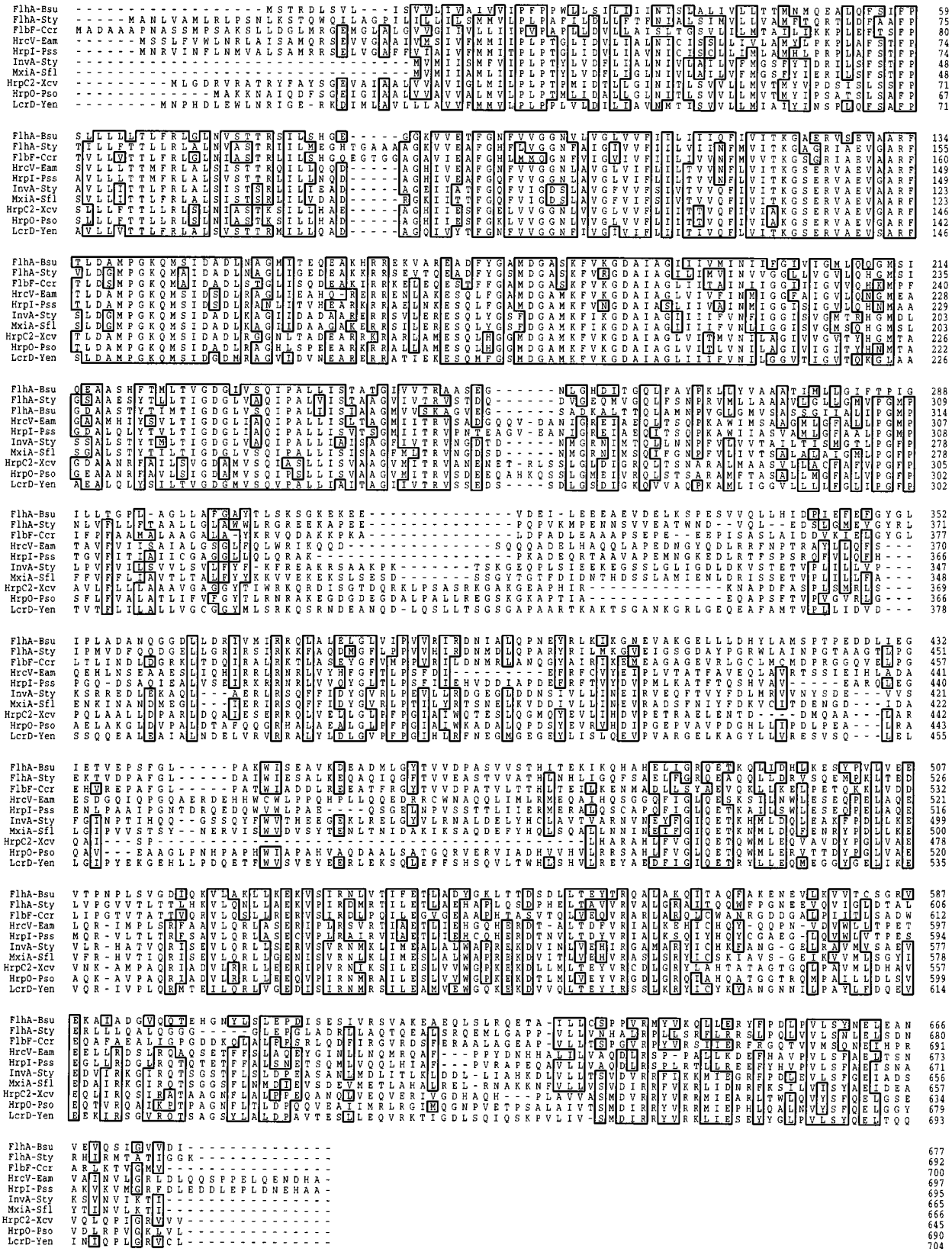

FIG. 5. Multiple alignment of predicted amino acid sequences for the HrcV or LcrD family used to construct the phylogenetic tree in Fig. 6. The multiple alignment was generated using the PILEUP program (21) of the GCG package. When sequences for the same gene from two or more species of the same genus are available, only the sequence most similar to the corresponding *E. amylovora* gene is included in the multiple alignment. Names of bacteria follow each protein name, separated by a hyphen, and are abbreviated as follows: Bsu, *B. subtilis*; Ccr, *C. crescentus*; Eam, *E. amylovora*; Pss, *P. syringae* pv. syringae; Pso, *P. solanacearum*; Sfl, *S. flexneri*; Sty, *S. typhimurium*; Xcv, *X. campestris* pv. vesicatoria; Yen, *Yersinia enterocolitica*. Dashes represent gaps in the alignment. Boxes indicate conserved residues.

