Research Note

Genetic Organization of the *hrp* Gene Cluster and *dspAE/BF* Operon in *Erwinia herbicola* pv. *gypsophila*

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Erwinia herbicola pv. *gypsophila* induces gall formation in gypsophila that is dependent on the existence of a pathogenicity plasmid (pPATH_{Ehg}). We previously demonstrated the presence of several *hrp* genes on this plasmid. By employing transposon mutagenesis and sequencing, a functional *hrp* gene cluster on the pPATH_{Ehg} has now been characterized completely. The *hrp* genes of *E. herbicola* pv. *gypsophila* are remarkably similar to and colinear with those of *Erwinia amylovora* and *Pantoea stewartii* and generally showed 60 to 90% nucleotide or deduced amino acid identity. *E. herbicola* pv. *gypsophila*, however, lacks *hrpW*, which is present in *E. amylovora*. Additionally, *E. herbicola* pv. *gypsophila* mutants deficient in harpin production retained pathogenicity and were slightly reduced in their ability to elicit a hypersensitive response (HR) in tobacco. The “disease specific” region, *dspA/EB/F*, exhibited 60 to 74% identity with the *dspA/EB/F* loci of *E. amylovora* and *P. stewartii*, respectively. Mutations in *dspA/E* abolished pathogenicity of *E. herbicola* pv. *gypsophila* but not HR elicitation on tobacco. Inactivation of *HrpL* reduced plant-induced transcription of *dspA/E* by three orders, indicating Hrp-dependent regulation.

The presence of *hrp* genes is essential for the pathogenicity of most gram-negative phytopathogenic bacteria. Mutations in *hrp* genes abolish pathogenicity in susceptible host plants and the ability to elicit the hypersensitive response (HR) in resistant hosts or nonhost plants (Bonas 1994). Genetic and biochemical studies have demonstrated that *hrp* genes encode components of a type III secretion pathway, regulatory proteins that allow the bacteria to sense the host environment, and harpins, a class of proteinaceous elicitors of the HR in nonhost plants (Lindgren 1997). In animal and plant pathogenic bacteria, the type III secretion pathway appears to be responsible for the delivery of effector proteins into the eukaryotic host

cells (Galan and Collmer 1999). In gram-negative phytopathogenic bacteria, including *Pseudomonas syringae*, *Erwinia amylovora*, *Erwinia carotovora*, *Erwinia chrysanthemi*, *Pantoea* (*Erwinia*) *stewartii* subsp. *stewartii*, *Xanthomonas campestris*, *Xanthomonas oryzae*, and *Ralstonia solanacearum*, the *hrp* genes are organized in clusters of 22 to 40 kb and contain at least six to eight complementation groups (Bonas 1994; Lindgren 1997). The nucleotide sequences of *hrp* gene clusters revealed high similarities between pathogens belonging to different genera, and certain genes appear to be highly conserved (Bogdanove et al. 1996a). Earlier studies have demonstrated that the *hrp* clusters of *E. amylovora* and *P. syringae* are closely related yet distinct from the *hrp* genes of *X. campestris* and *R. solanacearum*, which are similar and form a second lineage (Bonas 1994; Laby and Beer 1992).

A “disease-specific” (*dsp*) region next to the *hrp* gene cluster of *E. amylovora* is required for pathogenicity but not for HR elicitation. This region has been characterized (Bogdanove et al. 1998a; Gaudriault et al. 1997) and contains the 6.6-kb *dspA/EB/F* operon, which is homologous to *avrE* of *P. syringae* pv. *tomato*. The *avrE* locus in *trans* partially restored virulence to a *dspA/E* mutant strain of *E. amylovora* (Bogdanove et al. 1998a). The predicted DspA/E protein is large (198 kDa) opposed to the small protein encoded by *dspB/F* (16 kDa).

In the gall-forming bacterium *Erwinia herbicola* (*Pantoea agglomerans*) pv. *gypsophila*, we previously reported that *hrp* genes are homologous to and colinear with the *hrpJ* operon of *E. amylovora* (Nizan et al. 1997). *E. herbicola* generally is an epiphyte that is present on many different plants. Some strains of this bacterium such as *E. herbicola* pv. *gypsophila* and *E. herbicola* pv. *betae* induce gall formation on gypsophila (Cooksey 1986) or on gypsophila and beet (Burr et al. 1991), respectively. The pathogenicity of both pathovars is dependent on a similar pathogenicity plasmid (designated pPATH_{Ehg} or pPATH_{Ehb}), which is present exclusively in pathogenic strains (Manulis et al. 1991; I. Barash and S. Manulis, unpublished). The pPATH_{Ehg} contains genes involved in virulence of *E. herbicola* pv. *gypsophila*, including genes responsible for biosynthesis of indole-3-acetic acid and cytokinins (Clark et al. 1993; Lichter et al. 1995a; Lichter et al.

