The Presence of Diverse IS Elements and an *avrPphD* Homologue That Acts as a Virulence Factor on the Pathogenicity Plasmid of *Erwinia herbicola* pv. *gypsophila*

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The pathogenicity of *Erwinia herbicola* pv. *gypsophila* (Ehg) and *Erwinia herbicola* pv. *betae* (Ehb) is dependent on a native plasmid (pPATH_{Ehg} or pPATH_{Ehb}) that harbors the *hrp* gene cluster, genes encoding type III effectors, phytohormones, biosynthetic genes, and several copies of IS1327. Sequence analysis of the *hrp*-flanking region in pPATH_{Ehg} (cosmid pLA150) revealed a cluster of four additional IS elements designated as ISEhe1, ISEhe2, ISEhe3, and ISEhe4. Two copies of another IS element (ISEhe5) were identified on the upstream region of the indole-3-acetic acid operon located on the same cosmid. Based on homology of amino acids and genetic organization, ISEhe1 belongs to the IS630 family, ISEhe2 to the IS5 family, ISEhe3 and ISEhe4 to different groups of the IS3 family, and ISEhe5 to the IS1 family. With the exception of ISEhe4, one to three copies of all the other IS elements were identified only in pathogenic strains of *Erwinia herbicola* pv. *gypsophila* and *Erwinia herbicola* pv. *betae* whereas ISEhe4 was present in both pathogenic and non-pathogenic strains. An open reading frame that exhibited high identity (89% in amino acids) to *AvrPphD* of *Pseudomonas syringae* pv. *phaseolicola* was present within the cluster of IS elements. An insertional mutation in the *AvrPphD*_{Ehg} reduced gall size in *gypsophila* by approximately 85%. In addition, remnants of known genes from four different bacteria were detected on the same cosmid.

Additional keywords: cytokinins, *Pantoea agglomerans*.

Erwinia herbicola (*Pantoea agglomerans*) is widespread in nature as an epiphyte on many different plants (Gavini et al. 1989; Starr 1981). In addition to its prevalence on plant surfaces, it has been characterized as an endophyte in grapevine (Bell et al. 1995) and has been isolated from seeds, water, humans, and animals (Gavini et al. 1989). Some strains of *Erwinia herbicola* have evolved into tumorigenic bacteria that incite gall formation on *gypsophila* (*Gypsophila paniculata* L.) (Cooksey 1986) and beet (*Beta vulgaris* L.) (Burr et al. 1991). The host range of *Erwinia herbicola* pv. *gypsophila* (Ehg) is restricted to *gypsophila* (Volcani 1985) whereas the beet pathovar, *Erwinia herbicola* pv. *betae* (Ehb) elicits galls on both beet and *gypsophila* (Burr et al. 1991). Other pathovars of

Erwinia herbicola that cause galls on *Wisteria* (Opgenorth et al. 1994), Douglas fir (De Young et al. 1998) and cranberry (Vasanthakumar and McManus 2001) have also been reported. The pathogenicity of *Erwinia herbicola* pv. *gypsophila* or *Erwinia herbicola* pv. *betae* depends on a similar large indigenous plasmid (designated as pPATH_{Ehg} or pPATH_{Ehb}) that is present in all pathogenic, but not in nonpathogenic, strains (Manulis et al. 1991; I. Barash and S. Manulis, *unpublished data*). pPATH_{Ehg}, which has been extensively studied, has a size of approximately 150 kb. It harbors many virulence genes that can be divided into three groups: an *hrp* gene cluster (Mor et al. 2001; Nizan et al. 1997), genes encoding type III effectors that are involved in pathogenicity and host specificity (Ezra et al. 2000; Mor et al. 2001; Valinsky et al. 1998), and genes encoding biosynthetic pathways for indole-3-acetic acid (IAA) and cytokinins (Clark et al. 1993; Gafni et al. 1997; Lichter et al. 1995a). All these genes are located on three successive and overlapping cosmids that occupy approximately 55 kb of the pPATH_{Ehg} and that could be conceived as a pathogenicity island.

During recent years, the concept of pathogenicity islands (PAIs) has emerged to describe genomic regions of pathogens carrying virulence genes together with loci whose presence strongly indicates horizontal gene transfer between species or even genera (Hacker and Kaper 1999). PAIs are typically present in genomes of pathogenic bacteria but not in those of non-pathogenic members of the same or closely related species. The definition of PAIs originally included chromosomal location (Hacker et al. 1997), but the increasing volume of sequence data available for virulence plasmids supports the view that parts of them can also be considered to be PAIs (Hacker and Kaper 1999). Examples include plasmid-borne PAIs in bacteria pathogenic on animals (Iriarte and Cornelis 1999; Parsot and Sansonetti 1999) and on plants (Jackson et al. 1999; Winans et al. 1999).

