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Expression and Localization of HrpA1, a Protein of *Xanthomonas campestris* pv. vesicatoria Essential for Pathogenicity and Induction of the Hypersensitive Reaction

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The *hrp* cluster of the pepper and tomato pathogen *Xanthomonas campestris* pv. vesicatoria is required for both pathogenicity on susceptible host plants and induction of the hypersensitive reaction on resistant plants. The *hrpA* locus is located at the left end of the 25-kb *hrp* region and encodes a single 64-kDa Hrp protein, HrpA1, which belongs to the PulD superfamily of proteins involved in type II and type III protein secretion. In this study, we developed a defined medium without any plant-derived molecules that induces expression of *hrpA* in vitro. The *hrpA* transcription start site was mapped in the coding region of the *hrpB8* gene, which is the last gene of the *hrpB* operon. The inducible *hrpA* promoter shows no homology to known promoter elements or other *hrp* loci of *X. campestris* pv. vesicatoria. *hrpA* expression was shown to be independent of the *hrp* regulatory gene *hrpX*. The amino acid sequence of the HrpA1 protein is predicted to contain an N-terminal signal sequence and no further transmembrane domains and to be rich in β-sheet stretches. Expression of HrpA1 in *Escherichia coli* cells causes induction of the *psp* operon like some of its counterparts, suggesting some commonality of function and that HrpA1 forms multimers. The protein product of *hrpA1* was identified by using a specific polyclonal antibody. Cell fractionation studies demonstrated that the HrpA1 protein is localized in the outer membrane of *X. campestris* pv. vesicatoria. HrpA1 is the first component of the Hrp secretion system whose localization has been determined in the original organism.

Xanthomonas campestris pathovar vesicatoria is the causal agent of bacterial spot disease of pepper and tomato plants. Successful infection of a plant with *X. campestris* pv. vesicatoria, i.e., colonization of the intercellular spaces, is dependent on the *hrp* gene cluster (12). With *hrp* mutant strains, a pleiotropic phenotype is observed. The bacteria no longer cause any obvious macroscopic symptoms in a susceptible host plant, nor do they induce the hypersensitive reaction (HR) in resistant host or in nonhost plants. The HR is the result of a primary defense response leading to necrosis of the infected tissue (40). *hrp* genes have been identified in most major gram-negative plant-pathogenic bacteria by complementation of nonpathogenic mutants generated by transposon or chemical mutagenesis (reviewed in references 11 and 72). In *X. campestris* pv. vesicatoria, the *hrp* genes map to a 25-kb chromosomal region and are organized in six genetic loci, designated *hrpA* to *hrpF*. Transposon mutations in any one of the six *hrp* loci abolish symptom formation in susceptible and resistant host plants (12). When Tn*3-gus* insertions which carry a promoterless β-glucuronidase (GUS) gene as reporter gene were used, the expression of the *hrp* loci was found to be induced in planta and under certain in vitro conditions but suppressed in complex NYG (19) medium and in several minimal media tested (60, 61). Until now, and in contrast to other phytopathogenic bacteria, a defined medium for *hrp* gene induction in *X. campestris* pv. vesicatoria has not been available.

Sequence analysis gave some initial indications as to what the biochemical function of the *hrp* genes might be. Similarities between several predicted Hrp proteins, including HrpA1 of *X. campestris* pv. vesicatoria and proteins of mammalian pathogens, for example, Ysc and Spa/Mxi proteins of *Yersinia* spp. and *Shigella flexneri*, respectively, suggested that some Hrp proteins may be involved in protein secretion (23, 65). A novel type of protein secretion, the type III pathway, originally described for *Yersinia enterocolitica*, involves proteins that are thought to form a tunnel or a pore through which certain proteins lacking a typical signal sequence at their amino termini are specifically secreted without processing (18, 46). For *Erwinia amylovora* (68, 69), *Pseudomonas syringae* pv. syringae (30), and *Pseudomonas solanacearum* (7), Hrp-dependent secretion of proteins which induce the HR in nonhost plants has indeed recently been shown.

The HrpA1 protein also shows similarity to components of a second protein secretion system, the type II pathway, originally discovered in *Klebsiella oxytoca* (54), which secretes specific target proteins across the outer membrane to the extracellular milieu. While the other proteins that constitute the type II pathway are unrelated to those of the type III pathway described above, the PulD homologs are common to both. This protein family also includes a protein encoded by filamentous bacteriophages from *Escherichia coli* and *Pseudomonas aeruginosa* which is necessary for phage assembly and secretion. This protein, pIV, forms a large multimer in the outer membrane, induces the *psp* (stands for phage shock protein) operon, and has been proposed to form an exit channel through which the phage passes (39, 56). Functional similarities between pIV and several of the type II homologs suggest that the bacterial proteins also form export channels.

Hence, one function of *hrp* genes is most likely to establish a specific protein translocation apparatus in the bacterial envelope that is expressed only in the interaction with the host or

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TABLE 1. Strains and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristic(s)	Reference or source
Strains		
X. campestris pv. vesicatoria		
85-10	Pepper race 2; wild type; Rif^r	13
85E	*eps*::Tn*3-gus* insertion mutant of 85-10; *hrp*$^+$ Rif^r Km^r	K. Wengelnik
85EDAD	*hrpA* to *D4* deletion mutant of 85E; Rif^r Km^r Spec^r	This study
85X	hrpX insertion mutant of 85E; Rif^r Km^r Spec^r	K. Wengelnik
75-3	Tomato pathogenic; wild type; Rif^r	47
85-10::*hrpA* 22	*hrpA* mutant; Rif^r Km^r	60
E. coli		
DH5α	F$^-$ *recA* f80*dlacZ* DM15	Bethesda Research Laboratories, Bethesda, Md.
K561	HfrC *lacI*^{q}	39
Phage		
f1	Wild-type isolate	75
Plasmids		
pBluescript II KS	Phagemid, pUC derivative; Ap^r	Stratagene, La Jolla, Calif.
pGEX-2T	Protein expression vector; Ap^r	Pharmacia, Uppsala, Sweden
MAL-2p	Protein expression vector; Ap^r	New England Biolabs, Beverly, Mass.
pLAFR3	RK2 replicon Mob$^+$ Tra$^-$ Tc^r; contains p*lac*	63
pLAFR6	RK2 replicon Mob$^+$ Tra$^-$ Tc^r; multicloning site flanked by transcriptional terminators	13
pL6GUSB	Carries promoterless *gusA* gene in pLAFR6	42
pLA10	3.3-kb *hrpA1* fragment in pLAFR3, expressed under control of p*lac*	This study
pXV9	pLAFR3 *hrpA* to *E* clone from *X. campestris* pv. vesicatoria 75-3	12
pXV74	pLAFR3 *hrpA* to *F* clone from *X. campestris* pv. vesicatoria 75-3	R. Stall
pXV9::A14, pXV9::A22, pXV9::45	Tn*3-gus* insertion derivatives of pXV9	60
pK2	4.5-kb *Hin*dIII-*Eco*RV fragment of pXV9 in pBluescript II KS; contains *hrpA* and 1.5-kb downstream region	This study
pKE10b	5.5-kb *Eco*RI fragment of pXV9 in pBluescript II KS; contains part of *hrpB* and part of *hrpA*	This study
pK221	Deletion subclone of pK2; insert 3.3 kb	This study
pK235	Deletion subclone of pK2; insert 3.55 kb	This study

