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Identification of Two Novel *hrp*-Associated Genes in the *hrp* Gene Cluster of *Xanthomonas oryzae* pv. oryzae†

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We have cloned a *hrp* gene cluster from *Xanthomonas oryzae* pv. oryzae. Bacteria with mutations in the *hrp* region have reduced growth in rice leaves and lose the ability to elicit a hypersensitive response (HR) on the appropriate resistant cultivars of rice and the nonhost plant tomato. A 12,165-bp portion of nucleotide sequence from the presumed left end and extending through the *hrpB* operon was determined. The region was most similar to *hrp* genes from *Xanthomonas campestris* pv. vesicatoria and *Ralstonia solanacearum*. Two new *hrp*-associated loci, named *hpa1* and *hpa2*, were located beyond the *hrpA* operon. The *hpa1* gene encoded a 13-kDa glycine-rich protein with a composition similar to those of harpins and PopA. The product of *hpa2* was similar to lysozyme-like proteins. Perfect PIP boxes were present in the *hrpB* and *hpa1* operons, while a variant PIP box was located upstream of *hpa2*. A strain with a deletion encompassing *hpa1* and *hpa2* had reduced pathogenicity and elicited a weak HR on nonhost and resistant host plants. Experiments using single mutations in *hpa1* and *hpa2* indicated that the loss of *hpa1* was the principal cause of the reduced pathogenicity of the deletion strain. A 1,519-bp insertion element was located immediately downstream of *hpa2*. Hybridization with *hpa2* indicated that the gene was present in all of the strains of *Xanthomonas* examined. Hybridization experiments with *hpa1* and IS*1114* indicated that these sequences were detectable in all strains of *X. oryzae* pv. oryzae and some other *Xanthomonas* species.

The *hrp* ("harp") genes encode type III secretory pathways and are required by many phytopathogenic bacteria to elicit a hypersensitive response (HR) on nonhost or resistant host plants and for pathogenesis on susceptible hosts. The HR is a rapid localized death of the host cells that occurs upon pathogen infection and, together with the expression of a complex array of defense-related genes, is a component of plant resistance. The *hrp* genes were first identified in *Pseudomonas syringae* pv. phaseolicola, a bean pathogen (38). Since then, *hrp* genes from a variety of plant pathogenic bacteria, including *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas*, have been characterized (for reviews, see references 2, 9, and 11). The specific functions of the *hrp* pathway in pathogenesis are not known. However, type III secretion pathways of animal and plant pathogens have been demonstrated to mediate the secretion of virulence factors into the extracellular melieu. Some of the proteins ultimately end up in the host cell cytoplasm (reviewed in references 11, 25, and 37). In mediating the interaction of the bacterium and the host plant, the *hrp* pathway presumably acts to prevent or inhibit a general resistance response or otherwise enhance the colonization of the plant by the bacteria.

Given the importance of the type III systems to pathogenicity, it can be expected that analysis of the systems in different species will provide insight into the adaptation of the species to their respective host plants. The *hrp* gene clusters appear to group into two types on the basis of sequence relatedness and operon organization (reviewed in reference 9). The *hrp* genes of *Pseudomonas* and *Erwinia* comprise one group, and the *hrp* genes of *Xanthomonas campestris* pv. vesicatoria and *Ralstonia solanacearum* comprise the second group. Our present understanding of the group 2 *hrp* genes is based almost entirely on the characterization of two strains representing *R. solanacearum* and *X. campestris* pv. vesicatoria. The genus *Xanthomonas* itself is comprised of a large number of different species and pathovars that colonize over 392 species of plants (36). *Xanthomonas oryzae* pv. oryzae is the causal pathogen of bacterial leaf blight on rice (54). We report here the cloning of a *hrp* cluster from *X. oryzae* pv. oryzae and sequence analysis of the left end of the region. In the process of characterization, we identified two novel loci that are associated with the *hrp* cluster and an insertion sequence (IS) element not previously characterized in *X. oryzae* pv. oryzae.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in the experiments are listed in Table 1. *Escherichia coli* strains were grown in Terrific broth (TB) or on Luria agar plates at 37°C with the appropriate antibiotics. *X. oryzae* pv. oryzae strains were cultured on tryptone sucrose agar or in TB at 28°C. The genomic library of PXO86 in pHM1 was described previously (24). Carbenicillin, spectinomycin, and kanamycin were used at 100 μg/ml.

Recombinant DNA techniques. DNA manipulations were performed by standard procedures (6). Restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase I, and *Taq* polymerase were purchased from Life Technologies, Inc. (Gaithersburg, Md.) and Fisher Scientific (St. Louis, Mo.). Chemicals were purchased from Sigma Chemical (St. Louis, Mo.) and Fisher Scientific. BioTrace HP membrane (Gelman Sciences, Ann Arbor, Mich.) was used for Southern blot hybridization. *Eco*RI DNA fragments from p23-44, a cosmid clone containing *hrp* genes of *X. oryzae* pv. oryzae, were subcloned into pBluescript KS(+) (Stratagene, Inc., La Jolla, Calif.). The DNA sequences of the two strands of these subclones were determined by the DNA Sequencing Facility of Iowa State University. Amino acid alignments were constructed using Clustal W 1.7 (55). Similarity searches were performed using the BLAST program (4). Identity and similarity comparisons among proteins were performed using the Fasta program (45). Potential signal peptides at the N-terminal and transmembrane domains of the proteins were predicted by PSORT (41, 42) and TopPred2 (60),

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TABLE 1. Strains and plasmids used in study

Strain, phage, or plasmid	Relevent characteristics	Reference or source
Strains		
X. oryzae pv. oryzae		
PXO86	Wild-type strain, race 2	24
PXO99^A	5-Azacytidine resistant, race 6	24
PXO99^{A}2785mx	GPS-1 insertion in *hrpA*, Kn^r	This study
PXO99^{A}3230mx	GPS-1 insertion in *hrpA*, Kn^r	This study
PXO99^{A}8mx	*hrpB* mutant of PXO99^A, Kn^r	This study
PXO99^{A}9mx	*hrpC* mutant of PXO99^A, Kn^r	This study
PXO99^{A}56mx	*hrpD* mutant of PXO99^A, Kn^r	This study
PXO99^{A}Δ2	PXO99^A with 2-kb *Sal*I/*Xho*I deletion, Kn^r	This study
E. coli		
DH5α	F$^-$ *recA* ϕ80*dlacZ* ΔM15	Gibco-BRL
S17-1	*recA* Tra$^+$ Sp^r	53
TB1	*recA*$^+$ *C2110, Nal*^{r} *Rif*^{r} *polA1 rha his*	35
Phage λA1	Tn*5-gusA1* Kn^r Tet^r	51
Plasmids		
pBluescript II KS(+)	Phagemid, pUC derivative, Cb^r	Stratagene
pCR2.1-TOPO	Phagemid, Cb^r	Invitrogen
pUC4K	pUC7 with *nptII*	58
pHM1	Broad-host-range vector with pUC19 polylinker, Sp^r	R. Innis
p2-2	Cosmid clone of *X. oryzae* pv. oryzae *hrp* region	This study
p23-14	Cosmid clone of *X. oryzae* pv. oryzae *hrp* region	This study
p23-44	Cosmid clone of *X. oryzae* pv. oryzae *hrp* region	This study
pFWX10-F2	*avrXa10* in pHM1	65
pK4.0	4.0-kb *Kpn*I fragment from p23-44	This study
pK6.0	6.0-kb *Kpn*I fragment from p23-44	This study
pK6.0B	*Bgl*II/*Bam*HI deletion of pK6.0	This study
pK6.0BΔ2	2.0-kb *Sal*I/*Xho*I fragment replaced by 1.3-kb *Sal*I Kn^r fragment in pK6.0B	This study
p23-44Δ2	Cosmid with 2.0-kb *Sal*I/*Xho*I deletion, Kn^r	This study
pK6.0B-479	GPS-1 insertion at position 479, Kn^r	This study
pK6.0B-1116	GPS-1 insertion at position 1116, Kn^r	This study
pK6.0B-1139	1.3-kb fragment from pUC4K, Kn^r	This study
pK6.0B-1795	GPS-1 insertion at position 1795, Kn^r	This study

respectively. Specific motif searches were performed using the MOTIF program (43).