Type III secretion systems play an essential role in the virulence of most gram-negative pathogenic bacteria and are closely associated with PAIs (Hacker and Kaper 2000). Moreover, it is now assumed that bacteria acquire pathogenicity not by slow adaptive evolution but by “quantum leaps” that enable them to acquire complete virulence systems in the course of one or a few steps (Meccas and Strauss 1996). Consequently, the flanking regions of the *hrp*/type III gene clusters in phytopathogenic bacteria are of particular interest because they may provide valuable information on PAIs evolution and may help to identify type III effectors or other genes that could contrib-

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ute to virulence (Alfano et al. 2000). Cosmid pLA150 of the pPATH_{Ehg} has previously been shown to encompass the *hrpJ* operon (Nizan et al. 1997), a host-specific virulence gene *hsvG* (Valinsky et al. 1998), a cluster of IAA and cytokinin biosynthesizing genes (Clark et al. 1993; Lichter et al. 1995a), and two copies of insertion sequences (IS) designated as IS1327 that belong to the IS6 family (Lichter et al. 1996) (Fig. 1). Therefore, we considered the possibility that pLA150 might

possess additional loci associated with either virulence or sequences acquired by horizontal gene transfer. The present study supports the latter hypothesis and demonstrates that this cosmid contains additional multiple and diverse IS elements, an *avrPphD* homologue of *Pseudomonas syringae* pv. *phaseolicola* that acts as a virulence factor, and remnants of foreign genes from different bacteria. Furthermore, additional evidence was found that substantiates the presence of a PAI on pPATH_{Ehg}.

Fig. 1. Schematic map of the pathogenicity plasmid pPATH_{Ehg} and physical map of its cosmid clone pLA150. Previously identified genes and IS element: *iaaM* and *iaaH* (indole-3-acetic acid biosynthesizing genes); *pre-etz* and *etz* (cytokinin biosynthesizing genes); *hsvG*, *dspAE/BF*, *pthG* (type III virulence effectors); *hrpJ* operon, and IS1327. **A**, Enlarged sequenced DNA region (4 kb) upstream to *iaaM*. **B**, Enlarged sequenced *hrpJ*-flanking DNA region (10 kb) extending between *hrpJ* and IS1327. Open arrows signify the location and orientation of the newly identified IS elements: IS1-ISEhe1, IS2-ISEhe2, IS3-ISEhe3, IS4-ISEhe4, and IS5-ISEhe5 (two copies). IS1a and IS1b are two parts of ISEhe1 that were disrupted by ISEhe2, whereas IS3a and IS3b are two parts of ISEhe3 that were disrupted by ISEhe4. *avrPphD*_{Ehg} is a homologue of the *avrPphD* reported in *Pseudomonas syringae* pv. *phaseolicola*. Roman numerals I to IV in gray boxes indicate residual gene sequences acquired from a variety of bacteria. R = EcoRI, B = BamHI, P = PstI, H = HindIII, K = KpnI.

RESULTS

Characterization of new IS elements on cosmid pLA150.

To determine whether additional virulence genes or IS elements are present on pLA150, we sequenced the region upstream to the *iaaM* gene (Fig. 1A) and the flanking region of the *hrp* gene cluster between the *hrpJ* operon and IS1327 (Fig. 1B), as described below. In addition to two copies of the previously described IS1327 (Lichter et al. 1996), five new IS elements were characterized (Table 1). According to their distance from the *hrp* gene cluster, they were designated as ISEhe1 to ISEhe5 as suggested by the IS finder Internet website. Classification of the IS elements into families and groups was based on similarities in their putative transposases (Table 1), the presence of common domains (not shown), and similarities in genetic organization (arrangement of open reading frames [ORFs]) (Table 1) as described by Mahillon and Chandler (1998). Accordingly, as shown in Table 1, ISEhe1 was allocated to the IS630 family with the highest homology in amino acids (84%) to IS630 from *Shigella sonnei* (Hu-Shu and Malabi 1998); ISEhe2 to the IS5 family with the highest homology (52%) to IS427 from *Agrobacterium tumefaciens* (de Meirsmen et al. 1990); ISEhe3 to the IS3 family (group IS3) with the highest homology (59%) to an IS3-like element from *A. tumefaciens* (Otten and Schmidt 1998); ISEhe4 to IS3 (group IS407) with the highest homology (95%) to IS122 from *Enterobacter agglomerans* (Steibl and Lewecke 1995); and ISEhe5 to the IS1 family with the highest homology (47%) to IS1 of *Shigella dysenteriae* (Ohtsubo et al. 1981). With the exception of ISEhe1, which carries a single transposase, all the IS elements accommodate two transposases (Table 1).