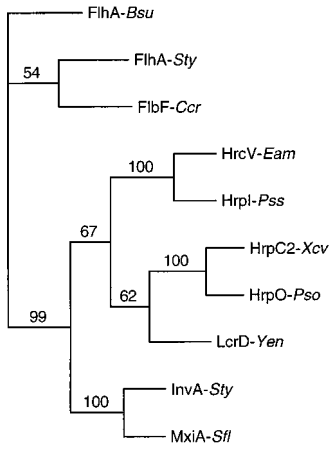

FIG. 6. Phylogenetic tree showing the relationships among HrcV and homologs based on the multiple alignment shown in Fig. 5. Names of bacteria are italicized and abbreviated as in Fig. 5. The tree was constructed with the PAUP computer program by the bootstrap method with heuristic search for 100 replicates. The number above each branch represents the number of replicate trees in which that branch was present.

between the pathogen and host cells. Translocation of YopN to the cell surface itself requires YscN (85), the putative energizer of the type III pathway in *Yersinia* spp. Perhaps YopN is involved in directing already assembled secretion complexes to the interaction zone or acts to open or activate complexes in contact with a host cell. YopN may act in concert with LcrG, a small, basic, extracellular protein. YopD is also required for polarized transfer of YopE (72). YopB may also be involved (72). Thus far, genes encoding homologs of these three proteins have not been discovered in *E. amylovora*. *hrpJ* is homologous with *hrpJ* of *P. syringae* pv. syringae (which, however, does not show significant similarity to *yopN*), but no gene with significant similarity to either *yopN* or *hrpJ* was detected in the published sequences of *P. solanacearum* or *X. campestris*.

Currently, there is no direct evidence of contact-dependent, polarized transfer of an effector protein or proteins from a plant pathogen into a plant cell. Our findings of extensive similarity between the *hrp* gene cluster of *E. amylovora* and virulence loci of *Yersinia* spp., and particularly the discovery of a YopN homolog in *E. amylovora*, certainly suggest the possibility. Direct transfer of effector proteins of plant-pathogenic bacteria to the plant cytosol may explain the difficulty experienced in isolating proteins such as *avr* gene products and pathogenicity factors from extracellular media. In this context, it is no longer puzzling that the predicted products of many of the plant disease resistance genes cloned to date appear to be cytoplasmic (73).

Harpin is sufficient and necessary to elicit an HR in nonhost plants, and the *E. amylovora hrpN* gene is required for full expression of disease. The role of harpin in disease, however, remains elusive. We have been unable to demonstrate a necrogenic effect of purified harpin on any of a variety of host tissues (14). The role of harpin in disease may be distinct from its role as an HR elicitor, and its activity in the host plant may depend on an intact, and possibly cell-contact-dependent, delivery system. Alternatively, harpin may be part of the delivery system. The target cells of a plant pathogen, unlike those of animal pathogens, are surrounded by rigid cell walls (of which glycine-rich proteins are a major constituent), a significant barrier to the bacterium. Conceivably, glycine-rich bacterial proteins such as harpin could play a role in breaching that barrier and facilitate the type III-dependent, polarized transfer of an effector or effectors.

Several investigators have noted the similar nature of the polarized transfer of Yops and the homologous process of the sequential export and assembly of flagellar components during flagellar biosynthesis (48, 65). In addition to allowing for delivery of subunits to the distal end, the structure of the flagellum must in someway accommodate information transfer from its terminus back to regulatory components within the cell (54). Is there a type III "secretion organelle" (72) akin to the flagellar basal body, and could there be a flagellum-like structure involved in polarized delivery of effector proteins, cell contact sensing, or both?