Transposon and deletion mutagenesis. Mutagenesis of p23-44 with Tn*5-gusA1* was performed in *E. coli* strain DH5αMCR. Cells growing exponentially in TB were infected with bacteriophage pλA1 carrying Tn*5-gusA1* (51). After 1 h of incubation at 37°C, which allowed the phage to be absorbed and phenotypic expression of kanamycin resistance, bacteria were plated on Luria agar medium supplemented with kanamycin and incubated overnight at 28°C. Plasmid DNA was extracted from single colonies, digested with *Eco*RI, and analyzed by electrophoresis on 1% agarose gels. Each plasmid with a transposon in the bacterial genomic DNA of the clone was transformed into PXO99^A by electroporation or by conjugation using the conjugal helper strain S17-1 (53). Marker exchange mutagenesis was performed using spontaneous homologous recombination and screening for kanamycin-resistant, spectinomycin-sensitive clones. Mutations were confirmed by Southern blot analysis of bacterial genomic DNA using ^{32}P-labeled probes for subclones of p23-44. Four isolates from each marker exchange were tested on tomato and rice plants. The p23-44 cosmid was reintroduced by electroporation into the *hrp* mutants for complementation tests.

Mutations in *hpa1*, *hpa2*, and *hrpA* were made using GPS-1 of the Genome Priming System (New England BioLabs, Beverly, Mass.). GPS-1 contains a modified Tn*7* with the *nptII* gene for resistance to kanamycin, and insertions were generated in vitro in pK4.0 and pK6.0B according to the instructions of the manufacturer (14). One mutation in *hpa1* was generated by introducing the gene for resistance to kanamycin from pUC4K into the *Eco*RI site of *hpa1* (58). Marker exchange mutations of *hrpA* were created by first introducing the GPS-1 mutations into p23-44 in *E. coli*. Recombinants were generated by introduction of both plasmids into the Rec$^+$ strain of *E. coli* TB1. Recombinant plasmids were rescued by electroporation into *E. coli* strain C2110 (35), which is deficient in polymerase I activity and does not permit replication of ColE1 replicons (pK4.0 or pK6.0B), and selection for resistance to spectinomycin and kanamycin. The resulting recombinant cosmids were transformed into *E. coli* strain S17-1 and moved from S17-1 into the PXO99^A strain of *X. oryzae* pv. oryzae by biparental mating. Four colonies were selected for marker exchange mutagenesis as described above.

The deletion in the left end of the *hrp* cluster covering *hpa1* and *hpa2* was created by replacing a 2.1-kb *Sal*I/*Xho*I fragment in pK6.0B with 1.3-kb *Sal*I fragment containing a kanamycin resistance gene from pUC4K (58). The resulting plasmid, pK6.0BΔ2, was introduced into p23-44 by homologous recombination in *E. coli* as described above for the GPS-1 *hrpA* mutations. The resulting cosmid, p23-44Δ2, was first transformed into *E. coli* strain S17-1 and then moved from S17-1 into *X. oryzae* pv. oryzae PXO99^A by biparental mating. Twenty colonies that were sensitive to spectinomycin and resistant to kanamycin after growth on nonselective media were obtained and tested for virulence and HR on rice and tomato.

Plant assays. Pathogenicity and hypersensitivity assays were performed as described previously (24). Tomato cv. VFN8 was used for the nonhost hypersensitivity test. Ten-day-old rice seedlings IRBB10 and IRBB7, containing corresponding resistance genes *Xa10* and *Xa7*, respectively, were used for race-specific resistance assays. IR24 was used for pathogenicity testing. All plants were grown in growth chambers at 28°C (daytime) and 25°C (nighttime) with a 14-h photoperiod and 85% humidity. For *hrp* phenotype assays, inoculum concentrations were adjusted to an optical density at 600 nm of 1.0 (approximately 2×10^9 CFU/ml) using a DU-64 spectrophotometer (Beckman Instruments). Inoculum concentrations for *hpa1* and *hpa2* mutation phenotype assays were adjusted to an optical density at 600 nm of 0.3. The differences between wild-type and mutant pathogenicity and hypersensitivity reactions were enhanced at the lower dilution. Growth of bacteria on rice after infiltration was monitored as previously described (24).

Sequence analysis of PXO99^A. A 651-bp region was amplified by PCR from PXO99^A using the primers 5′-GATTGTCTGCGGAAAATAG-3′ (IS99FOR) and 5′-GGTACGCAGCAGATCTGGG-3′ (IS99REV), cloned into pCR2.1-TOPO (52), and sequenced. The parameters used for PCR were as follows: step 1, 95°C for 2 min; step 2, 50°C for 30 s; step 3, 72°C for 90 s; step 4, 95°C for 30 s; step 5, 35 cycles from step 2 to step 4; step 6, 72°C for 2 min.

Southern hybridization analysis. Total DNA isolation and Southern hybridization analysis were as previously described (34). Probes were prepared by either PCR (49) or gel purification and random priming (6). The 408-bp *Eco*RI/*Xho*I fragment from *hpa1* was used as a probe for *hpa1*-related sequences. A 321-bp probe for *hpa1* was generated by PCR using internal primers 5′-AACA GGATCCAGATTGCTTCGAAGAGGCTGCC-3′ (HPA2F1) and 5′-AACAA

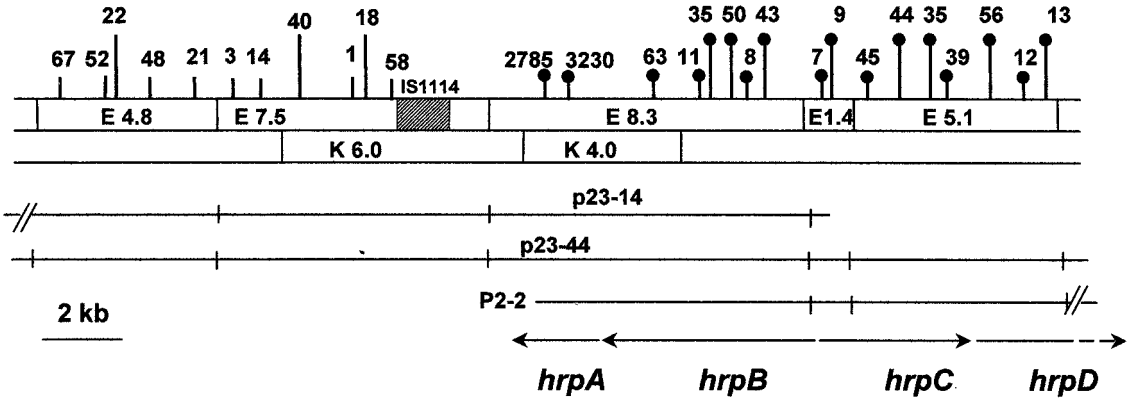

FIG. 1. Restriction fragment map of the *hrp* region in *X. oryzae* pv. oryzae. The name of each fragment also indicates the size in kilobases. Vertical lines above the map indicate positions of Tn*5-gusA1* insertion. Lines with filled circles indicate that the insertion abolished *hrp* function; lines without circles indicate insertions that did not cause loss of *hrp* activity. Arrows indicate positions and orientations of *hrp* operons. E, *Eco*RI; K, *Kpn*I.

GGATCCGCATATTTATCACGCTCC-3′ (HPA2R1). A 1522 base pair probe for IS1114 was amplified using primers 5′-AGTCGCCCCTGAAAAACCCCCA G-3′ (ISHRPF1) and 5′-AAGTCGCCCCTGAAAAACCCTC-3′ (ISHRPR1). PCR conditions for both probes were as described above. Probes were labeled using a Rediprime random-priming labeling kit (Amersham, Arlington Heights, Ill.).

Nucleotide sequence accession numbers. The DNA sequence for the *hpa2*-to-*hrpB* region has GenBank accession no. AF232057. The sequence for the region including IS*1114* from PXO86 is under GenBank accession no. AF232058. The sequence of the corresponding region of IS*1114* insertion in PXO99^{A} is under GenBank accession no. AF232714.

RESULTS

Isolation of the *hrp* region of *X. oryzae* pv. oryzae. Cosmids p23-14, p23-44, and p2-2 were recovered from a genomic library of strain PXO86 by using p83-15, which contained a portion of the *X. campestris* pv. vesicatoria *hrp* region (10), as a probe. The maps of the clones were characterized by endonuclease restriction digests, Southern blotting, and DNA sequence analysis and shown to cover the regions corresponding to *hrpA*, *hrpB*, *hrpC*, and *hrpD* of *X. campestris* pv. vesicatoria (Fig. 1). Thirty-five strains with Tn*5-gusA1* insertions in the region covered by p23-44 were generated from strain PXO99^{A} of *X. oryzae* pv. oryzae. Eleven strains, with insertions in the 4.8- and 7.5-kb *Eco*RI fragments, had unaltered *hrp* gene function. Twenty-four strains, with insertions in the 8.3-, 1.4-, and 5.1-kb *Eco*RI fragments, lost the ability to elicit disease on rice and an HR on tomato (Fig. 1). The mapping data indicated that the insertions in *hrp* loci were located in the *hrpB*, *hrpC*, and *hrpD* operons. None of the Tn*5-gusA1* insertions appeared to have inserted into the *hrpA* operon. Therefore, two insertions in *hrpA* were generated in pK4.0 by using pGPS-1 and recombined into the *hrp* region (Fig. 1, insertions 3230 and 2785). Both mutations resulted in *hrp* mutant phenotypes. The phenotypes of a representative *hrp* mutant after inoculation on rice and tomato are shown in Fig. 2A. Introduction of p23-44 into selected *hrp* mutants restored the ability to elicit disease on rice and an HR on tomato (Fig. 2A). In planta growth of a representative *hrp* mutant was reduced compared to that of the wild-type strain, while the levels of in planta growth of the mutant strains carrying p23-44 were similar to that of the parent strain PXO99^{A} (Fig. 2B).