The G+C contents of ISEhe1, ISEhe2, ISEhe3, ISEhe4, and ISEhe5 (in mole percentages) were 49.2, 51.2, 47.7, 53.9, and 43.9, respectively, as compared with G+C contents of 55.1 to 56.8 mole% for *Erwinia herbicola* (*Pantoea agglomerans*) (Gavini et al. 1989). The continuities of ISEhe1 and ISEhe3 were interrupted by insertion of ISEhe2 and ISEhe4, respectively (Fig. 1B). The latter sequence organization could indicate that the horizontal transfer of ISEhe1 and ISEhe3 into the pPATH_{Ehg} preceded that of ISEhe2 and

ISEhe4. It is also noteworthy that ISEhe5 was present in two copies on pLA150 (Fig. 1A).

In addition to the IS elements and the *avrPphD* homologue (i.e., *avrPphD_{Ehg}*) to be described below, four remnants of known gene sequences (designated I to IV) from various bacteria were identified (Fig. 1). These include residual sequences that exhibited 75% homology (per 193 amino acids [aa]) with the coagulase/fibrinolysin precursor from *Yersinia pestis* (accession number AL109969), 50% homology (per 120 aa) with hypothetical protein from *Xylella fastidiosa* (accession number AE004024), 68% homology (per 290 aa) with conserved hypothetical protein 1 of *Actinobacillus pleuropneumoniae* (accession number AF282240) (Cheng et al. 2000), and 52% homology (per 115 aa) with a putative transposase from *Escherichia coli* (accession number AE005345).

Distribution of the IS elements in pathogenic and nonpathogenic strains of *Erwinia herbicola*.

The presence and the copy number of newly identified IS elements in pathogenic (*Ehg*824-1, *Ehg*3-1a, *Ehb*4188) and nonpathogenic (*Eh*3-1, *Eh*24-8, *Eh*K4, *Eh*27-3, *Eh*717-2, *Eh*3-106) strains of *Erwinia herbicola* were determined by digesting the total DNA of each strain with *Eco*RI, *Hind*III, and *Bam*HI, and then performing a Southern blot hybridization with IS-specific probes. An example of a Southern blot hybridization obtained with *Hind*III digests of three pathogenic and two nonpathogenic strains are illustrated in Figure 2. Results summarized in Table 2 indicate that ISEhe1, ISEhe2, ISEhe3, and ISEhe5 were present only in pathogenic strains, whereas ISEhe4 was present in both pathogenic and nonpathogenic strains. The nonpathogenic strain *Eh*3-106 is a Tn5 derivative of *Ehg*824-1 with a deletion of 40 to 50 kb in the pPATH_{Ehg} region upstream to the *etx* gene (Lichter et al. 1995b). Thus it does not contain the 2 copies of ISEhe5 located upstream to the *iaaM* gene (Fig. 1A) but still accommodates more than 100 kb of the pPATH_{Ehg} on which the other IS elements were present. All the IS elements also appeared in the beet pathovar (*Ehb*4188). The copy numbers of the various IS elements among the different *Erwinia* strains ranged from one to three with the exception of ISEhe3 in *Ehb*4188, which

Table 1. New IS elements characterized on the pLA150 cosmid of pPATH_{Eh}

IS elements/ accession no.	ORF ^a (bp)	Inverted repeats	Family	Group	Possible sources (homology) ^b	Reference or accession no.
ISEhe1 AF326767	525	21/21	IS630	IS630	<i>Shigella a sonnei</i> IS630 (84%)	Hu-Shu and Malabi 1998
					<i>Salmonella choleraesuis</i> (62%) <i>Salmonella typhimurium</i> (61%) <i>Streptomyces coelicolor</i> IS1648 and IS1650 (50%)	BAA01531.1 Krause et al. 1991 Redenbach et al. 1996
ISEhe2 AF327444	A-449 B-515	23/22	IS5	IS427	<i>Agrobacterium tumefaciens</i> IS427 (52%) <i>Mycobacterium tuberculosis</i> ISMt1 (42%) <i>Erwinia amylovora</i> ISEam1 (51%)	de Meirsmen et al. 1990 Mariani et al. 1993 McGhee and Jones 2000
ISEhe3 AF327445	A-284 B-839	20/20	IS3	IS3	<i>Agrobacterium tumefaciens</i> IS3-like (59%) <i>Yersinia enterocolitica</i> IS1329 (43%) <i>Shigella sonnei</i> IS600 (40%)	Otten and Schmidt 1998 Carniel et al. 1996 Matsutani et al. 1987
ISEhe4 AF324174	A-263 B-824	26/25	IS3	IS407	<i>Enterobacter agglomerans</i> IS1222 (95%) <i>Pseudomonas cepacia</i> IS407 (69%) <i>Xanthomonas campestris</i> pv. <i>campestris</i> (58%) <i>Xanthomonas campestris</i> pv. <i>amaranthicola</i> (59%) <i>Rhizobium</i> spp ISR1 (59%) <i>Shigella dysenteriae</i> IS1 (47%)	Steibl and Lewecke 1995 Wood et al. 1991 Lee and Chiu 1998 Gomez et al. 1997 Priefer et al. 1989 Ohtsubo et al. 1981
ISEhe5 AF327446	A-275 B-506	30/30	IS1		<i>Escherichia coli</i> IS1 (46%)	Umeda and Ohtsubo 1991

^a Open reading frames (ORF) of transposases. With the exception of ISEhe1, all IS elements contained two putative transposases, designated A and B.