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Nucleotide and/or amino acid sequence data is in the GenBank database as accession nos. AF272053, AF271716, and AF271717.

1995b), genes determining host specificity of *E. herbicola* pv. *gypsophillae* on gypsophila (Ezra et al. 2000; Valinsky et al. 1998), and *hrp* genes (Nizan et al. 1997). The plasmid also contains several copies of an insertion element, IS1327, which may have played a role in the horizontal transfer of the *hrp* genes (Lichter et al. 1996). The discovery of *hrp* genes in *E. herbicola* pv. *gypsophillae* and *E. herbicola* pv. *betae* was the first report that a type III secretion system is an important determinant of pathogenicity in gall-forming bacteria. It is noteworthy that the tumorigenic bacterium *Agrobacterium tumefaciens* does not possess a *hrp* cluster (Laby and Beer 1992). The aims of this study were to demonstrate that a full and functional *hrp*–*dsp* gene cluster resides on the pPATH_{Ehg} plasmid and to determine the role of harpin and the *dspA*/EB/F operon in pathogenicity of *E. herbicola* pv. *gypsophillae*.

The bacterial strains, cosmids, and plasmid constructs used in this study are presented in Table 1. Bacteria were grown in

Luria-Bertani (LB) broth or LB agar at 28°C for *E. herbicola* pv. *gypsophillae* and 37°C for *Escherichia coli*. Mutagenesis was carried out on two cosmid clones of pPATH_{Ehg}, pLA352 and pLA244 (Fig. 1), which were obtained from a pLAFR3 library of *E. herbicola* pv. *gypsophillae* strain Ehg824-1 (Lichter et al. 1995b). Insertional mutagenesis with the transposon reporter Tn3-Spice (Lindgren et al. 1989) or mini-Tn10 (De Lorenzo et al. 1990) and construction of marker exchange mutants of the wild-type strain were performed as previously described (Lichter et al. 1995a; Nizan et al. 1997). The marker-exchange mutants were tested for pathogenicity on cuttings of *Gypsophila paniculata* cv. *perfecta*, essentially as described previously (Lichter et al. 1995a). After removal of an approximately 3-mm section from the bottom of the stem, the cutting was inoculated by dipping into a bacterial suspension of 10⁷ CFU/ml and placed in vermiculite-filled trays. The greenhouse temperature was maintained at 26 to 29°C and