under plant-mimicking conditions. An important question, therefore, concerns localization of the Hrp proteins. This paper is the first report on expression and localization of a Hrp protein in a plant-pathogenic bacterium. The HrpA1 protein of *X. campestris* pv. vesicatoria was detected with polyclonal antibodies. Cell fractionation studies indicated that 90% of the HrpA1 protein is localized in the outer membrane of *X. campestris* pv. vesicatoria. HrpA1 induces the *psp* operon in *E. coli*, although it does not form mixed multimers with the pIV protein. In addition, a new medium that allows efficient *hrpA* induction in vitro has been developed. The transcription start site was determined by primer extension analysis, and the promoter region of the *hrpA* locus was defined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are described in Table 1. Plasmids were introduced into *E. coli* by electroporation and into *Xanthomonas* spp. by conjugation, using pRK2013 as a helper plasmid in triparental matings (22, 25). *E. coli* cells were cultivated at 37°C in Luria-Bertani medium, and *Xanthomonas* strains were cultivated at 28°C in NYG broth (19) or on NYG 1.5% agar. The XVM2 medium is based on XVM1 (61) and has the following composition: 20 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4, 0.01 mM $FeSO_4$, 10 mM fructose, 10 mM sucrose, 0.03% Casamino Acids (pH 6.7). Antibiotics were added to the media at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; tetracycline, 10 μg/ml; rifampin, 100 μg/ml; spectinomycin, 100 μg/ml.

Construction of deletion mutant 85EDAD. The 15.3-kb *Bam*HI fragment spanning *hrpA1-hrpD4* in pXV9 was deleted, replaced by the omega cassette conferring spectinomycin resistance (52), and introduced into *X. campestris* pv. vesicatoria 85E to generate the mutant 85EDAD by marker gene exchange. The deletion was verified by Southern blot analysis. As expected, this strain is Hrp$^-$, i.e., nonpathogenic on pepper ECW and unable to induce the HR on ECW-10R, and was complemented by plasmid pXV9.

Plant material and plant inoculations. Inoculation of tomato Moneymaker and the near-isogenic pepper cultivars ECW and ECW-10R and reisolation of bacteria from plant tissue 3 days after inoculation were performed as previously described (12).

Sequence analysis. For sequence analysis, a series of nested deletions of pKE10b and pK2 were generated by using DNase I as previously described (13). The DNA sequences of both strands of overlapping deletion subclones were determined by using commercial primers (Stratagene) or custom primers and the T7 DNA polymerase sequencing kit (Pharmacia, Uppsala, Sweden). Tn*3-gus* insertion sites and promoter-*gusA* fusions were sequenced by using an oligonucleotide complementary to a sequence in the N terminus of the GUS gene (5′ GATTTCACGGGTTGGGG 3′). Standard molecular techniques were used (59). Sequence data were analyzed with the University of Wisconsin Genetics Computer Group package (version 8.0 [21]) and TBLAST (5).

Primer extension analysis. Bacteria were grown for 16 h in NYG or XVM2 broth or recovered from susceptible pepper plants 3 days after whole-plant infiltration. Bacterial RNA was extracted with hot phenol as previously described (2). The following oligonucleotides were used (positions refer to Fig. 2): no. 76, (5′-GGTGGCGATGGACTGGCTGGCGGACAAATC-3′; positions 429 to 458); no. 83, (5′-GGGGAGGTTTCGAATTTGCCGCTCAGGGTGCCG-3′; positions 479 to 511); no. 84, (5′-GGGCGGCGTCGGCATGTGGCGACAA CAGCGG-3′; positions 330 to 360); no. 107, (5′-GGCGGTGGGCGGTGGTA CAGG-3′; positions 268 to 288). Primer extension was done as previously described (8). Briefly, 15 μg of RNA and a ^{32}P-labelled oligonucleotide were annealed for 90 min at 65°C, and the extension reaction was performed in the presence of actinomycin D with 200 U of Moloney murine leukemia virus-

Superscript II reverse transcriptase (Gibco-BRL) for 1 h at 42°C. The resulting extension products were analyzed on 6% denaturing polyacrylamide gels.

Construction of promoter subclones. To determine promoter activity, fragments were subcloned into promoter probe plasmid pL6GUSB. pPA1 contains a 312-bp *Not*I (polylinker site)-*Eco*RI fragment isolated from the deletion subclone pK221 (see Fig. 2). From plasmid pK235, a 518-bp *Not*I-*Eco*RI fragment and a 269-bp *Not*I-*Rsa*I fragment were isolated, resulting in pPA2 and pPA3, respectively. All fragments were first cloned into pBluescript II KS, and their identity was checked by sequencing the 3′ end of the insert before cloning into pL6GUSB. To analyze the origin of the activity of Tn*3-gus* insertion no. 45, we subcloned a *Bam*HI fragment containing the *gusA* gene and 371-bp upstream sequence from the Tn*3-gus* insertion plasmid pXV9::45 into pLAFR3 (pL345; *gusA* gene under control of p*lac*) and pLAFR6 (pL645). All constructs were transferred into *X. campestris* pv. vesicatoria 85-10 or 85E to determine GUS activities after growth of the bacteria under different conditions as previously described (60).