^b Percent homology in amino acid

reached five or six copies (Fig. 2, Table 2). Interestingly, the nonpathogenic *Eh3-1* is apparently an isogenic strain of *Ehg3-1a* that resulted from the loss of pPATH_{Ehg} (Table 3). Thus, the IS elements that are present in *Ehg3-1a* and are absent in *Ehg3-1* are most likely located on pPATH_{Ehg}.

The *AvrPphD*_{Ehg} and its effect on pathogenicity.

DNA sequence analysis of the *hrp* flanking region between ISEhe3 and IS1327 (Fig. 1B) revealed an ORF of 2,118 bp encoding a deduced peptide product of 706 aa with a predicted molecular mass of 75.45 kDa. A putative *hrp* box was identified 42 bp upstream of the start codon. The ORF showed 89% identity in amino acids to *AvrPphD* of *Pseudomonas syringae* pv. *phaseolicola*, which contains an ORF of 2,130 bp and encodes a peptide product of 710 aa (Arnold et al. 2001). The G+C content of the *avrPphD*_{Ehg} was 52.3%, compared with 55% of *Pseudomonas syringae* pv. *phaseolicola*. The nucleotide sequence of the *avrPphD*_{Ehg} was submitted to GenBank as accession number AF462346. This gene has also been isolated from pPATH_{Ehb}, and the two ORFs share 95% homology.

In order to examine the effect of *avrPphD*_{Ehg} on the pathogenicity of *Erwinia herbicola* pv. *gypsophylae*, the marker exchange mutant *Ehg824-1*Mx(Km-*avrPphD*_{Ehg}) was generated by inserting a kanamycin-resistant (Km^r) cassette into the ORF of *avrPphD*_{Ehg}. The obtained mutant was then tested for pathogenicity on gypsophila cuttings. The results illustrated in Figure 3 clearly demonstrated that this mutant substantially reduced gall size in gypsophila compared with that in the wild-type strain. Measurements on six replicates per treatment showed that the galls in the wild-type *Ehg824-1* weighed an average of 50±18 mg after 12 days compared with 7.5±0.2 mg in the mutant. Similar reductions of approximately 85% in gall size were obtained in three independent experiments. Pathogenicity of the mutant could be restored almost to the wild-type level by complementation with pLA150 in trans (Fig. 3). The marker exchange mutant *Ehg824-1*Mx(Km-*avrPphD*_{Ehg}) was also tested for hypersensitive response (HR) elicitation on

tobacco leaves (*Nicotiana tabacum* L. Hicks) as described previously (Nizan et al. 1997). The mutant elicited the HR response, as did the wild-type strain (results not shown).

DISCUSSION

The presence of an assembly of six different IS elements, including IS1327 (Lichter et al. 1996), in the flanking regions of the *hrp* gene cluster and of the IAA operon on pPATH_{Ehg} (Fig. 1) provides strong evidence that an intensive horizontal gene transfer may have taken place during the evolution of the pathogenicity plasmid. Studies of the mechanisms of bacterial pathogenesis have demonstrated associations between ISs and many pathogenic and virulence functions. Such associations have been observed in animal pathogens, e.g., *Bacillus* (Mahillon et al. 1994), *Yersinia* (Filippov et al. 1990), and *Escherichia* (Hu and Lee 1988); plant pathogens, e.g., *Agrobacterium* (Otten et al. 1992) and *Pseudomonas* (Yamada et al. 1986); and symbionts, e.g., *Rhizobium* (Freiberg et al. 1997). The clustering of various IS elements in "islands" within plasmid genomes has also been observed frequently (Mahillon and Chandler 1998) and may serve to promote plasmid integration, excision, and other rearrangements.

We have previously shown that multiple copies of IS1327 are found only in pPATH_{Ehg} and pPATH_{Ehb} (Lichter et al. 1996). The observation that multiple copies of ISEhe1, ISEhe2, ISEhe3, and ISEhe5 are present only in pathogenic strains of *Erwinia herbicola* pv. *gypsophylae* and *Erwinia herbicola* pv. *betae* and are not present in nonpathogenic strains that lack pPATH (Table 2) could imply that they are confined to the pathogenicity plasmids in both pathovars. Comparison of IS elements in the pathogenic strain *Ehg3-1a* with those in its isogenic and nonpathogenic counterpart *Eh3-1* corroborates this hypothesis. The latter results may suggest that these IS elements participated in the evolution of pPATH. In contrast, ISEhe4 was present in both pathogenic and nonpathogenic strains and might not necessarily have been involved in the formation of pPATH. The apparent specificity