Table 1. Bacterial strains, cosmids, and plasmids used in this study

Strain, cosmid, plasmid	Relevant characteristics ^a	Reference, source
<i>Escherichia coli</i> DH5α	F [−] , ϕ80 <i>dlacZ</i> ΔM15, Δ(<i>lacZYA</i> -argF), U169, <i>deoR</i> , <i>RecA1</i> , <i>endA1</i> , <i>hsdR17</i> (r _K [−] , m _K ⁺) <i>supE44</i> , λ [−] , <i>thi</i> -1, <i>gyrA96</i> , <i>relA1</i>	D. Hanahan 1983
<i>Erwinia herbicola</i> <i>Ehg824</i> -1	Wild-type pathogenic strain Rif ^r serotype 1	Manulis et al. 1991
<i>Ehg824</i> -1Mx14, <i>Ehg824</i> -1Mx41	Marker exchange mutants of <i>hrpN</i>	This study
<i>Ehg824</i> -1Mx39, <i>Ehg824</i> -1Mx23, <i>Ehg824</i> -1Mx25	Marker exchange mutants of <i>dspA/E</i>	This study
<i>Ehg824</i> -1Mx39(Mx <i>hrpL::aphA3</i>)	Marker-exchange double mutant in <i>hrpL</i> and <i>dspA/E</i>	This study
Cosmids		
pLAFR3	Tc ^r , broad-host-range vector IncP-1 <i>rixRK2</i> ⁺ <i>lacZa</i> Tra [−] Mob ⁺ cos	Staskawicz et al. 1987
pLA352	Tc ^r , a 23.5-kb cosmid of pPATH in pLAFR3	Nizan et al. 1997
pLA244	Tc ^r , a 25-kb cosmid of pPATH in pLAFR3	This study
pMBN-H6.4	Tc ^r , a 6.4-kb <i>Hind</i> III fragment from pLA352 in pLAFR3	This study
pMBN-H4.2	Tc ^r , a 4.2-kb <i>Hind</i> III fragment from pLA352 in pLAFR3	This study
pMBZ-H6	Tc ^r , a 6-kb <i>Hind</i> III fragment from pLA244 in pLAFR3	This study
Plasmids		
pTn3-Spice	Amp ^r , Sp ^r , and clone containing the <i>inaZ</i> reporter gene in Tn3	Ditta et al. 1980
pMBZ-H-R1.3	Amp ^r , a 1.3-kb <i>Hind</i> III- <i>Eco</i> RI fragment from pLA352 in pBluescript containing <i>hrcN</i>	This study
PMBZ-R-H2.9	Amp ^r , a 2.9-kb <i>Eco</i> RI- <i>Hind</i> III fragment from pLA352 in pBluescript containing <i>hrcV</i> , <i>hrpQ</i> , and <i>hrcN</i>	This study
pMBN-H-R2.5	Amp ^r , a 2.5-kb <i>Hind</i> III- <i>Eco</i> RI fragment from pLA352 in pBluescript containing <i>hrpJ</i> and <i>hrpL</i>	This study
pMBN-R2.1	Amp ^r , a 2.1-kb <i>Eco</i> RI fragment from pLA352 in pBluescript containing <i>hrpX</i> and <i>hrpY</i>	This study
pMBN-R-H1.8	Amp ^r , a 1.8-kb <i>Eco</i> RI- <i>Hind</i> III fragment from pLA352 in pBluescript containing <i>hrpS</i>	This study
pMBZ-H-R2.3	Amp ^r , a 2.3-kb <i>Hind</i> III- <i>Eco</i> RI fragment from pLA244 in pBluescript containing <i>hrpB</i> , <i>hrpD</i> , and <i>hrpE</i>	This study
pMBH-P1.5	Amp ^r , a 1.5-kb <i>Pst</i> I fragment from pLA244 in pBluescript containing <i>hrpF</i> , <i>hrpG</i> , and <i>hrcC</i>	This study
pMBH-P3	Amp ^r , a 3-kb <i>Pst</i> I fragment from pLA244 in pBluescript containing <i>hrcC</i> , <i>hrpT</i> , <i>hrpV</i> , and <i>hrpN</i>	This study
pMBH-P1.3	Amp ^r , a 1.3-kb <i>Pst</i> I fragment from pLA244 in pBluescript containing <i>hrpN</i> and <i>dspE/A</i>	This study
pMBH-H-P0.7	Amp ^r , a 0.7-kb <i>Hind</i> III- <i>Pst</i> I fragment from pLA244 in pBluescript containing <i>dspA/E</i>	This study
pMBH-H2.5	Amp ^r , a 2.5-kb <i>Hind</i> III fragment from pLA244 in pBluescript containing <i>dspA/E</i>	This study
pMBH-H0.5	Amp ^r , a 0.5-kb <i>Hind</i> III fragment from pLA244 in pBluescript containing <i>dspA/E</i>	This study
pMBH-H-P1.3	Amp ^r , a 1.3-kb <i>Hind</i> III- <i>Pst</i> I fragment from pLA244 in pBluescript containing <i>dspA/E</i>	This study
pMBH-P-H2.3	Amp ^r , a 2.3-kb <i>Pst</i> I- <i>Hind</i> III fragment from pLA244 in pBluescript containing <i>dspA/E</i>	This study
pMBH-K1.5	Amp ^r , a 2.3-kb <i>Kpn</i> I fragment from pLA244 in pBluescript containing <i>dspA/E</i>	This study

^a Antibiotics used per ml: 100 μg of ampicillin, 50 μg of kanamycin, 50 μg of spectinomycin, and 100 μg of rifampicin.

high humidity was generated by computer-controlled mist sprinklers. Gall formation was scored 14 days after inoculation, as described elsewhere (Lichter et al. 1995a). Hypersensitivity tests were carried out in tobacco leaves (*Nicotiana tabacum* L. "Hicks"), according to Nizan et al. (1997). Isolation of plasmid DNA from *E. coli*, restriction enzyme digestions, subcloning, transformations, Southern analysis, Western blots, and polymerase chain reaction were carried out by standard procedures (Ausubel et al. 1995) or as recommended by the suppliers. Primers were synthesized by GIBCO-BRL (Glasgow, Scotland).

The *hrp* gene cluster initially was mapped on the overlapping cosmids pLA352 and pLA244 (Fig. 1) by saturation mutagenesis with Tn3-Spice and mini-Tn10 (results not shown). The location of the insertions in mutants that were not pathogenic in gypsophila and failed to elicit HR in tobacco (i.e., with a Hrp⁻ phenotype) was determined, as previously described (Nizan et al. 1997). DNA fragments from the region containing the Hrp⁻ mutations were subcloned into pBluescript (KS⁺) for sequencing, and the resulting series of plasmids were designated pMBH, pMBN, and pMBZ, with a plasmid number designating the size of the insert (Table 1). Unidirectional deletions of the cloned fragment were generated with the Erase-a-Base kit (Promega, Madison, WI, U.S.A.). Automated sequencing with *Taq* DNA polymerase was carried out at the Laboratories for Biological Services, Tel-Aviv University. When necessary, the sequence was completed by walking with custom primers. Analysis of sequence data for DNA and deduced protein sequences was performed with the Wisconsin Package, version 9 (Genetic Computer Group, Madison, WI, U.S.A.).