Protein expression in *E. coli* and antibody preparation. To express *hrpA1* under the control of the *lac* promoter, the 3.3-kb *Not*I-*Hin*dIII insert of pK221 was subcloned into pLAFR3 to generate pLA10. For antibody production against HrpA1, a 2,055-bp *Nco*I fragment was isolated from pK2 and ligated into the filled-in *Bam*HI site of pGEX-2T (clone pGA26) and into the filled-in *Eco*RI site of pMAL-2p (clone pMA1). The fusion sites of both constructs, which carry the entire open reading frame (ORF) plus 235 bp downstream, were sequenced. pGEX-2T allows in-frame fusions to the C terminus of the glutathione *S*-transferase, and pMAL-2p allows in-frame fusions to the maltose-binding protein. Fusion proteins were expressed in *E. coli* cells after induction of an early log culture for 1 h by IPTG (isopropyl-β-thiogalactopyranoside; 0.1 mM) at 30°C. The HrpA1–glutathione *S*-transferase fusion protein was enriched from total extracts of DH5α(pGA26) by using affinity glutathione Sepharose 4B according to the manufacturer's instructions (Pharmacia). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels, the fusion protein was visualized by shaking the gel in cold 0.25 M KCl, cut out, dialyzed against 50 mM Tris-HCl, pH 7.5, and frozen. New Zealand White rabbits were immunized with the powderized protein in acrylamide (Eurogentec, Seraing, Belgium). The serum taken after the third booster injection was depleted against total protein extracts from induced *E. coli* DH5α(pGEX-2T) and from *X. campestris* pv. vesicatoria 85EDAD grown in NYG medium.

Immunoprecipitation. Membrane proteins from [^{35}S]methionine-labelled *E. coli* cells were extracted in 1% Triton X-100 as described previously (39, 57). Immunoprecipitation of the Triton X-100 extracts by antiserum to pIV (encoded by the *E. coli* filamentous phage f1) or of SDS-denatured whole-cell lysates by antiserum to HrpA1 or the *E. coli* protein PspA were carried out with Staph A protein-Sepharose beads as described previously (57).

Subcellular fractionation. Subcellular fractionation was carried out on cells from exponentially growing *X. campestris* pv. vesicatoria cultures. Bacteria were harvested, washed once with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; buffer pH 7.4), and resuspended at 4°C in the following buffer (1 ml/g of cells): 10 mM HEPES (pH 7.4), 20% sucrose, RNase A (10 μg/ml), DNase (10 μg/ml), phenylmethylsulfonyl fluoride (100 μg/ml). The cells were disrupted at 4°C by three to four passages through a French pressure cell. Unlysed cells and large debris were removed by centrifugation. Cell extracts were separated into soluble and insoluble material in the presence of 5 mM EDTA by centrifugation at 300,000 × *g* for 3 to 4 h at 4°C. Total membranes were isolated by centrifugation of 1.5 to 2 ml of total extract onto a 60% sucrose cushion (0.8 ml of 60% sucrose, overlaid with 2.5 ml of 25% sucrose [wt/vol]) and collected for further analysis. For separation of inner and outer membranes in a sucrose step gradient, 1 to 2 ml of total membranes was applied in 25% sucrose to a preformed gradient (percents refer to sucrose [wt/vol]) of 0.5 ml, 70%; 1 ml, 65%; 1 ml, 60%; 1.6 ml, 55%; 1.6 ml, 50%; 1.6 ml, 45%; 1 ml, 40%; and 0.5 ml, 35%, in Beckman SW41 tubes.

Flotation gradients were prepared as previously described by Poquet et al. (50). Briefly, 1 to 2 ml of total membranes in 60% sucrose (wt/wt) was loaded on a 0.5-ml cushion of 63% sucrose and overlaid with 56, 53, 50, 47, 44, 41, 35, and 28% sucrose (wt/wt).

Both gradients were centrifuged at 210,000 × *g* at 6°C for a minimum of 90 h. Fractions were collected from the top of the gradients. Samples from each fraction were assayed for sucrose concentration with a refractometer and for protein content with the Bradford assay (Bio-Rad) in the presence of 0.1 N NaOH. NADH oxidase and succinate dehydrogenase activity and the concentration of 2-keto-3-deoxyoctonate were measured as previously described (48, 49).

After electrophoresis on SDS-polyacrylamide gels (10 or 15% polyacrylamide), the proteins were stained with Coomassie brillant blue or were transferred to nitrocellulose. Western blots (immunoblots) were reacted with antiserum against HrpA1 or *E. coli* OmpA protein (kindly provided by J. Stierhof and U. Henning, Tübingen, Germany). Bound antibody was visualized after incubation of the blots with alkaline phosphatase-coupled anti-rabbit immunoglobulin G (Promega) and color development with nitroblue tetrazolium and 5-bromo-4-chloro-indolyl phosphate.

The protein quantity of HrpA1 was estimated on Western blots. A series of fivefold dilutions of protein samples were loaded on a gel and blotted, and the signal strengths of the HrpA1 band were compared.

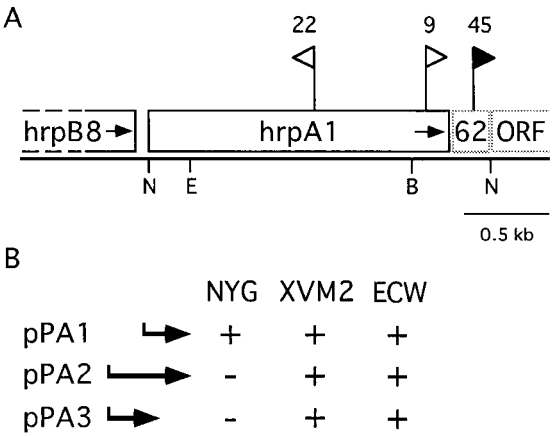

FIG. 1. Transcriptional organization of the *hrpA* region in *X. campestris* pv. vesicatoria. (A) Schematic drawing showing the *hrpA1* gene, two ORFs (ORF62 and an ORF of unknown size) downstream of *hrpA1*, and the end of the *hrpB* operon. The orientation and position of Tn*3-gus* insertions no. 22, 9, and 45, which have been sequenced, are indicated by flags; open flags indicate insertions resulting in loss of pathogenicity and HR induction, while the filled-in flag refers to a wild-type reaction of the insertion mutant. The following restriction sites are indicated: B, *Bam*HI; E, *Eco*RI; N, *Nco*I. (B) Promoter subclones in pL6GUSB and their GUS activities under different conditions. pPA1 is derived from the deletion clone pK221, and plasmids pPA2 and pPA3 are derived from pK235; the arrows indicate the region and orientation in front of the promoterless *gusA* gene (for details, see Fig. 2 and the text). The plasmids were introduced into *X. campestris* pv. vesicatoria 85-10. GUS activity was measured in two to three independent experiments after growth in NYG broth (16 h), XVM2 medium (16 h), and pepper cultivar Early Calwonder (ECW; 40 h) upon two to three samples in each case. +, active; −, inactive (see the text).

Nucleotide sequence accession number. The nucleotide sequence of *hrpA* has been deposited together with that of the *hrpB* operon and can be retrieved from GenBank (accession no. U33548). Position 1 in Fig. 2 corresponds to position 5771 in the sequence deposited.