Fig. 2. Southern blot hybridization showing the distribution of ISEhe1, ISEhe2, ISEhe3, ISEhe4, and ISEhe5 in different *Erwinia herbicola* strains. Total DNA was digested with *Hind*III and hybridized with probes of each of the IS elements (ISEhe1 to ISEhe5). *Ehg 824-1*, *Ehg 3-1a*, and *Ehb 4188* are pathogenic, whereas *Eh 3-1* and *Eh3-106* are nonpathogenic strains. Left lane, λ *Bsr* EI DNA digest.

of IS1327 and ISEhe1, ISEhe2, ISEhe3, and ISEhe5 to the pPATH could result from their inability to transpose to other plasmids or to chromosomal DNA. Alternatively, these IS elements may have been actively distributed on the pPATH prior to its acquisition by *Erwinia herbicola*, in which they became inactive. At present, we do not have evidence to support current transposition of these IS elements, although copies of IS1327 have been transcribed and their transcripts may accommodate the transposase size (Lichter et al. 1996). Detailed research would be required to determine whether the observed polymorphism in IS elements between pPATH_{Ehg} and pPATH_{Ehb}, which is manifested in fragment size in the case of IS1327 and in copy number in the

case of IS1327 (Lichter et al. 1996) and ISEhe3 (Table 2), could result from active transposition or from rearrangements of plasmids after segregation of the pathovars. Nevertheless, the hypothesis of a common origin of pPATH in the two pathovars (Ezra et al. 2000) is supported by the presence of the same IS elements as well as by the presence of common virulence genes on both plasmids (Lichter et al. 1995a; Mor et al. 2001; Valinsky et al. 1998; I. Barash and S. Manulis unpublished data).

AvrPphD, which was originally isolated from *Pseudomonas syringae* pv. *phaseolicola*, is widely distributed in almost all *Pseudomonas syringae* pathovars (Arnold et al. 2001). Homologues of *avrPphD* have also been identified in *Xanthomonas*

Table 2. Number of copies of IS elements in pathogenic and nonpathogenic *Erwinia herbicola* strains

IS	Number of copies of IS elements ^a								
	Pathogenic strains			Nonpathogenic strains					
	<i>Ehg824-1</i>	<i>Ehb4188</i>	<i>Ehg3-1a</i>	<i>Eh3-1</i>	<i>Eh24-8</i>	<i>EhK4</i>	<i>Eh27-3</i>	<i>Eh717-2</i>	<i>Eh3-106</i>
ISEhe1	1	1	1	0	0	0	0	0	1
ISEhe2	2	1	2	0	0	0	0	0	2
ISEhe3	1	5 to 6	1	0	0	0	0	0	1
ISEhe4	3	2	3	1	0	1	2	2	3
ISEhe5	2	2	2	0	0	0	0	0	0

^a The total DNA of pathogenic strains *Ehg* 824-1, *Ehg* 3-1a, and *Ehb* 4188 and nonpathogenic strains *Eh* 3-1, *Eh*24-8, *Eh* K4, *Eh* 27-3, *Eh* 717-2, and *Eh*3-106 was digested with *Eco*RI, *Hind*III, and *Bam*HI, separately. Following gel electrophoresis, each digest was subjected to Southern blot hybridization with probes for each of the five IS elements. The number of copies were determined from the number of bands observed after Southern blotting. *Eh*3-106 is a Tn5 derivative of *Ehg*824-1 with a deletion of 40 to 50 kb in the pPATH_{Ehg} upstream to the *etz* gene.