The flagellar switch proteins FliM and FliN of *E. coli* and *S. typhimurium* have been localized to a cytoplasmic ring complex associated with the basal body (27). FliN is homologous with the C terminus of HrcQ. The N-terminal sequence of the *B. subtilis* switch protein FliY is similar to FliM and the C-terminal sequence of FliY is similar to FliN (12). FliY is required for flagellar assembly and is likely to be a component of a cytoplasmic ring complex like the one described in *E. coli*. HrcQ is homologous to FliY with similarity distributed over the length of the protein. Thus, HrcQ may be part of a similar cytoplasmic ring complex. FliF, (whose N terminus is similar to YscJ [57] and HrcJ of *E. amylovora* [47]) is present in the inner-membrane-bound MS (membrane and supramembrane) ring of the flagellar basal body (27). FlhA, FliP, FliQ, FliR, and FlhB are homologous with HrcV and HrcR to HrcU, respectively. Whether any of these putative inner membrane proteins required for flagellar biosynthesis is associated with the basal body, however, has not been determined. These proteins may form an extended complex that generates the basal body. FliN, the homolog of HrcQ, may link the MS ring and the export machinery (54).

Many of the proteins exported via type III pathways, including harpin, are found in association with the bacterial cell surface as well as in the culture medium (20, 70, 80). Ipa proteins of *S. flexneri* were observed to form regular, extracellular filamentous structures when produced by a derepressed strain in culture (61). Appendages correlated with invasiveness have been observed in *S. typhimurium* and were shown to be dependent on the type III gene *invC*, a *yscN* homolog (29). Whether the generation of cell-associated, flagellum-like structures is a characteristic feature of type III secretion and how such structures might function in pathogenicity await further investigations of several type III systems.

We explored the phylogeny of type III secretion with a cladistic analysis of the HrcV or LcrD family of proteins. As expected, the flagellar biosynthetic proteins and the virulence or pathogenicity proteins form distinct groups. Surprisingly, LcrD of *Y. entercolitica* groups with the homologs in plant pathogens, rather than with the homologs in the other animal pathogens *S. typhimurium* and *S. flexneri*. Furthermore, the Hrp proteins form two distinct groups, those from *E. amylovora* and *P. syringae* and those from *P. solanacearum* and *X. campestris*. The *E. amylovora* and *P. syringae* group is roughly equidistant from the *P. solanacearum* and *X. campestris* group and the *Y. entercolitica* homolog.

The flagellar genes are clustered and chromosomal, as are the *hrp* genes of *E. amylovora* and *P. syringae*, and the *spa* and *inv* genes of *S. typhimurium*. The *hrp* genes of *P. solanacearum*, the *spa* genes of *S. flexneri*, and the *ysc* genes of *Yersinia* spp. are all plasmid-borne. Generally, the flagellar genes from the gram-positive bacterium *B. subtilis* are more similar in sequence to the pathogenicity-associated genes, all from gram-negative pathogens, than are the flagellar genes of *E. coli* and

S. typhimurium. In particular, whereas FliP of *E. coli* has a classic N-terminal signal peptide (55), FliP of *B. subtilis* (19) more closely resembles its pathogenicity-associated homologs in its lack of such a sequence. Also, FliY of *B. subtilis* (12), in contrast with FliN of *E. coli* and *S. typhimurium*, aligns with its pathogenicity-associated homologs over their entire lengths. The *B. subtilis* genes *fliP*, *fliQ*, *fliR*, and *flhB* (19) are colinear with the corresponding *hrc*, *ysc*, and *spa* genes (and with the corresponding *hrp* genes of *P. syringae*); the homologous flagellar genes of *E. coli* and *S. typhimurium* (54) and the homologous *hrp* genes of *P. solanacearum* (75), however, are only partially so. It is tempting to speculate that a duplicated set of flagellar genes, possibly in a gram-positive ancestor of *B. subtilis*, evolved to export nonflagellar proteins. Some of these proteins may have enabled the bacterium to exploit, in novel ways, the nutritional resources and protected environments within living eukaryotic hosts, conferring a significant selective advantage. This complex of secretory genes could have become, in effect, a "pathogenicity cassette" that may have been acquired independently by a variety of bacterial species and adapted over evolutionary time to export diverse proteins under diverse specific environmental conditions.

Harpin of *E. amylovora* is the only HR elicitor clearly shown to play a determinative role in disease. The striking similarity (at the genetic level) between the harpin export system of *E. amylovora* and the Yop export system of *Yersinia* spp. underscores the fundamental importance of type III pathways in the bacterial pathogenesis of both plants and animals. Further investigation of the harpin secretion system of *E. amylovora* likely will contribute to our overall understanding of type III secretion, and ultimately, aid in developing strategies for more effective control of bacterial diseases of agricultural and clinical importance.

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