X. oryzae pv. oryzae interacts with rice plants in a race-specific manner. PXO99^{A} with *avrXa10* or *avrXa7* elicits an HR on rice cultivars with the corresponding resistance gene *Xa10* or *Xa7* (24). Three *hrp* mutants of *X. oryzae* pv. oryzae, PXO99^{A}8mx, PXO99^{A}9mx, and PXO99^{A}56mx, whose inser-

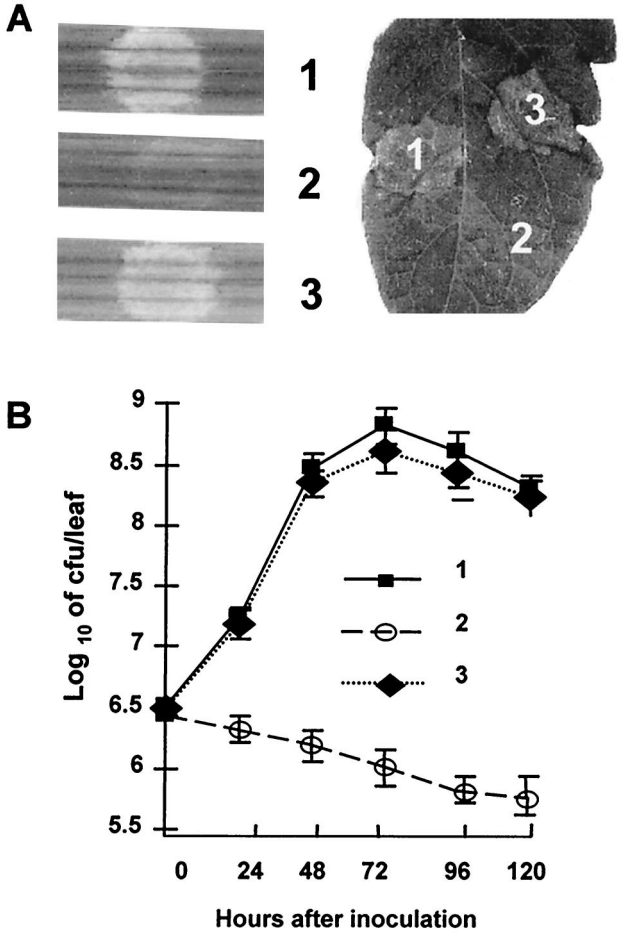

FIG. 2. Effects of mutations in the *hrp* genes of *X. oryzae* pv. oryzae. (A) Phenotype of rice (cultivar IR24) and tomato leaf (VFN8) reactions to the following strains: 1, PXO99^{A}; 2, PXO99^{A}9mx (*hrpC*); 3, PXO99^{A}9mx (p23-44). Water soaking and disease symptoms were evidenced by discoloration at the inoculation site on rice leaves (left). Inoculation in the *hrp* mutants resulted in no change in leaf coloration (leaf 2). HR on tomato leaf (right) is indicated by light gray patches (leaves 1 and 3). (B) Effect of *hrp* mutation on growth of bacteria in rice leaves. Numbering as for panel A.

TABLE 2. Phenotypes of interaction between *X. oryzae* pv. oryzae strains and plants

Strain	Phenotype[a] on:	
	Rice (IRBB10[b])	Tomato
PXO99^A	WS	HR
PXO99^{A}8mx	NR	NR
PXO99^{A}9mx	NR	NR
PXO99^{A}56mx	NR	NR
PXO99^{A}8mx(p23-44)	WS	HR
PXO99^{A}9mx(p23-44)	WS	HR
PXO99^{A}56mx(p23-44)	WS	HR
PXO99^A(pFWX10-F2)	HR	HR
PXO99^{A}8mx(pFWX10-F2)	NR	NR
PXO99^{A}9mx(pFWX10-F2)	NR	NR
PXO99^{A}56mx(pFWX10-F2)	NR	NR

[a] WS, water soaking (pathogenesis reaction); NR, no reaction.
[b] IRBB10 is rice cultivar containing resistance gene *Xa10*.

tions could be mapped in the *hrpB*, *hrpC*, and *hrpD* operons, respectively, were unable to elicit a race-specific HR when carrying *avrXa10* (Table 2). Identical results were obtained with *avrXa7* (data not shown).

Two novel *hrp*-associated loci are located in the left end of the *hrp* cluster. The DNA sequence was determined for 12,165 bp from the *Bgl*I site in the 7.5-kb *Eco*RI fragment (E7.5) and extending 713 bp into the 1.4-kb *Eco*RI fragment of p23-44 (Fig. 3). The sequence data were organized into two portions. The first portion, starting at the second *Sal*I site in E7.5, contained 10,096 bp and included transcription units A and B and two additional open reading frames, tentatively termed *hpa1* and *hpa2* (Fig. 3). Table 3 gives the positions and properties of the noted features in the first portion. The second portion, containing 2,075 bp and the IS element IS*1114*, extended upstream from the *Sal*I site to a *Bgl*II site in the E7.5 fragment (Fig. 3).

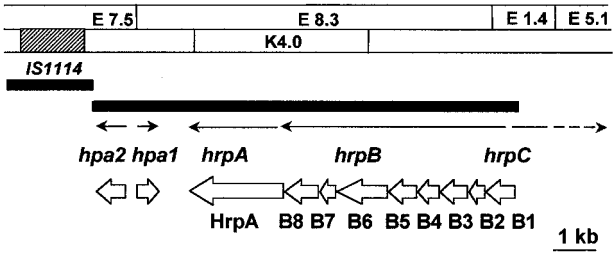

FIG. 3. Region of sequence analysis of the left *hrp* region of PXO86. Transcriptional units and open reading frames of the *hrp* genes are indicated by lines and open arrows, respectively. The direction of transcription and translation is indicated by direction of the arrow. Filled bars indicate sequenced regions. E, *Eco*RI; K, *Kpn*I.

The *hrpA* operon contained a single coding sequence for HrpA (HrcC, using the unified nomenclature [8]), which started at position 4123 and continued to position 2306. The sequence predicted a protein of 605 amino acids with 96% similarity to HrpA1 of *X. campestris* pv. vesicatoria (63). HrpA from *X. oryzae* pv. oryzae lacked two alanine residues corresponding to positions 295 and 296 in HrpA1 from *X. campestris* pv. vesicatoria (63) and was, therefore, two amino acids shorter than HrpA1 from *X. campestris* pv. vesicatoria. The *hrpB* locus of *X. oryzae* pv. oryzae contained eight coding regions extending from position 9820, which is the start of HrpB1, to the end of HrpB8 at 4211. The putative GTG start codon for HrpB1 was based on the alignment to HrpB1 of *X. campestris* pv. vesicatoria, which has the usual ATG start codon (18). A PIP box (PIP-3), which consists of two direct repeats (TTCGC) separated by 15 nucleotides, was located 81 bp upstream of the putative HrpB1-coding sequence and was similar to the PIP box found in *X. campestris* pv. vesicatoria (18) and *R. solanacearum* (21). PIP-3 was separated by 14 nucleotides from another PIP box (PIP-4) in the opposite direction. PIP-4 was

TABLE 3. Summary of features of the sequence of the left *hrp* region of *X. oryzae* pv. oryzae