Table 3. Bacterial strains, cosmids, and plasmids^a

Strain/plasmid	Relevant characteristics	Reference or source
<i>Escherichia coli</i> DH5 α	RecA, lacZ Δ , M15	Hanahan 1983
<i>Erwinia herbicola</i> (Eh)		
Ehg824-1	Rif ^r , wild-type pathogenic strain, gypsophila pathovar	Manulis et al. 1991
Ehb4188	Rif ^r , wild-type pathogenic strain, beet pathovar	Burr et al. 1991
Ehg3-1a	Wild-type, pathogenic strain, gypsophila pathovar	Manulis et al.1991
Eh3-106	Ehg824-1::Tn5, Km ^r , nonpathogenic, a 40 to 50-kb deletion in pPATH _{Ehg}	Lichter et al.1995b
Eh3-1	Nonpathogenic strain, a derivative of Ehg3-1a	Lichter et al.1995a
Eh24-8	Wild-type, nonpathogenic strain	Manulis et al.1991
EhK4	Wild-type, nonpathogenic strain	Manulis et al.1991
Eh 27-3	Wild-type, nonpathogenic strain	Manulis et al.1991
Eh717-2	Wild-type, nonpathogenic strain	Manulis et al.1991
Ehg824-1Mx (Km-avrPphD _{Ehg})	Km ^r , marker exchange mutant of Km ^r cassette in avrPphD _{Ehg}	This study
Cosmids		
pLAFR3	Tc ^r , broad-host-range vector IncP-1 rlx RK2 ⁺ lacZ α Tra ⁻ Mob ⁺ cos	Staskawicz et al. 1987
pLA150	Tc ^r , a 28-kb cosmid clone in pLAFR3	Lichter et al.,1995a
pLG101	Tc ^r , 7.5-kb <i>Eco</i> RI fragment from pLA150 cloned into pLAFR3, covering <i>iaaM</i> , <i>iaaH</i> , and part of cytokinin operon	Clark et al. 1993
pLA-H2.4, pLA-H1.8, pLA-H9, pLA-H7.3, pLA-H2.8, and pLA-H4.1	Tc ^r , <i>Hind</i> III-digested fragments of pLA150 subcloned into pLAFR3	This study
pLA-B/H3.8	Tc ^r , a 3.8-kb <i>Bam</i> HI/ <i>Hind</i> III fragment of pLA-H7.3 subcloned into pLAFR3	This study
pLA-B/H3.0	Tc ^r , a 3.0-kb <i>Bam</i> HI/ <i>Hind</i> III fragment of pLA-H7.3 subcloned into pLAFR3	This study
pLA-B4.5	Tc ^r , a 4.5-kb <i>Bam</i> HI fragment of pLG101subcloned into pLAFR3	This study
pLA-R/H5.5	Tc ^r , a 5.5 <i>Eco</i> RI/ <i>Hind</i> III fragment of pLA-H9 subcloned into pLAFR3	This study
Plasmids		
pBluescript KS	Amp ^r , subcloning and sequencing vector	Stratagene La Jolla, CA, U.S.A.
pMBG-H/P2.5	Amp ^r , a 2.5-kb <i>Hind</i> III/ <i>Pst</i> I fragment of pLA-R/H5.5 subcloned into into KS ⁺ (pBS)	This study
pMBG-H/P1.7	Amp ^r , a 1.7-kb <i>Hind</i> III/ <i>Pst</i> I fragment of pLA-B/H3.8 subcloned into KS ⁺ (pBS)	This study
pMBG-R/B2.8	Amp ^r , a 2.8-kb <i>Eco</i> RI/ <i>Bam</i> HI fragment of pLA-B/H3.8 subcloned into KS ⁺ (pBS)	This study
pMBG-B/H3.0	Amp ^r , a 3.0-kb <i>Bam</i> HI/ <i>Hind</i> III fragment of pLA-H7.3 subcloned into KS ⁺ (pBS)	This study
pMBG-R/H1.1	Amp ^r , a 1.1-kb <i>Eco</i> RI/ <i>Hind</i> III fragment of pLA-H2.8 subcloned into KS ⁺ (pBS)	This study
pMBG-R/H1.5	Amp ^r , a 1.5-kb <i>Eco</i> RI/ <i>Hind</i> III fragment of pLA-H2.8-kb subcloned into KS ⁺ (pBS)	This study
pMBG-B/K2.4	Amp ^r , a 2.4-kb <i>Bam</i> HI/ <i>Kpn</i> I fragment of pLA-B4.5 subcloned into KS ⁺ (pBS)KS ⁺ (pBS)	This study
pMBG-B/K2.5	Amp ^r , a 2.5-kb <i>Bam</i> HI/ <i>Kpn</i> I fragment of pLA-B4.5 subcloned into KS ⁺ (pBS)	This study
pMBG-H2.4	Amp ^r , a 2.4 <i>Hind</i> III fragment of pLA-H2.4 subcloned into KS ⁺ (pBS)	This study
pMBG-H1.8	Amp ^r , a 1.8 <i>Hind</i> III 1.8kb fragment of pLA-H1.8 subcloned into KS ⁺ (pBS)	This study

^a Antibiotics were used in the following concentrations (μ g/ml): ampicillin (Amp), 100; kanamycin (Km), 50; rifampicin (Rif), 100; and tetracycline (Tc), 12.

campestris pv. *vesicatoria* (Noel et al. 2001) and in the genome sequence of *Ralstonia solanacearum*. However, in spite of its prevalence in three major phytopathogenic bacterial species, the involvement of *avrPphD* in virulence has never been reported. In the present study, we have not only demonstrated the presence of an *avrPphD*_{Ehg} in a new bacterial species but have also shown that it functions as a virulence factor in *Erwinia herbicola* pv. *gypsophila*. Interestingly, gall reduction in gypsophila (85%) obtained by the *avrPphD*_{Ehg} mutant was significantly higher than the approximately 50% reduction obtained by mutants deficient in production of either cytokinins or IAA, or both (Manulis et al. 1998). The presence of a *hrp* box in the promoter region of the gene suggests that it acts as a type III virulence effector. Further work will be required to elucidate the mode of action of *AvrPphD*_{Ehg} in gall formation by *Erwinia herbicola* pv. *gypsophila*.