RESULTS

DNA sequence analysis of the *hrpA1* gene. The *hrpA* locus has been genetically defined by transposon insertions as being the last locus in the left part of the *hrp* cluster (12). DNA sequence analysis of this region revealed one long ORF (Fig. 1), designated *hrpA1*. The first possible translation start codon of this ORF is the ATG at position 258 (see Fig. 2), which is preceded by a ribosome binding site and is, therefore, most likely used as a translation start site. A TGA stop codon is at position 2079. The predicted protein is 607 amino acids long, with a molecular mass of 64 kDa. The N-terminal 34 amino acids contain positively charged residues followed by a hydrophobic, α-helical stretch typical of prokaryotic signal peptide sequences (67), with a putative cleavage site between amino acids 33 and 34, both alanine residues (Fig. 2), thus predicting a mature protein of 574 amino acids (60.4 kDa). A putative transcription termination sequence (GGTAGC-N_5-GCTACC) is located 15 bp downstream of the stop codon but has been shown not to be effective (see below).

Tn*3-gus* insertions no. 22 and no. 9, which abolish *hrpA* function, interrupt the ORF after amino acid positions 332 and 558, respectively. Tn*3-gus* insertion no. 45, which has no effect on pathogenicity, is inserted 126 bp downstream of the stop codon of the *hrpA1* gene, indicating that this locus contains only one *hrp* gene. In the 578-bp sequence that was determined downstream of the *hrpA1* stop codon, one possibly expressed ORF starts at bp 28, with a coding capacity for a 62-amino-acid peptide (ORF62); two ORFs whose lengths are unknown start

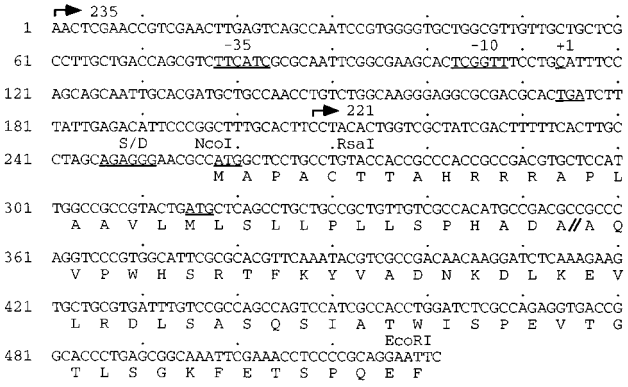

FIG. 2. DNA sequence of the upstream region and the beginning of *hrpA1*. The sequence starts with the first nucleotide of deletion clone pK235. S/D refers to a possible ribosome binding site. The TGA stop codon of *hrpB8* and the first two ATG start codons of *hrpA1* are underlined. The sequence of the deduced translation product is given in the single-letter code below the DNA sequence; the putative cleavage site of the signal sequence is marked by a double bar (//). Arrows refer to the first nucleotides of deletion clones. Restriction sites used for subcloning experiments are indicated.

at 232 and 410 bp downstream of the *hrpA1* stop codon, respectively. That this region might belong to the *hrpA* transcription unit is suggested by the inducible GUS activity of the Tn*3-gus* insertion no. 45 (60) that is integrated in ORF62. To analyze the origin of this activity, we subcloned a fragment containing the *gusA* gene and 371-bp upstream sequence from insertion no. 45 into pLAFR3 (pL345) and pLAFR6 (pL645). Only pL345 revealed GUS activity which was constitutive, i.e., expressed under the control of the *lacZ* promoter that is constitutive in *X. campestris* pv. vesicatoria. pL645 did not show any GUS activity. These results exclude transcription termination downstream of the *hrpA1* gene (see above) and indicate that the region within or downstream of the *hrpA1* gene does not contain a promoter. Hence, the plant-inducible expression observed for pXV9::45 (60) must be due to the upstream *hrpA* promoter.

TABLE 2. Sequence similarities to the *X. campestris* pv. vesicatoria HrpA1 protein

Protein group	Amino acid identity and similarity (%)[a]	Bacterium or phage	Reference
1			
HrpA	48, 66	*Pseudomonas solanacearum*	28
HrpH	29, 52	*Pseudomonas syringae* pv. syringae	34
YscC	34, 55	*Yersinia enterocolitica*	46
MxiD	28, 50	*Shigella flexneri*	4
InvG	29, 52	*Salmonella typhimurium*	38
SepD	29, 54	*Escherichia coli*	37
NolW	29, 51	*Rhizobium fredii*	45
2			
PulD	23, 46	*Klebsiella oxytoca*	20
OutD	25, 47	*Erwinia chrysanthemi*	17
XpsD	24, 49	*Xanthomonas campestris* pv. campestris	32
ORF E	23, 47	*Haemophilus influenzae*	64
PilQ	24, 50	*Pseudomonas aeruginosa*	44
3			
pIV	25, 48	Bacteriophage f1	31

[a] Similarities are based on sequence comparisons with BESTFIT (21), allowing gaps in the aligned sequences.

HrpA1 is a member of what is now recognized as the PulD family of proteins involved in protein secretion (23, 26) (Table 2) that can be divided into three groups on the basis of the relatedness of their N-terminal domains (26, 44). Several recent papers describe sequence comparisons of HrpA1-related proteins (26, 38, 44). HrpA1 belongs to group 1 and is most similar to HrpA in *P. solanacearum* (28) and to YscC in *Yersinia* spp. (46). The C-terminal domain of the group 1 proteins contains several highly conserved motifs of yet-unknown function (26).

Studies of the *hrpA* promoter. To identify regulatory sequences needed for *hrpA* expression, the region upstream of *hrpA1* was subcloned into promoter probe plasmid pL6GUSB containing the *gusA* gene as a reporter. Fig. 1B shows the fragments subcloned and their promoter activity in *X. campestris* pv. vesicatoria under different growth conditions. To facilitate expression studies in *X. campestris* pv. vesicatoria and to avoid the use of tomato conditioned medium (60), the quality of which varied, a new in vitro medium, XVM2, was developed for induction of *hrp* gene expression. This medium is similar to XVM1 (61) but contains fructose in addition to sucrose and Casamino Acids instead of methionine. In contrast to XVM1, XVM2 medium allowed, for the first time, efficient induction of *hrpA* and the other five *hrp* loci in a synthetic medium (71).

Assays of GUS activity after growth of the bacteria under different conditions (16 h in NYG or XVM2 medium and 40 h in pepper ECW) were performed as described previously (60). pPA2 and pPA3 showed very low levels of expression in complex medium NYG (0.02 to 0.2 U/10^{10} CFU) and induction in XVM2 medium or in the susceptible pepper plant ECW (1.3 to 2.8 U/10^{10} CFU). These values were of the same order of magnitude as for *hrpA* transcriptional fusions with Tn*3-gus* (60). In both pPA2 and pPA3, a fragment containing 257 bp upstream of the putative translation start site is sufficient for regulated promoter activity. Surprisingly, the GUS activity of construct pPA1 containing only 48 bp upstream of the putative translation start site was constitutive (2 to 6 U/10^{10} CFU). As the larger promoter constructs pPA2 and pPA3 are clearly inducible, the activity of pPA1 could be due to generation of a new promoter in the 5′ region of this particular construct.