Sequence position[a]	Feature	Properties (aa, MW)[b]	Relatedness[d]		Hrc nomenclature[e]	Flagellar homolog[f]
			Xcv	Rs		
569–129	Hpa2	146[c], 16.5				
884–908	PIP-1					
976–1000	PIP-2					
1136–1567	Hpa1	143, 14.0				
4123–2306	HrpA	605, 64.0	96/99	HrpA, 48/54	HrcC	
5036–4208	HrpB8	236[c], 24.9	96/99	HrpC, 46/81	HrcT	FliR
5542–5033	HrpB7	169, 18.8	95/100	HrpD, 25/60		
6863–5535	HrpB6	422, 47.2	98/99	HrpE, 59/89	HrcN	FliI
7554–6853	HrpB5	233, 25.3	100/100	HrpF, 25/54	HrcL	FliH[g]
8168–7539	HrpB4	209, 22.2	97/100	HrpH, 27/60		
8937–8176	HrpB3	232[c], 24.9	94/100	HrpI, 56/78	HrcJ	FliF
9331–8939	HrpB2	110[c], 11.8	98/100	HrpJ, 31/73		
9820–9365	HrpB1	151, 15.9	96/99	HrpK, 32/80		
9901–9926	PIP-3					
9941–9965	PIP-4					
10035	HrpC1	ND[h]				

[a] Nucleotide position according to GenBank accession no. AF232057.
[b] aa, number of amino acid residues; MW, molecular mass in kilodaltons.
[c] Protein size after cleavage of putative signal peptide.
[d] Relatedness to proteins of the same name from *X. campestris* pv. vesicatoria (Xcv) (17, 18) or to proteins from the *hrp* region of *R. solanacearum* (Rs) (21). The first number indicates the percentage of identical amino acid matches. The second number indicates the percentage of similar amino acid matches.
[e] Classification within the highly conserved members of the Hrp pathway proteins according to Bogdanove et al. (8).
[f] Related component of flagellar biosynthetic pathway (8).
[g] HrpB5 is weakly related to YscL (18) and BscL (64), which in turn are related to FliH (59).
[h] ND, not determined.

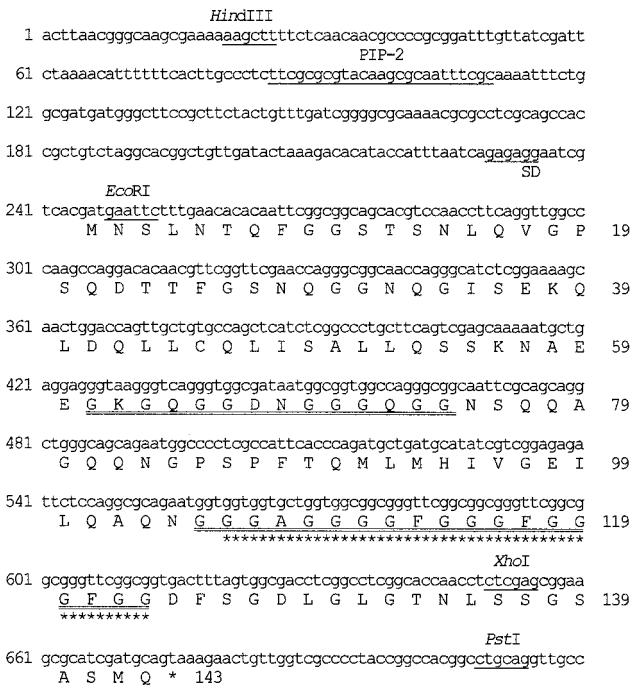

FIG. 4. DNA sequence of *hpa1*. Sequences for a PIP box, restriction sites, and a ribosome binding site (SD) are underlined. The sequence of the deduced translation product is given in the single-letter code below the DNA sequence. The glycine-rich regions are double underlined. Asterisks indicate a potential transmembrane motif.

located 155 nucleotides away from the start codon of the putative HrpC1-coding sequence. (Complete sequence analysis of the *hrpC* operon will be presented later.)

Sequence analysis at the left end of the 8.3-kb *Eco*RI fragment revealed an open reading frame for a protein of 143 amino acid residues, starting at position 1136, that had not previously been described for *Xanthomonas* and was tentatively named *hpa1* (Fig. 4). A perfect consensus PIP box was located at position 975 and 135 bp upstream of *hpa1*. The putative protein encoded by *hpa1* is glycine rich (26% glycine), particularly in the middle and C-terminal portions, and has no high degree of sequence similarity to proteins in the databases.

The *hpa2* open reading frame was oriented in the opposite direction to *hpa1*. The putative protein product has a high degree of similarity at the amino acid level to a group of lysozyme-like proteins starting at position 569 (Fig. 5A). An imperfect PIP box (with C replaced by a T in the second TTCGC consensus repeat) was located upstream of *hpa2* at position 884 and 165 bp upstream from the first ATG (Fig. 5B). The putative start codon for Hpa2 was unclear. The first upstream ATG was 150 bp from the region of similarity to lysozyme-like proteins (Fig. 5B). A consensus secretion signal sequence was identified within 54 bp upstream from the start of the sequence similarity to lysozyme-like proteins (Fig. 5B). The putative cleavage site was predicted to occur immediately before the start of the sequence similarity.

An IS element is located adjacent to *hpa2* in PXO86. An IS element was identified 254 bp from the end of *hpa2*. The element is 1,519 bp long and bounded by an 18-bp perfect inverted repeat. IS*1114* was similar in sequence to two recently described insertion elements from *Rhizobium* and *X. campestris* pv. campestris (Fig. 6A) (13, 50). The repeat was located within a 6-bp direct repeat (TAAAA) that may have been generated by the insertion process (Fig. 6B). Strain PXO99^A did not contain IS*1114* adjacent to *hpa2*, and the sequence analysis of the same region revealed only the TAAAA sequence with no duplication (Fig. 6B). The facts that the TAAAA sequence, which is duplicated at the ends of IS*1114* in PXO86, was not duplicated in PXO99^A and that no remnant of IS*1114* was found at the corresponding region of PXO99^A suggest that the insertion is unique to the PXO86 lineage. The sequence from the *Bgl*II site to the end of *hpa2* without the IS element did not match any entries in the GenBank database. Therefore, the element also does not appear to have inserted into an identifiable genetic locus. Genetic loci that are involved in pathogenicity have been associated with transmissible genetic elements (30). These so-called pathogenicity islands may also have G+C compositions that differ from the G+C content of the bulk of the chromosomal DNA, reflecting the transfer among different species of bacteria (reviewed in reference 22). However, no evidence for a dramatic shift in the G+C content at the element's boundary with the *hrp* region was observed. We therefore could not find that the element had any relevance to *hrp* function. We believe that IS*1114* is present near the *hrp* region in some lineages and probably inserted into the region by chance.

Deletion of the left end affects virulence. A deletion mutant (PXO99^{A}Δ2) covering the *hpa1-hpa2* region was constructed by replacing the region between the *Sal*I and *Xho*I sites of pK6.0B with the gene for resistance to kanamycin and subsequent recombination into the chromosome of PXO99^A to give strain PXO99^{A}Δ2 (58). PXO99^{A}Δ2 was therefore missing *hpa2* entirely, and *hpa1* was truncated. Upon inoculation to rice, PXO99^{A}Δ2 was found to cause reduced disease symptoms and, when harboring *avrXa10*, elicited a weak HR on rice plants with resistance gene *Xa10* in terms of the intensity of browning (Fig. 7A). The reduced disease symptoms were accompanied by reduced bacterial populations in the leaf tissue (Fig. 7B). Similarly, the HR of the mutant on tomato was delayed and weaker in terms of the area that collapsed after inoculation (not shown). The reduced pathogenicity of the mutant could be complemented by reintroduction of p23-44 (Fig. 7B).

Insertions and one deletion were generated in pK6.0B, which by itself restored full pathogenicity to PXO99^{A}Δ2, and the mutants were tested for the ability to restore pathogenicity (Fig. 8). The insertions in *hpa2* (pK6.0B-479) and immediately downstream from *hpa1* (pK6.0B-1795) did not affect the ability of pK6.0B to restore water soaking to PXO99^{A}Δ2 (Table 4). Plasmids pK6.0B-1116 and pK6.0B-1139, on the other hand, failed to restore water soaking (Table 4). The insertion in pK6.0B-1116 was located 20 bp upstream of the *hpa1* start codon and interrupted the presumed promoter elements from the coding sequence of *hpa1*. The plasmid pK6.0B-1139 had an insertion at the *Eco*RI site in the coding sequence of *hpa1*. Thus, plasmids with *hpa1* mutations were unable to restore water soaking to PXO99^{A}Δ2, indicating that the loss of *hpa1* was the principal cause of the reduced virulence of PXO99^{A}Δ2.