Hacker and associates (1997) defined PAIs as gene clusters that exhibit the following characteristics: (i) they include many virulence genes; (ii) they are present in pathogenic but not in closely related nonpathogenic strains; (iii) they have a different G+C content from that of the host bacteria DNA; (iv) they occupy large chromosomal regions; (v) they contain (often cryptic) 'mobility' genes (IS elements, transposases, integrases); (vi) there is association with tRNA genes or IS elements, or both, in their boundaries; and (vii) they are unstable. Current and previous data on the organization and nature of the mobile elements and virulence genes on the pPATH_{Ehg} plasmid comply with the major criteria for a PAI definition and unequivocally

confirm the presence of a PAI. Evidence that substantiates this conclusion is based on the following findings: (i) as indicated above, all the virulence genes that have so far been characterized on the pPATH_{Ehg}, as well as the cluster of IS elements demonstrated in the present study, are assembled on three consecutive overlapping cosmids that occupy approximately 55 kb of the plasmid (Fig. 1), (ii) with the exception of ISEhe4, all the IS elements are present in pathogenic but not in nonpathogenic strains, and (iii) the range of the G+C content of ISEhe1, ISEhe2, ISEhe3, and ISEhe5 extends from 51.24 to 43.93% and is significantly lower than the characteristic G+C contents of the species (55 to 56.8%) (Gavini et al. 1989). Similarly, the G+C contents of all the characterized virulence genes are significantly different from those of the species. Thus, the range of G+C content of the various virulence genes are: operons of the *hrp* cluster, from 51.8 to 47.8% (Mor et al. 2001); IAA operon, 50.3 to 52.8% (Gafni et al. 1997); cytokinin operon, 34.8 to 41.3%; (Lichter et al. 1995a); *hsvG*, 65.4% (Valinsky et al. 1998); *pthG*, 51.58% (Ezra et al. 2000); *dspAE/BF*, 49.9% (Mor et al. 2001); and *avrPphD*_{Ehg}, 52.6%. Further evidence of the foreign nature of the DNA that forms the PAI on pPATH_{Ehg} was provided by the identification of remnants from known genes of *Yersinia*, *Xylella*, *Actinobacillus* and *Escherichia* spp. (Fig. 1). The existence of the type III system (Mor et al. 2001) reinforces the characterization of the PAI on pPATH_{Ehg}. Type III systems have been shown to act as major components in many PAIs of gram-negative bacteria that are pathogenic on animals and plants (Galan and Collmer 1999; Hueck 1998). The possibility that additional genetic loci associated with virulence will be discovered prevents the precise delimitation of the plasmid-borne PAI in *Erwinia herbicola* pv. *gypsophila*.

Erwinia herbicola is widespread in numerous and diverse natural microenvironments (Gavini et al. 1989). It appears that many different bacteria that presumably encountered *Erwinia herbicola* in its various habitats have left their fingerprints on its genome via mobile elements such as plasmids and insertion sequences and have been instrumental in its conversion from an epiphyte into a biotrophic and gall-forming pathogen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacterial strains and plasmids used in the present study are listed in Table 3. Bacteria were grown in Luria Bertani (LB) broth or on LB agar plates, with appropriate antibiotics, at 28°C for *Erwinia herbicola* and at 37°C for *E. coli*. Genetic manipulations were carried out in *Escherichia coli* DH5α (Hanahan 1983).

Pathogenicity tests.

Pathogenicity tests on cuttings of *Gypsophila paniculata* cvs. Festival or Golan were essentially as described previously (Lichter et al. 1995a). After removal of an approximately 3-mm

Fig. 3. Phenotypic expression of insertional kanamycin-resistant cassette in *avrPphD*_{Ehg} following inoculation of gypsophila cuttings. **A**, water control; **B**, wild type, *Ehg824-1*; **C**, marker exchange mutant *Ehg824-1Mx* (*Km-avrPphD*_{Ehg}); and **D**, complementation of *Ehg824-1Mx* (*Km-avrPphD*_{Ehg}) with pLA150.