Mapping of the *hrpA* transcription start site. To define the *hrpA* promoter more precisely, the transcription start site was determined by primer extension analysis. With the wild-type strain, very weak signals were detectable and difficult to reproduce. To increase the level of *hrpA*-specific transcripts, we used strain 85E(pXV74), a wild-type strain that carries two to three additional copies of the entire *hrp* cluster and shows unaltered reactions when inoculated into susceptible and resistant pepper lines. As a negative control, a null mutant was used. For this purpose, the *hrpA1-hrpD4* region was deleted, replaced by the omega cassette, and introduced into *X. campestris* pv. vesicatoria 85E to generate the mutant 85EDAD by marker gene exchange.

Figure 3 shows the result of a typical primer extension experiment, using primer no. 84, which is complementary to the sequence at positions 330 to 360. A specific signal is present in XVM2-induced RNA of 85E(pXV74) but not in RNA of strain 85EDAD grown in NYG medium. A corresponding signal is also obtained with three other oligonucleotides (data not shown), indicating that transcription starts at position 114 (position +1; Fig. 2). A second but weaker transcription start site could be 8 nucleotides upstream at position 106. A corresponding extension product was detected with all four oligonucleotides tested but was low in abundance. Analysis of RNA from

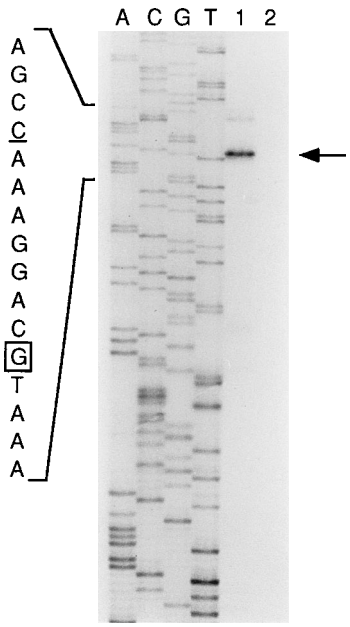

FIG. 3. Mapping of the transcription start site of *hrpA* by primer extension analysis. The RNAs were extracted from strain 85E(pXV74) (lane 1) and 85EDAD (lane 2) after growth in XVM2 and NYG medium (16 h), respectively, annealed with oligonucleotide no. 84, and used as templates for reverse transcriptase. The sequence is the reverse complement of the coding strand and was obtained with plasmid pKS2 as the template and oligonucleotide no. 84 as the primer. The boxed nucleotide refers to the transcription start site, indicated by an arrow on the right-hand side; the nucleotide corresponding to the second, weaker signal is underlined.

bacteria induced by growth for 3 days in planta (data not shown) showed extension products identical to those seen with XVM2-induced cells, thus demonstrating that the same transcription start site is used under natural conditions, i.e., during growth in the host.

Inspection of the sequence upstream of the +1 site revealed only weak homology to canonical −10 and −35 promoter elements present in sigma 70-dependent promoters of *E. coli* and showed no homology to sigma 54-regulated promoters. No homology to the PIP-box sequence in other *X. campestris* pv. vesicatoria *hrp* promoters (24) was found, nor was homology to the "harp" box or to the upstream sequence described for *hrp* genes and avirulence genes whose expression is *hrp* dependent in pathovars of *P. syringae* (36, 58, 62, 63, 74) found.

Expression of *hrpA* is not controlled by the *hrp* regulatory gene *hrpX*. The *hrpX* gene whose product directly or indirectly activates expression of several *X. campestris* pv. vesicatoria *hrp* loci was recently isolated (71). To assess the role of *hrpX* in induction of *hrpA* expression, the activity of promoter construct pPA3 in the *hrpX* wild-type and mutant strains was measured. As shown in Table 3, the *hrpA* promoter is still induced in the *hrpX* mutant, 85X. The same was observed for the GUS activity of pXV9::A14, in which Tn*3-gus* is inserted into *hrpA1* with the *gusA* gene under the control of the *hrpA* promoter (data not shown). These results demonstrate that expression of *hrpA* is independent of the *hrpX* gene. It was then tested whether expression of *hrpA* depends on an intact copy of the locus. Strain 85-10::A22 and the *hrpA-D4* deletion mutant 85EDAD were used as recipient strains for pPA3. Strain 85-10::A22 carries a Tn*3-gus* insertion with the *gusA* gene in an orientation opposite to that of transcription of *hrpA* (Fig. 1) and has no GUS activity. Activity of pPA3 in both strains was similar to that observed in the wild-type background, thus excluding an influence of genes in the region *hrpA* to *hrpD4* on *hrpA* expression (Table 3).

TABLE 3. *hrpA* promoter activity in different genotypes of *X. campestris* pv. vesicatoria

X. campestris pv. vesicatoria strain	Genotype	GUS activity of pPA3 after growth in medium (U/10^{10} CFU):	
		NYG	XVM2
85E	*hrp*$^+$	0.03	5.2
85X	*hrpX*	0.07	3.1
85EDAD	Δ*hrpA1-D4*	0.05	2.6
85-10::*hrpA*22	*hrpA*	0.04	2.4

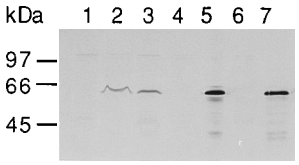

FIG. 4. Identification of the HrpA1 protein. Proteins were separated by SDS-PAGE (12% polyacrylamide), transferred to nitrocellulose, and reacted with anti-HrpA1 antibodies (dilution, 1:1,500). Bound antibody was visualized with secondary anti-rabbit antibodies conjugated with alkaline phosphatase. Lanes 1 and 2, total extracts of *X. campestris* pv. vesicatoria 85-10 grown in NYG medium and pepper ECW, respectively. Lane 3, *X. campestris* pv. vesicatoria 75-3 grown in tomato. Lanes 4 to 7, mutant 85EDAD, 85EDAD(pLA10), mutant 85-10::A22, and 85-10::A22(pLA10), respectively, grown in XVM2 medium. Because of overexpression of HrpA1, the samples loaded in lanes 5 and 7 were diluted fivefold.