Distribution of *hpa1*, *hpa2*, and IS*1114* in species of *Xanthomonas*. To determine the prevalence of the newly identified elements in a variety of other strains of *X. oryzae* pv. oryzae and *X. campestris*, gene-specific and element-specific probes were generated from *hpa1*, *hpa2*, and IS*1114* by PCR or gel purification of an internal restriction fragment. The probes were then used in Southern analyses of genomic DNA. Both *hpa1* and IS*1114* hybridized to DNAs from all of the strains of *X. oryzae* pv. oryzae and the one strain of *X. oryzae* pv. oryzicola that were tested (Fig. 9A and B, lanes 1 to 5). Signal for *hpa1* was detected in DNA from *X. campestris* pv. alfalfae KX-1, *X. campestris* pv. malvacearum H, and *X. campestris* pv. phaesoli

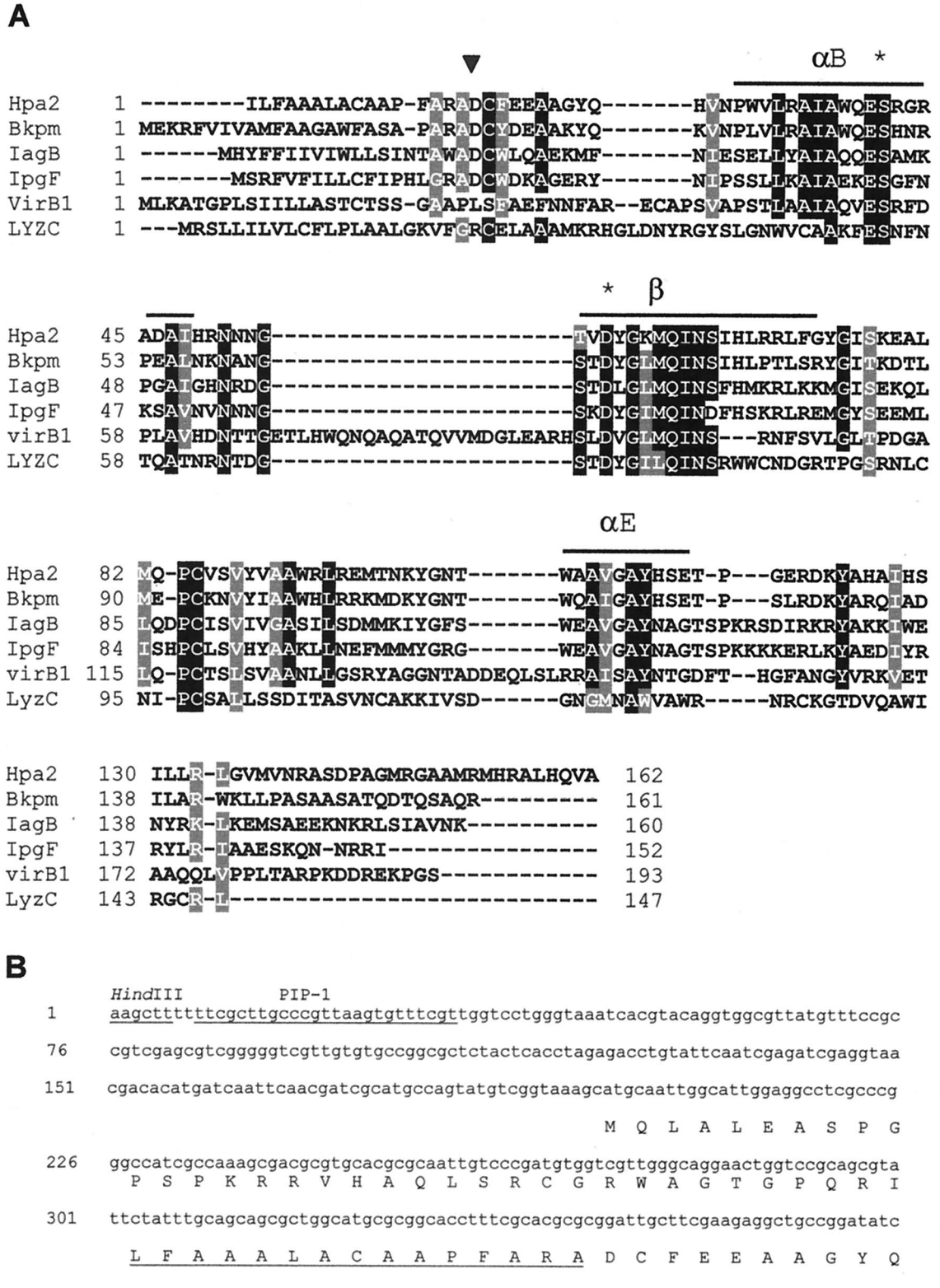

FIG. 5. Sequence analysis of *hpa2*. (A) Sequence alignment of Hpa2 and selected proteins. Bkpm, *Burkholderia pseudomallei* (GenBank accession no. AAD05172); IagB, *Salmonella enterica* serovar Typhimurium (39); IpgF, *Shigella flexneri* (3); VirB1, *Agrobacterium tumefaciens* (40); LyzC, chicken (28). The conserved A and E α-helices (αB and αE) and β-sheet (β) in the structure of lysozyme are overlined. Asterisks indicate conserved asparagine and aspartate residues in the catalytic region of lysozyme. Gaps in the alignment are represented by dashes. Hpa2 is shown as starting from the isoleucine that is 16 residues upstream of the putative cleavage site. The triangle indicates the putative cleavage site. (B) Promoter region of *hpa2*. A *Hin*dIII site and imperfect PIP box (PIP-1) are underlined. Only amino acids starting from the last methionine before the conserved region are indicated. The predicted signal peptide in the protein product is underlined.

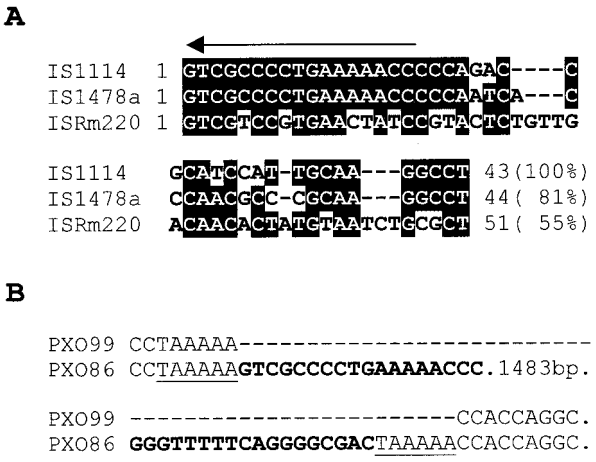

FIG. 6. Sequence analysis of IS*1114*. (A) Sequence similarity of IS*1114* to insertion elements IS*1478a* (13) and IS*Rm220-13-5* (50). The alignment shows only 43 to 51 bases of sequence from one end, with the overall sequence identity of the entire element to IS*1114* given in parentheses. The arrow indicates an 18-bp repeat. Identities in two of the three elements are shaded. (B) Sequence alignment of genomic DNA from strain PXO99^A with the region of insertion in strain PXO86. Only the 18-bp direct repeats are shown (boldface). Dashes indicate spaces introduced for optimal alignment. A direct 6-bp repeat at the site of insertion in PXO86 is underlined.

SC-4A and was not detected in *X. campestris* pv. vesicatoria 81-23, *X. campestris* pv. campestris KXXC-1, or *X. campestris* pv. holcicola (Fig. 9A, lanes 6 to 11). Outside *X. oryzae*, IS*1114* was detected only in *X. campestris* pv. campestris KXXC-1 and *X. campestris* pv. malvacearum H (Fig. 9B, lanes 7 and 10). In contrast to *hpa1* and IS*1114*, *hpa2* was detected in DNAs from all of the tested strains (Fig. 9C).

DISCUSSION

The results presented here demonstrate that a type III secretory system, also known as the *hrp* system, has a critical role for pathogenicity of *X. oryzae* pv. oryzae on rice. Type III secretory systems play central roles in the ability of many gram-negative bacteria to colonize plant and animal hosts. In general, the systems are envisaged to direct the assembly of a supramolecular secretory apparatus similar in structure to the flagellar apparatus, which is also the product of a type III secretory system. Some components of the type III systems are conserved due to the structural requirements, while other components can be expected to reflect the adaption of the system to the particular niche of the bacterium (26). The *hrpA* and *hrpB* operons represent part of the conserved core of genes necessary for the assembly of the *hrp* system. The predicted proteins of the *hrpA* and *hrpB* operons had 94% or higher amino acid residue identity with the proteins from *X. campestris* pv. vesicatoria (10, 57). HrpA is a member of the HrcC class of *hrp* proteins and is localized to the outer membrane, where it is thought to function the transporter past the outer membrane layer (63). As a class, HrpA proteins have similarity to a variety of proteins from other type III systems, and in fact, HrpA ancestry can be traced to other secretory systems (63).