Table 4. Polymerase chain reaction (PCR) primers used for obtaining IS probes

Primer	Sequence (5'-3')	Source ^a	IS element	IS probe (bp)
1A	ATGGTGAAACTGGGCTGC	pMBG-R/H1.1	ISEhe1	575
1B	ATCACCAGCCGACTGAG	pMBG-R/H1.1		
2A	TGTCCCTTAATCACCTGAG	pMBG-B/H3.0	ISEhe2	598
2B	TCATGCTGCCGTTCTTAC	pMBG-B/H3.0		
3A	TCCAGCGTTGCAACACGTC	pMBG-B/H3.0	ISEhe3	655
3B	GAACAGATCAACAACACGG	pMBG-B/H3.0		
4A	CCTGCAGGCTGGCAGGTCTG	pMBG-R/B2.8	ISEhe4	738
4B	TGCAAATTCAGACGGCGTC	pMBG-R/B2.8		
5A	GTTACCGTCCTCCCGTGC	pMBG-H2.4	ISEhe5	479
5B	TGCATATCACAGCCAGCG	pMBG-H2.4		

^a Subclones used as templates for PCR are described in Table 3.

section from the bottom of the stem, cuttings were inoculated by dipping into a bacterial suspension of 10^8 CFU/ml and placed in vermiculite-filled trays. The greenhouse temperature was held at 26 to 28°C, and computer-controlled mist sprinklers maintained high humidity, as described previously (Lichter et al. 1995a). Gall formation was scored 14 days after inoculation on three or four replicates per strain in three independent experiments.

DNA manipulations.

Isolation of plasmid DNA from *Escherichia coli*, restriction enzyme digestions, subcloning, transformation, Southern blot analyses, polymerase chain reactions (PCRs), and miniprepations of total DNA from *Erwinia herbicola* were carried out by standard procedures (Ausubel et al. 1995). A DNA probe for each IS element was prepared by PCR, with primers synthesized by Gibco BRL (Glasgow, U.K.) (Table 4). Following electrophoresis, DNA product was extracted from the gel with a Glass Powder DNA isolation kit (Biological Industries Co., Beth Halmek, Israel) and was Dig-labeled using a Random Primer Labeling Kit (Roche Molecular Biochemicals, Mannheim, Germany).

Insertional inactivation of *avrPphD*_{Ehg} was carried out by introducing a Km^r cassette. pMBG-H/P1.7 (Table 3) was digested by *Eco*RI and self ligated to obtain a deletion of 316 bp. The Km^r cassette was then inserted into the *Eco*RI site of the deleted pMBG-H/P1.7 as described elsewhere (Lichter et al. 1995a). This construct was digested with *Asp*718 and *Nor*I, and the cohesive termini of the restriction sites were then filled with Klenow enzyme to form blunt ends. The resulting fragment was cloned into pLAFR3 that had been predigested with *Pst*I and treated with Klenow enzyme to obtain blunt ends. The mutated *avrPphD* in pLAFR3 was mobilized to *Ehg*824-1 by triparental mating followed by plasmid curing as described previously (Clark et al. 1993) to obtain the marker exchange mutant *Ehg*824-1Mx (Km-*avrPphD*_{Ehg}). Isolation of *avrPphD*_{Ehb} from the beet pathovar was carried out with the cosmid pLE142 from the plasmid library of *Ehb*4188, which forms part of pPATH_{Ehb} (Ezra et al. 2000).

DNA sequencing.

The *Hind*III-digested fragments from pLA150 were cloned into pLAFR3 to produce subclones designated as pLA-H2.4, pLA-H1.8, pLA-H9.0, pLA-H7.3, pLA-H2.8, and pLA-H4.1, (Table 3). pLA-H7.3 was further digested with *Bam*HI and *Hind*III into two fragments of 3.8 and 3 kb that were cloned into pLAFR3 to yield pLA-B/H3.8 and pLA-B/H3, respectively. A 4.5-kb fragment obtained from pLG101 (Table 3) following digestion with *Bam*HI was cloned into pLAFR3 to generate pLA-B4.5. Similarly, a 5.5-kb fragment obtained by digesting pLA-H9 with *Eco*RI and *Hind*III was cloned into pLAFR3 to generate pLA-R/H5.5. The DNA inserts from the above mentioned cosmids were subcloned into pBluescript (KS⁺) for sequencing, and the resulting plasmids were designated as pMBG, with a plasmid number indicating the size of the insert (Table 3). The plasmids pMBG-B/K2.4 and pMBG-B/K2.5 were used for obtaining the sequence described in Figure 1A, whereas pMBG-H/P2.5, pMBG-H/P1.7, pMBG-R/B2.8, pMBG-B/H3.0, pMBG-R/H1.1, pMBG-R/H1.5, pMBG-H2.4, and pMBG-H1.8 were used for obtaining the sequence described in Figure 1B. Automated sequencing with Taq DNA polymerase was carried out at the Laboratories for Biological Services, Tel-Aviv University. Whenever required, walking with custom primers completed the sequence. Sequence data were analyzed with the Wisconsin Package, version 9 (Genetic Computer Group, Madison, WI, U.S.A.), and sequences were then compared with data in the Gene Bank by means of DNA or protein Blast tools at NCBI.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

INRA IS Finder Database: <http://www-is.biotoul.fr>
 National Center of Biotechnology Information website: www.ncbi.nlm.nih.gov/
Ralstonia solanacearum BLAST Server: sequence.toulouse.inra.fr/ralsto/public/doc/RalstoForm.html