HrpA1 protein expression. To raise polyclonal antibodies, the *hrpA1* gene was expressed in *E. coli* cells. Translational fusions between HrpA1 and the C termini of the glutathione *S* transferase (clone pGA26) and of the maltose-binding protein (clone pMA1) were obtained. The construct pGA26 was superior in producing a fusion protein of the expected molecular weight of about 90 kDa (61 kDa plus 27 kDa of glutathione *S*-transferase) which was purified for immunization of a rabbit. Specificity of the obtained antiserum was demonstrated by Western blotting with protein extracts of *E. coli* carrying pGA26 or the pMAL construct pMA1. In the latter case, the antibody specifically reacted with a 100-kDa protein which is the expected size of the maltose-binding protein-HrpA1 fusion protein (data not shown).

In total extracts of induced *X. campestris* pv. vesicatoria, the antibody detects a protein of 64 kDa, which is in good agreement with the predicted molecular mass, suggesting that this protein corresponds to HrpA1 (Fig. 4, lanes 2 and 3). The protein is absent in the extracts of uninduced 85-10 wild type and of induced *hrpA* mutants 85-10::*hrpA*22 and 85EDAD (Fig. 4; compare lanes 2 and 3 with lanes 1, 4, and 6). *hrpA1* was constitutively expressed under the control of the *lac* promoter in plasmid pLA10 (Fig. 4, lanes 5 and 7). pLA10 expresses a functional HrpA1 protein as shown by complementation of mutant 85-10::*hrpA*22: the transconjugant was pathogenic on pepper ECW and induced an HR on pepper ECW-10R.

HrpA1 expression in *E. coli* induces the *psp* operon. The filamentous phage pIV protein, a HrpA1 homolog, induces the *psp* operon in *E. coli* cells (16). This induction depends on multimerization of pIV (55). To determine whether HrpA1 induces the expression of the *E. coli psp* operon, the *hrpA1* construct pLA10 was introduced into *E. coli* K561 cells. Figure

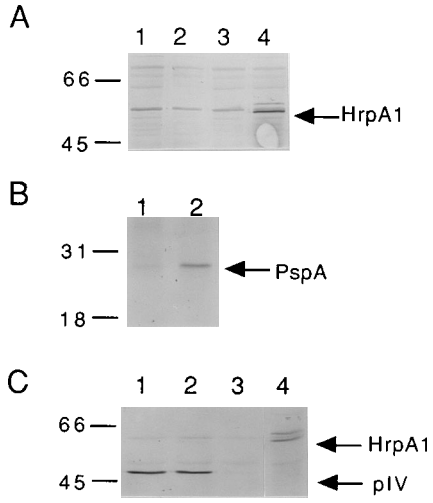

FIG. 5. Expression of the HrpA1 protein in *E. coli* cells. (A) Total cell proteins from K561 (lanes 1 and 2) and K561(pLA10) (lanes 3 and 4) grown in the absence (lanes 1 and 3) or in the presence (lanes 2 and 4) of 1 mM IPTG were separated, transferred to nitrocellulose, and reacted with anti-HrpA1 antibodies as described in the legend to Fig. 4. (B) K561(pLA10) cells were grown in minimal medium lacking methionine (15). Culture samples labelled with [^{35}S]methionine in the absence of IPTG (lane 1) or 15 min after the addition of IPTG (lane 2) were precipitated in cold 10% trichloroacetic acid, pelleted, resuspended in 3% SDS, and precipitated with antiserum to PspA and Staph A-Sepharose beads (57). The immune precipitates were electrophoresed on a 12% polyacrylamide–SDS gel and exposed to X-ray film. (C) Wild-type filamentous phage f1 and/or IPTG was added to K561 or K561(pLA10) cells 30 min prior to the addition of [^{35}S]methionine. The cells were lysed, and the membrane pellets were suspended in 50 mM Tris (pH 8.0)–1% Triton X-100–10 mM EDTA (39). The solubilized membrane proteins from f1-infected K561 (lane 1), f1-infected K561(pLA10) (lane 2), and uninfected K561(pLA10) (lane 3) cells were precipitated with antiserum to f1 pIV and Staph A-Sepharose beads, and the immune precipitates were electrophoresed on a 10% polyacrylamide–SDS gel and exposed to X-ray film. The HrpA1 marker (lane 4) is an immune precipitate of denatured proteins from K561(pLA10) cells labelled in the presence of IPTG with antiserum to HrpA1.

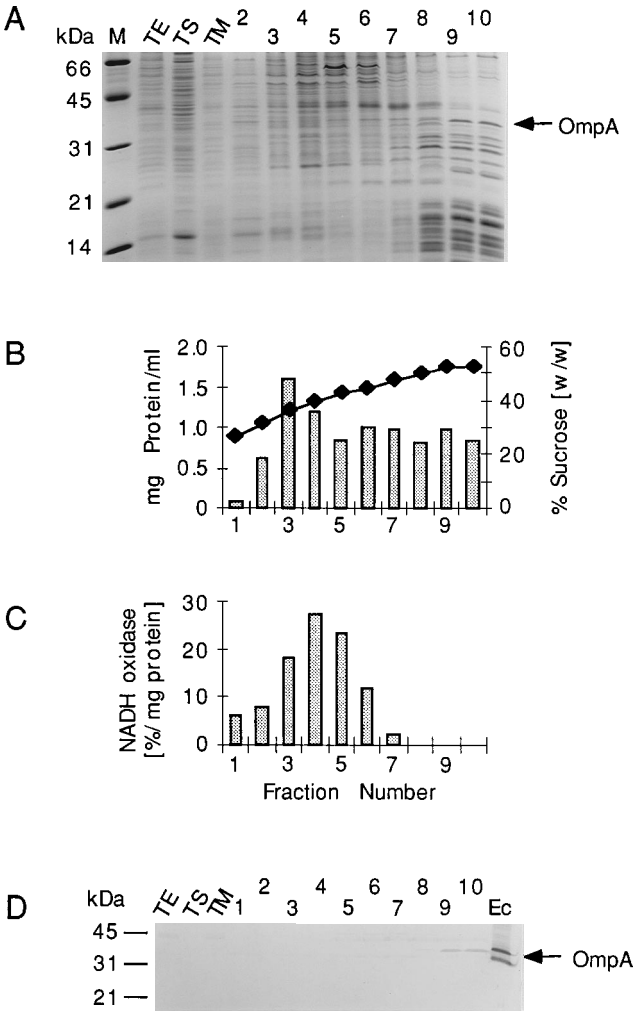

FIG. 6. Fractionation of protein extract from *X. campestris* pv. vesicatoria by a sucrose step gradient. Strain 85E(pXV74) grown in XVM2 medium was fractionated as described in Materials and Methods. (A) Proteins corresponding to total extract (TE), total soluble (TS), and total-membrane proteins (TM) and to fractions 2 to 10 collected from the gradients were separated on a 15% polyacrylamide gel and stained with Coomassie brillant blue. Three micrograms of protein was loaded in each lane except for fraction 2 (0.3 μg). The OmpA protein present in fractions 9 and 10 is indicated by an arrow. M, protein size marker. (B) The sucrose concentration in fractions 1 to 10 (diamonds) was measured with a refractometer. Protein concentration was determined by the Bradford assay (bars). (C) NADH oxidase activity in fractions 1 to 10. (D) OmpA detection in a Western blot of the samples shown in panel A with an *E. coli*-specific antibody. Ec refers to total protein extract of *E. coli* DH5α as a control. Numbers indicate the number of the fraction obtained in the step gradient. Before being loaded onto the gel, the samples were treated at 65°C for 10 min. TE, TS, and TM are as defined above.