HrpB3, HrpB5, HrpB6, and HrpB8 of the *hrpB* operon are related to components of other type III systems, including the flagellar assembly pathway (8). HrpB8 and HrpB3, for example, are similar in amino acid sequence to FliR and FliF, which have been determined to be components of the inner membrane basal body and M-ring portion of the flagellar secretory

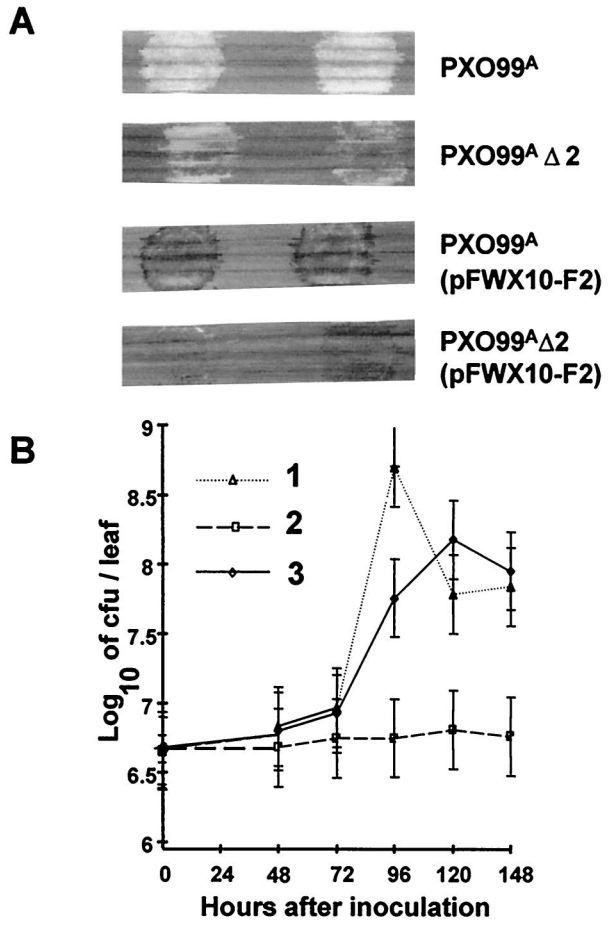

FIG. 7. Effect of an *hpa1-hpa2* deletion on virulence and *avrXa10* activity. (A) Phenotypes of PXO99^{A}Δ2 and PXO99^{A}Δ2(pFWX10-F2), which contains the avirulence gene *avrXa10*, on susceptible (IR24) and resistant (BB10) rice cultivars. Susceptible leaves (upper two leaves) and resistant leaves (lower two leaves) were photographed 3 days after inoculation with the strain indicated at the right. (B) Growth of PXO99^{A}Δ2 in susceptible rice cultivar IR24. 1, PXO99^A; 2, PXO99^{A}Δ2; 3, PXO99^{A}Δ2(p23-44).

apparatus, respectively (16, 27). HrpB1, HrpB2, HrpB4, and HrpB7 are proteins that, on the basis of sequence similarity, are unique to *Xanthomonas* and *R. solanacearum*. These proteins either have diverged from the ancestral secretion system or represent unique adaptations of the type III system in *Xanthomonas* and *R. solanacearum*. Divergence between the latter four proteins in *X. oryzae* pv. oryzae and *X. campestris* pv.

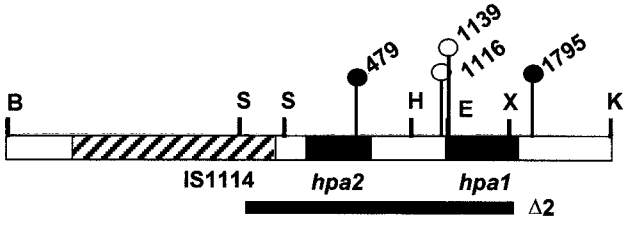

FIG. 8. Map of single-gene mutations in *hpa1* and *hpa2*. Insertion sites are indicated by lines with circles at the top. Filled circles indicate no loss of virulence; open circles indicate insertions that reduced virulence. The filled bar indicates the region missing in deletion Δ2. IS*1114* is indicated by the hatched bar. B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I; X, *Xho*I.

TABLE 4. Effects of mutations in the left end of the *hrp* region of *X. oryzae* pv. oryzae

Strain	Reaction[a]
PXO99^A(pHM1)	+
PXO99^{A}9mx(pHM1)	−
PXO99^{A}Δ2(pHM1)	±
PXO99^{A}Δ2(p23-44)	+
PXO99^A(pFWX10-F2)	HR
PXO99^{A}Δ2(pFWX10-F2)	WHR
PXO99^{A}Δ2(pK6.0B)	+
PXO99^{A}Δ2(pK6.0B-479)	+
PXO99^{A}Δ2(pK6.0B-1116)	±
PXO99^{A}Δ2(pK6.0B-1139)	±
PXO99^{A}Δ2(pK6.0B-1795)	+

[a] +, water-soaking reaction on rice comparable to wild type; −, no water soaking; ±, weak or delayed water-soaking symptoms; HR, HR on rice with *Xa10*; WHR, weak HR.

vesicatoria was no greater than that for proteins with recognizable counterparts in other type III systems, and they therefore appear not to have diverged along species lines as a possible consequence of adaptation to the particular host plants.

The *hrp* mutations in *X. oryzae* pv. oryzae resulted in the loss of pathogenicity and prevented the elicitation of the nonhost HR on tomato and the race-specific HR on incompatible rice plants due to the avirulence genes *avrXa10* and *avrXa7*, which are homologs of *avrBss3* from *X. campestris* pv. vesicatoria (24). Activity due to *avrBs3* was also lost when *X. campestris* pv. vesicatoria was *hrp* deficient (32), and dependence on *hrp* function for avirulence activity could be bypassed when *avrBs3* and other members of the family from *X. campestris* pv. malvacearum were expressed in the plant cells (15, 56). In a separate study, we have observed a 75% reduction in the number of transformation foci after particle bombardment of resistant rice leaves with a plant-expressed copy of *avrXa10* (66). Therefore, the protein products of *avrXa10* and *avrXa7* along with possible virulence factors are likely to be secreted by a *hrp*-encoded type III secretory apparatus into the cells of the rice plant.

Despite the high degree of similarity, the analysis of the left end of the *hrp* region of *X. oryzae* pv. oryzae revealed two genes, named *hpa1* and *hpa2*, that were not found in *X. campestris* pv. vesicatoria. The putative *hpa1* product has an amino acid composition similar to the compositions of the harpin proteins of *P. syringae* pathovars and *Erwinia* species and the harpin-like PopA protein of *R. solanacearum* (5, 20, 23, 61). These proteins share regions of high glycine content and are secreted via the type III pathway. Whether Hpa1 is secreted is unknown at present. Conditions for *hrp*-dependent secretion by *X. campestris* pv. vesicatoria have recently been determined (48). Thus, it may be possible to adapt the conditions to *X. oryzae* pv. oryzae and determine if the *hpa1* product is secreted in a *hrp*-dependent manner. Like *popA*, *hpa1* has a PIP box immediately upstream of the coding sequence and is likely to be regulated by the *hrpXo* gene product, which is a member of the AraC class of transcription factors (29, 44, 62).

Harpins gained attention by the fact that some harpins and PopA can elicit hypersensitive reactions on certain plants simply by injection of the proteins into the leaf tissue (5, 23, 46, 61). The protein product of *hrpW* has similarity to both harpin and pectate lyase (12, 20, 31). However, the elicitor properties are not shared by all harpins, and in the case of harpin from *P. syringae* pv. tomato, the protein elicited an HR on the host plant (46). Mutations in *hrpZ*, *hrpW*, and *popA* have no observable effect on the pathogenicity (1, 5, 12). Therefore, the biological relevance of the latter genes or the elicitor activities of their products is unknown. On the other hand, mutations in *hrpN*, from which harpins were originally named, abolished pathogenicity of *Erwinia amylovora* (61). Mutations in *hpa1* reduced pathogenicity due to *X. oryzae* pv. oryzae. *hpa1* is the only gene from *Xanthomonas* with a harpin-like product. However, sequence similarity with the *hrpW* gene from *P. syringae* pv. tomato in the DNAs from several *Xanthomonas* species was recently reported (12). Thus, *Xanthomonas*, like *P. syringae* and *Erwinia* species, may produce a variety of harpin-like proteins depending on the species or strain.

The role of the *hpa2* locus, which encodes a lysozyme-like protein, is unclear. However, an argument can be made that the locus is another core component of the type III system. The locus appears to be present in all of the species of *Xanthomonas* that were examined, and related genes can be found in association with a variety of secretory systems, including type III systems of animal pathogens. On the one hand, the mutation of *hpa2* had no apparent effect on pathogenicity under the conditions of the assay. Indeed, whether this protein is produced in vivo remains in question. The lack of a phenotype

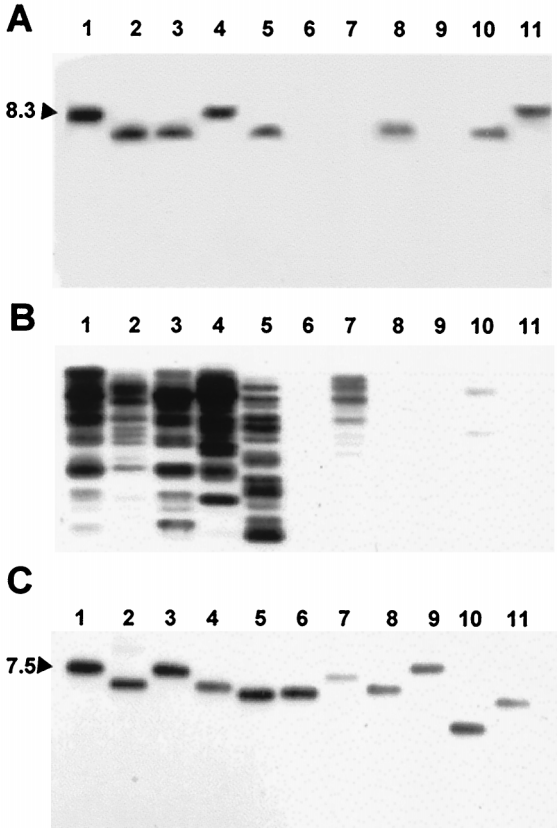

FIG. 9. Southern analysis of *hpa1*, *hpa2*, and IS*1114*. (A) DNA probed with the *Eco*RI/*Xho*I fragment of *hpa1*. (B) DNA probed with the PCR fragment from IS*1114*. (C) DNA probed with the PCR fragment from *hpa2*. Genomic DNA was extracted from 11 strains of *Xanthomonas* spp. and digested with *Eco*RI (A and B) or *Sal*I (C). Lanes 1, *X. oryzae* pv. oryzae PXO86; lanes 2, *X. oryzae* pv. oryzae PXO99^A; lanes 3; *X. oryzae* pv. oryzae PXO177; lanes 4, *X. oryzae* pv. oryzae C191; lanes 5, *X. oryzae* pv. oryzicola BLS 303; lanes 6, *X. campestris* pv. vesicatoria 81-23; lanes 7, *X. campestris* pv. campestris Kxxc1; lanes 8, *X. campestris* pv. alfalfae KX-1; lanes 9, *X. campestris* pv. holcicola; lanes 10, *X. campestris* pv. malvacearum H; lanes 11, *X. campestris* pv. phaesoli SC-4A. See Materials and Methods for PCR primers and conditions. Numbers at the left indicate kilobase pairs.

with the *hpa2* mutants may reflect the lack of a requirement for this gene under the conditions of testing or environment, yet the locus may be required under natural infection conditions. None of the lysozyme-related proteins appear to be essential for the related systems under the conditions of testing. Mutations in *virB1* severely reduced but did not eliminate tumor formation (40). Similarly, mutations in gene 19, which encodes a lysozyme-like protein in the R1-16 plasmid conjugation pathway, reduced but did not eliminate conjugative transfer of R1-16 (7). A mutation in *ipgF*, which is a locus in a type III pathway of *Shigella flexneri*, was reported to have no effect on pathogenicity (3). One obvious possibility for the function of the lysozyme-like proteins would be in the degradation of the peptidoglycan layer of the bacterial cell wall to accommodate the respective secretory apparatuses or associated pili (19, 33, 47). This possibility remains to be tested.

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REFERENCES

1. **Alfano, J. R., D. W. Bauer, T. M. Milos, and A. Collmer.** 1996. Analysis of the role of the *Pseudomonas syringae* pv. *syringae* HrpZ harpin in elicitation of the hypersensitive response in tobacco using functionally non-polar *hrpZ* deletion mutations, truncated HrpZ fragments, and *hrmA* mutations. Mol. Microbiol. **19:**715–728.
2. **Alfano, J. R., and A. Collmer.** 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: trafficking harpins, Avr proteins, and death. J. Bacteriol. **179:**5655–5662.
3. **Allaoui, A., R. Menard, P. J. Sansonetti, and C. Parsot.** 1993. Characterization of the *Shigella flexneri ipgD* and *ipgF* genes, which are located in the proximal part of the *mxi* locus. Infect. Immun. **61:**1707–1714.
4. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
5. **Arlat, M., F. Van Gijsegem, J. C. Huet, J. C. Pernollet, and C. A. Boucher.** 1994. PopA1, a protein which induces a hypersensitive-like response on specific Petunia genotypes, is secreted via the *Hrp* pathway of *Pseudomonas solanacearum*. EMBO J. **13:**543–553.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1991. Current protocols in molecular biology. Greene Publishing Associates-Wiley Interscience, New York, N.Y.
7. **Bayer, M., R. Eferl, G. Zellnig, K. Teferle, A. J. Dijkstra, E. Kordyum, G. Koraimann, and G. Hogenauer.** 1995. Gene 19 of Plasmid R1 is required for both efficient conjugative DNA transfer and bacteriophage R17 infection. J. Bacteriol. **177:**4279–4288.
8. **Bogdanove, A. J., S. V. Beer, U. Bonas, C. A. Boucher, A. Collmer, D. L. Coplin, G. R. Cornelis, H.-C. Huang, S. W. Hutcheson, N. J. Panopoulos, and F. Van Gijsegem.** 1996. Unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. Mol. Microbiol. **20:**681–683.
9. **Bonas, U.** 1994. *hrp* genes of phytopathogenic bacteria, p. 79–98. *In* J. L. Dangl (ed.), Bacterial pathogenesis of plants and animals. Springer-Verlag, Berlin, Germany.
10. **Bonas, U., R. Schulte, S. Fenselau, G. V. Minsavage, B. J. Staskawicz, and R. E. Stall.** 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.
11. **Bonas, U., and G. Van den Ackerveken.** 1997. Recognition of bacterial avirulence proteins occurs inside the plant cell: a general phenomenon in resistance to bacterial diseases? Plant J. **12:**1–7.
12. **Charkowski, A. O., J. R. Alfano, G. Preston, J. Yuan, S. Y. He, and A. Collmer.** 1998. The *Pseudomonas syringae* pv. tomato HrpW protein has domains similar to harpins and pectate lyases and can elicit the plant hypersensitive response and bind to pectate. J. Bacteriol. **180:**5211–5217.
13. **Chen, J. H., Y. Y. Hsieh, S. L. Hsiau, T. C. Lo, and C. C. Shau.** 1999. Characterization of insertions of IS*476* and two newly identified insertion sequences, IS*1478* and IS*1479*, in *Xanthomonas campestris* pv. *campestris*. J. Bacteriol. **181:**1220–1228.
14. **Craig, N. L.** 1996. Transposon Tn7. Curr. Top. Microbiol. Immunol. **204:** 27–48.
15. **de Feyter, R., H. McFadden, and L. Dennis.** 1998. Five avirulence genes from *Xanthomonas campestris* pv. *malvacearum* cause genotype-specific cell death when expressed transiently in cotton. Mol. Plant-Microbe Interact. **11:**698–701.
16. **Fan, F., K. Ohnishi, N. R. Francis, and R. M. Macnab.** 1997. The FliP and FliR proteins of *Salmonella typhimurium*, putative components of the type III flagellar export apparatus, are located in the flagellar basal body. Mol. Microbiol. **26:**1035–1046.
17. **Fenselau, S., I. Balbo, and U. Bonas.** 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.
18. **Fenselau, S., and U. Bonas.** 1995. Sequence and expression analysis of the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. Mol. Plant-Microbe Interact. **8:**845–854.
19. **Fullner, K. J., J. C. Lara, and E. W. Nester.** 1996. Pilus assembly by *Agrobacterium* T-DNA transfer genes. Science **273:**1107–1109.
20. **Gaudriault, S., M. N. Brisset, and M. A. Barny.** 1998. HrpW of *Erwinia amylovora*, a new Hrp-secreted protein. FEBS Lett. **428:**224–228.
21. **Gijsegem, F., C. Gough, C. Zischek, E. Niqueux, M. Arlat, S. Genin, P. Barberis, S. German, P. Castello, and C. Boucher.** 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.
22. **Hacker, J., G. Blum-Oehler, I. Muhldorfer, and H. Tschape.** 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23:**1089–1097.
23. **He, S. Y., H.-C. Huang, and A. Collmer.** 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.
24. **Hopkins, C. M., F. F. White, S. H. Choi, A. Guo, and J. E. Leach.** 1992. A family of avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. Mol. Plant-Microbe Interact. **5:**451–459.
25. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62:**379–433.
26. **Huguet, E., and U. Bonas.** 1997. *hrpf* of *Xanthomonas campestris* pv. *vesicatoria* encodes an 87-kDa protein with homology to NoIX of *Rhizobium fredii*. Mol. Plant-Microbe Interact. **10:**488–498.
27. **Jones, C. J., M. Homma, and R. M. Macnab.** 1989. L-, P-, and M-ring proteins of the flagellar basal body of *Salmonella typhimurium*: gene sequences and deduced protein sequences. J. Bacteriol. **171:**3890–3900.
28. **Jung, A., A. E. Sippel, M. Grez, and G. Schutz.** 1980. Exons encode functional and structural units of chicken lysozyme. Proc. Natl. Acad. Sci. USA **77:**5759–5763.
29. **Kamdar, H. V., S. Kamoun, and C. I. Kado.** 1993. Restoration of pathogenicity of avirulent *Xanthomonas oryzae* pv. *oryzae* and *X. campestris* pathovars by reciprocal complementation with the *hrpXo* and *hrpXc* genes and identification of HrpX function by sequence analyses. J. Bacteriol. **175:**2017–2025.
30. **Kearney, B., P. C. Ronald, D. Dahlbeck, and B. J. Staskawicz.** 1988. Molecular basis for evasion of plant host defense in bacterial spot disease of pepper. Nature **332:**541–543.
31. **Kim, J. F., and S. V. Beer.** 1998. HrpW of *Erwinia amylovora*, a new harpin that contains a domain homologous to pectate lyases of a distinct class. J. Bacteriol. **180:**5203–5210.
32. **Knoop, V., B. Staskawicz, and U. Bonas.** 1991. Expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. vesicatoria is not under the control of *hrp* genes and is independent of plant factors. J. Bacteriol. **173:** 7142–7150.
33. **Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S.-I. Aizawa.** 1998. Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. Science **280:**602–605.
34. **Leach, J. E., M. L. Rhoads, C. M. Vera Cruz, F. F. White, T. W. Mew, and H. Leung.** 1992. Assessment of genetic diversity and population structure of *Xanthomonas oryzae* pv. *oryzae* with a repetitive DNA element. Appl. Environ. Microbiol. **58:**2188–2195.
35. **Leong, S. A., G. S. Ditta, and D. R. Helinski.** 1982. Heme biosynthesis in *Rhizobium*. J. Biol. Chem. **257:**8724–8730.
36. **Leyns, F., M. De Cleene, J. G. Swings, and J. De Ley.** 1984. The host range of the genus *Xanthomonas*. Bot. Rev. **50:**308–356.
37. **Lindgren, P. B.** 1997. The role of *hrp* genes during plant-bacterial interactions. Annu. Rev. Phytopathol. **35:**129–152.
38. **Lindgren, P. B., R. C. Peet, and N. J. Panopoulos.** 1986. Gene cluster of *Pseudomonas syringae* pv. "phaseolicola" controls pathogenicity on bean plants and hypersensitivity on nonhost plants. J. Bacteriol. **168:**512–522.
39. **Miras, I., and D. Hermant.** 1995. Nucleotide sequence of *iagA* and *iagB* genes involved in invasion of HeLa cells by *Salmonella enterica* subsp. *enterica* ser. Typhi. Res. Microbiol. **146:**17–20.
40. **Mushegian, A. R., K. J. Fullner, E. V. Koonin, and E. W. Nester.** 1996. A family of lysozyme-like virulence factors in bacterial pathogens of plants and animals. Proc. Natl. Acad. Sci. USA **93:**7321–7326.
41. **Nakai, K.** 1991. Predicting various targeting signals in amino acid sequences.