5A shows that a protein of the expected size is recognized by HrpA1 antibodies in samples from pLA10-containing *E. coli* cells grown in the presence of 1 mM IPTG (lane 4) but not in the absence of IPTG (lane 3) or in cells that lack the plasmid (lanes 1 and 2). An additional band that migrates slightly more slowly than HrpA1 may be residual unprocessed HrpA1. Figure 5B shows results of an experiment in which K561(pLA10) cells were immune precipitated with PspA-specific antiserum. The level of PspA was higher in cells treated with IPTG (Fig. 5B, lane 2) indicating that HrpA1 induces the *psp* operon of *E. coli*. To test whether HrpA1 and the filamentous phage protein pIV could form mixed multimers, the two proteins were expressed either separately or together in the same cells (Fig. 5C). Membrane proteins were extracted under conditions previously shown to maintain the pIV multimer (39) and immune precipitated with antibodies to f1 pIV. HrpA1 did not coprecipitate with pIV (Fig. 5C, lane 2), indicating that mixed multimers do not form between these homologs.

Subcellular localization of HrpA1 in *X. campestris* pv. vesicatoria. To study the localization of the HrpA1 protein in *X. campestris* pv. vesicatoria, total protein extracts were used for cell fractionation experiments and analysis by Western blotting. To facilitate detection but avoid possible artifacts in localization when HrpA1 is overproduced, as with pLA10, we used strain 85E carrying the entire *hrp* region on pXV74. Total extracts of cultures grown in XVM2 medium were separated into soluble and insoluble (total-membrane) proteins. HrpA1 was found mainly in the membranes. Therefore, total membranes recovered from sucrose cushions were fractionated further with sucrose gradients (see Materials and Methods). Figure 6 shows results of a typical fractionation experiment using a sucrose step gradient. Fractions were analyzed for their content in protein and sucrose (Fig. 6A and B). As markers for inner and outer membranes, respectively, NADH oxidase activity (Fig. 6C) and the amount of OmpA protein (Fig. 6D) were determined. Most NADH oxidase activity was present in fractions 3 to 6. The OmpA protein of *X. campestris* pv. vesicatoria was detected with an antibody specific for the *E. coli* OmpA protein (Fig. 6D). In the *E. coli* extract, two protein

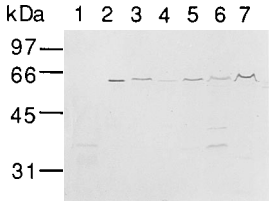

FIG. 7. Localization of the HrpA1 protein. Proteins were separated by SDS-PAGE (10% polyacrylamide), transferred to nitrocellulose, and reacted with anti-HrpA1 antibodies as for Fig. 4. Lanes 1 to 3 show total protein extracts of 85EDAD, 85EDAD(pLA10), and 85E (pXV74), respectively, grown in XVM2 medium. The latter was fractionated into total soluble proteins (lane 4), total membranes (lane 5), inner membranes (lane 6; fraction 4 of step gradient shown in Fig. 6), and outer membranes (lane 7; fraction 9 of step gradient shown in Fig. 6). About 3 μg was loaded in each lane, except for lane 2 (0.3 μg).

bands are visible, which can be explained by the heat modifiability of OmpA (41). In *X. campestris* pv. vesicatoria fractions 9 and 10 (and to a lesser extent, fraction 8), the OmpA antibody detected a protein of a size similar to that of the more slowly migrating species in *E. coli*. We, therefore, assume that this 34-kDa protein corresponds to *X. campestris* pv. vesicatoria OmpA. Fractions 8 to 10 were obviously enriched for OmpA, since the protein is undetectable in total extracts and total membranes. The distribution of NADH oxidase and OmpA indicated a good separation between inner and outer membranes, which was confirmed by using succinate dehydrogenase (mostly in fractions 4 to 6) and 2-keto-3-deoxyoctonate (fractions 7 to 10) as additional markers for inner and outer membranes, respectively (data not shown). Fractions 4 and 9 of sucrose step gradients were chosen as representative of inner and outer membranes, respectively, and tested for the presence of the HrpA1 protein. Figure 7 shows the subcellular distribution of HrpA1 in *X. campestris* pv. vesicatoria. The protein was detected in the total extract (Fig. 7, lane 3), which was separated into soluble proteins (lane 4) and total-membrane proteins (lane 5). While very little HrpA1 was found in the soluble fraction, more than 90% of the total amount of HrpA1 was estimated to be present in the membranes. Further fractionation into inner (Fig. 7, lane 6) and outer (lane 7) membranes by sucrose step gradients revealed that the majority of HrpA1 was present in the outer membrane. It was estimated by a series of dilutions that 80 to 90% of the total amount of the HrpA1 protein in *X. campestris* pv. vesicatoria is localized to the outer membrane. Similar results were obtained in flotation gradients of the same total-membrane sample (data not shown).

DISCUSSION

The *hrpA* locus of *X. campestris* pv. vesicatoria is at the left border of the 25-kb *hrp* cluster and encodes one Hrp protein, HrpA1, which belongs to the PulD superfamily of proteins (Table 2). The region downstream of *hrpA1* does not contain other *hrp* genes, because insertions have no effect on pathogenicity or HR. Likewise, the *hrpA1* homolog of the *P. solanacearum hrp* cluster, which in this region is colinear to the *X. campestris* pv. vesicatoria region, is also at the border of the cluster (66). However, it cannot be ruled out that in *X. campestris* pv. vesicatoria at least one more ORF (ORF62) downstream of *hrpA1* belongs to the *hrpA* locus, because expression of Tn*3-gus* insertion no. 45 is dependent on the *hrpA* promoter. While the deduced amino acid sequences of the ORFs identified downstream of *hrpA1* do not share any similarity with known proteins, the upstream region contains the end of the *hrpB8* gene (24). The HrpB8 protein is most related to HrpC of *P. solanacearum* (66) and to YscT of *Yersinia pseudotuberculosis* (9). Surprisingly, the colinearity between the *hrp* clusters of *X. campestris* pv. vesicatoria and *P. solanacearum* (10) is interrupted between *hrpB8* and *hrpA* by the presence of the regulatory gene *hrpB* in *P. solanacearum* (66).