Bull. Inst. Chem. Res. Kyoto Univ. **69:**269–291.
42. **Nakai, K., and M. Kanehisa.** 1991. Expert system for predicting protein localization sites in Gram-negative bacteria. Proteins Struct. Funct. Genet. **11:**95–110.
43. **Ogiwara, A., I. Uchiyama, T. Takagi, and M. Kanehisa.** 1996. Construction and analysis of a profile library characterizing groups of structurally known proteins. Protein Sci. **5:**1991–1999.
44. **Oku, T., A. M. Alvarez, and C. I. Kado.** 1995. Conservation of the hypersensitivity-pathogenicity regulatory gene hrpX of *Xanthomonas campestris* and *X. oryzae*. DNA Sequencing **5:**245–249.
45. **Pearson, W. R.** 1990. Rapid and sensitive sequence comparison with FASTP and FASTA. Methods Enzymol. **183:**63–98.
46. **Preston, G., H.-C. Huang, S. Y. He, and A. Collmer.** 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. Mol. Plant-Microbe Interact. **8:**717–732.
47. **Roine, E., W. Wei, J. Yuan, E.-L. Nurmiaho-Lassila, N. Kalkkinen, M. Romantschuk, and S. Y. He.** 1997. Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. Proc. Natl. Acad. Sci. USA **94:**3459–3464.
48. **Rossier, O., K. Wengelnik, K. Hahn, and U. Bonas.** 1999. The *Xanthomonas* Hrp type III system secretes proteins from plant and mammalian bacterial pathogens. Proc. Natl. Acad. Sci. USA **96:**9368–9373.
49. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
50. **Selbitschka, W., S. Zekri, G. Schroeder, A. Puehler, and N. Toro.** 1999. The *Sinorhizobium meliloti* insertion sequence (IS) elements ISRm102F34-1/ISRm7 and ISRm220-13-5 belong to a new family of insertion sequence elements. Fed. Eur. Microbiol. Soc. Microbiol. Lett. **172:**1–7.
51. **Sharma, S. B., and E. R. Signer.** 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn*5-gusA*. Genes Dev. **4:**344–356.
52. **Shuman, S.** 1994. Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. J. Biol. Chem. **269:**32678–32684.
53. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnology **1:**784–791.
54. **Swings, J., M. Van den Mooter, L. Vauterin, B. Hoste, M. Gillis, T. W. Mew, and K. Kersters.** 1990. Reclassification of the causal agents of bacterial blight (*Xanthomonas campestris* pv. *oryzae*) and bacterial leaf streak (*Xanthomonas campestris* pv. *oryzicola*) of rice as pathovars of *Xanthomonas oryzae* (ex Ishiyama 1922) sp. nov., nom. rev. Int. J. Syst. Bacteriol. **40:**309–311.
55. **Thompson, J. D., D. G. Higginsand, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
56. **Van den Ackerveken, G., E. Marois, and U. Bonas.** 1996. Recognition of the bacterial avirulence protein *AvrBs3* occurs inside the host plant cell. Cell **87:**1307–1316.
57. **Van Gijsegem, F., C. Gough, C. Zischek, E. Niqueux, M. Arlat, S. Genin, P. Barberis, S. German, P. Castello, and C. Boucher.** 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.
58. **Vieira, J., and J. Messing.** 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. Gene **100:**189–194.
59. **Vogler, A. P., M. Homma, V. M. Irikura, and R. M. Macnab.** 1991. *Salmonella typhimurium* mutants defective in flagellar filament regrowth and sequence similarity of FliI to F0F1, vacuolar, and archaebacterial ATPase subunits. J. Bacteriol. **173:**3564–3572.
60. **von Heijne, G.** 1992. Membrane protein structure prediction, hydrophobicity analysis and the positive-inside rule. J. Mol. Biol. **225:**487–494.
61. **Wei, Z.-M., R. J. Laby, C. H. Zumoff, D. W. Bauer, S. Y. He, A. Collmer, and S. V. Beer.** 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. Science **257:**85–88.
62. **Wengelnik, K., and U. Bonas.** 1996. HrpXv, an AraC-type regulator, activates expression of five of the six loci in the hrp cluster of *Xanthomonas campestris* pv. *vesicatoria*. J. Bacteriol. **178:**3462–3469.
63. **Wengelnik, K., C. Marie, M. Russel, and U. Bonas.** 1996. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. vesicatoria essential for pathogenicity and induction of the hypersensitive reaction. J. Bacteriol. **178:**1061–1069.
64. **Yuk, M. H., E. T. Harvill, and J. F. Miller.** 1998. The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. Mol. Microbiol. **28:**945–959.
65. **Zhu, W., B. Yang, J. M. Chittoor, L. B. Johnson, and F. F. White.** 1998. AvrXA10 contains an acidic transcriptional activation domain in the functionally conserved C terminus. Mol. Plant-Microbe Interact. **11:**824–832.
66. **Zhu, W., B. Yang, N. Wills, L. B. Johnson, and F. F. White.** 1999. The C terminus of AvrXa10 can be replaced by the transcriptional activation domain of VP16 from the herpes simplex virus. Plant Cell **11:**1665–1674.