Promoter studies and mapping of the *hrpA* transcription start site revealed that a region of 113 bp upstream of the transcription start site is sufficient for inducible promoter activity in the plant and in XVM2 medium. It should be noted, however, that suppression of expression in NYG medium was less clear for *hrpA* than for other *hrp* loci. The basal activity of the loci *hrpB* to *hrpF* in NYG medium is 10-fold lower and is considered to be in the range of background (60). The constitutive activity of promoter construct pPA1 is probably due to a promoter created during construction, because the transcription start site of *hrpA* has been shown to be located outside of the sequence present in pPA1.

The inducible *hrpA* promoter shows no homology to promoter elements present in other *hrp* loci of *X. campestris* pv. vesicatoria (24) or other plant-pathogenic bacteria. Moreover, we show here that induction of *hrpA* expression in the *hrp*-inducing medium XVM2 is independent of *hrpX*. This gene was recently found to be the positive regulator of other *hrp* loci in the *X. campestris* pv. vesicatoria *hrp* cluster (71). Our data strongly suggest that at least one more regulatory gene is involved in *hrp* gene regulation, regulating at least *hrpA*, since autoregulation of *hrpA* does not seem to play a role. This putative second regulatory gene must be located outside of the *hrp* cluster, in contrast to the *hrp* regulatory genes of *P. solanacearum* (27) and *P. syringae* (29, 73).

An interesting feature of the *hrpA* transcript is its long 5′ untranslated region of 144 nucleotides. This leads to an overlap in expression of the two flanking *hrp* loci *hrpB* and *hrpA*. HrpB8 translation stops 62 bp downstream of the transcription initiation site of *hrpA*, thus localizing the *hrpA* promoter within the coding region of *hrpB8*. No typical transcription termination sequence is present for *hrpB8*. Overlap of *hrpB* transcription and translation with *hrpA* transcription initiation might be an explanation for the weak expression of *hrpA*, which is at least fivefold lower than for *hrpB* (1 [“promoter occlusion”], 60).

Expression of the *hrpA* locus is induced in the plant (60). Here, we describe that XVM2 medium efficiently induces *hrpA* expression in vitro. Moreover, the other five *hrp* loci of *X. campestris* pv. vesicatoria are also efficiently expressed in this medium (71). This is a novel finding, proving that plant-derived molecules are not needed for inducible expression, in contrast to what has been speculated (61). The *hrp* expression in an artificial medium without any plant-specific molecule has also been described for other plant-pathogenic bacteria (for example, see references 6, 35, and 70); these various minimal media are, however, different from XVM2 medium.

As shown in this study, the HrpA1 protein was detected in *X. campestris* pv. vesicatoria with specific polyclonal antibodies. This is, to our knowledge, the first report on an antibody specific to a component of the Hrp secretion apparatus in a plant pathogen. Furthermore, biochemical fractionation studies clearly showed that the HrpA1 protein is localized in the outer membrane of *X. campestris* pv. vesicatoria. Analyzing the amino acid sequence with secondary-structure predictions revealed an N-terminal signal sequence and a high number of β-sheets but no hydrophobic domain long enough to span the cytoplasmic membrane. Thus, the localization of the HrpA1 protein in *X. campestris* pv. vesicatoria is in good agreement with these predictions.

A common feature of HrpA1-homologous proteins is that they are components of protein secretion systems. The most detailed studies have been performed with the HrpA1-related XpsD of *X. campestris* pv. campestris (33), the PulD protein of *K. oxytoca* (20), and the bacteriophage protein pIV (15, 39, 57). These proteins represent the three groups in this protein superfamily (Table 2). Like HrpA1, they and several other homologous proteins have been localized to the outer membrane. Proteins secreted via the type III, *sec*-independent pathway, for example, the Yops of *Yersinia* spp. (18) or Ipas in *Shigella flexneri* (4), lack a classical signal sequence and are translocated directly across both bacterial membranes without being processed (18). The cell surface pili and hydrolytic enzymes secreted by the type II, *sec*-dependent pathway have conventional, cleaved signal sequences (54), and the latter are first translocated into the periplasm, where they must fold to be competent for export across the outer membrane (14, 53). A special case is the assembly and secretion of filamentous phage from the host cell, which requires the HrpA1 and PulD homolog pIV but is not known to need proteins that resemble other components of either the type II or type III systems (15, 56). So far, neither the specific roles of the components nor the structure of the postulated transport apparatus is known.

The pIV protein forms a large homomultimer in the outer membrane which has been postulated to serve as an exit channel for the phage (39, 56). Formation of a large channel in the outer membrane could account for the observation that multimeric pIV (but not a pIV mutant defective in multimerization) induces *psp*, an operon that is also induced by hyperosmotic shock (16, 55). OutD (57) and other type II homologs also induce *psp* (3, 51), and OutD has been shown to form mixed multimers with pIV when both are expressed in *E. coli* cells (39). These observations strongly suggest that the type II homologs form homomultimers which could play a role similar to that of pIV. HrpA1 is the first type III homolog whose properties have been examined in this regard. Induction of *psp* appears to be a response to the presence of multimeric pIV in the outer membrane (55). Hence, the ability of HrpA1 to stimulate PspA synthesis in *E. coli* suggests that it also forms a multimer. Mixed multimers composed of pIV and HrpA1 were not detected. This may be due to their evolutionary distance: pIV and HrpA1 share only 25% identity and 48% similarity (Table 2), while the values for f1 pIV and OutD are 32 and 55%, respectively. Nonetheless, the existence of homomultimers of HrpA1 remains to be established.

It should be possible to address this and other such questions with the specific HrpA1 antibody described in this report. In case of a suprastructure in the outer membrane, one might detect this by immunoelectron microscopy. First attempts to localize HrpA1 in bacterial sections were unsuccessful, most likely because of the low level of protein present per cell (43). Given its localization and presumed role in protein secretion, the following questions remain. Which other proteins does HrpA1 interact with to link the cytoplasm with the bacterial surface? Which are the substrates to be secreted? The nature of the secreted virulence factors and elicitors in *X. campestris* pv. vesicatoria is still enigmatic. In conclusion, the antibody specific to a component of the Hrp secretion apparatus (HrpA1) will allow further dissection of the structure-function relationship.

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