We previously reported that the sequence of a 3.8-kb *Hind*III DNA fragment from pLA352 (GenBank accession no. X99768) contained six complete and two partial open reading frames (ORFs) with high homology and colinearity to the following genes in the HrpJ operon of *E. amylovora*: HrpO, HrpP, HrcQ, HrcR, HrcS, HrcT, and HrcU (Table 2) (Nizan et al. 1997). These proteins appeared to be components of a type

III secretion system on the basis of their homology to *E. amylovora* Hrp proteins (Bogdanove et al. 1996a; Bogdanove et al. 1996b). To delineate the complete *hrp* gene cluster of *E. herbicola* pv. *gypsophila*, the region containing the Hrp⁻ mutants was sequenced. The pLA352 and pLA244 subclones used for sequencing are described in Table 1. The sequences of the regulatory genes (*hrpL*, *hrpXY*, and *hrpS*), *hrpN* and *dspA/EB/F* operon were submitted to GenBank as accession nos. AF272053, AF271716, and AF271717 respectively.

The results shown in Table 2 indicate that the *E. herbicola* pv. *gypsophila* *hrp* gene cluster and *dspA/EB/F* operon are remarkably similar in their organization and sequence to that of the *E. amylovora* *hrp* cluster. With few exceptions, the percent identities of *E. herbicola* pv. *gypsophila* nucleotides and deduced proteins with those of *E. amylovora* ranged from 60 to 96%. Similar high identities also were observed with available *hrp* gene sequences of *P. stewartii* DC283 (Table 2). The *hrp* gene cluster of *E. herbicola* pv. *gypsophila* appeared to contain genes for all required Hrp functions, namely protein secretion via the type III system (i.e., *hrpJ*, *hrpA*, and *hrpC* operons), regulation (i.e., *hrpXY*, *hrpS*, and *hrpL* operons) and HR elicitation on nonhost plants (i.e., *hrpN*) (Kim et al. 1997). The nine broadly conserved genes designated *hrc* (Bogdanove et al. 1996a) also were present (Table 2). Comparison of the *hrp* genes of *E. herbicola* pv. *gypsophila* with other plant and animal pathogenic bacteria showed that the highest homologies were obtained with *E. amylovora* or *P. stewartii* subsp. *stewartii*.

The only major difference between the *hrp* cluster of *E. herbicola* pv. *gypsophila* and *E. amylovora* is located in the space between the *hrpN* and *dspA/EB/F* (Fig. 1). In *E. herbicola* pv. *gypsophila*, this region was 1 kb compared with 4 kb in *E. amylovora*, where four ORFs are arranged in two operons (Kim and Beer 1998). One of the ORFs in *E. amylovora* is *hrpW*. The HrpW protein resembles harpin in its high glycine content, but it also has homology to pectate lyase. The N-terminal domain of HrpW in *E. amylovora* was sufficient to elicit HR, but the C-terminal pectate lyase domain did not

Fig. 1. Physical map of the *hrp* gene cluster and *dspA/EB/F* operon carried on plasmid pPATH_{Ehg}. Schematic genetic organization of the *hrp* gene cluster and *dspA/EB/F* operon showing cosmid clones of pLA352 and pLA244. Locations of transposon Tn3-Spice mutations in *hrpN* (*EhgMx14* and *EhgMx41*) and *dspA/EB/F* (*EhgMx39*, *EhgMx23*, and *EhgMx25*) are indicated by numbers. Borders between *hrp* genes within the operons are indicated by vertical lines.

exhibit any enzymatic activity (Kim and Beer 1998). Although HrpW appears to be distributed widely among several *Erwinia* species (Kim and Beer 1998), only a remnant of this gene (50 bp) was detected in *E. herbicola* pv. *gypsophila*. Although HrpW may contribute to HR elicitation in nonhosts, its function is not entirely clear because *hrpW* mutants in either *E. amylovora* or *P. syringae* pv. *tomato* retained wild-type ability to elicit the HR in nonhosts and to cause disease (Charkowski et al. 1998; Kim and Beer 1998).

In order to assess the role of harpin in pathogenicity of *E. herbicola* pv. *gypsophila*, pLA244 was digested with *Hind*III and a 6-kb fragment was cloned into pLAFR3 to yield pMBZ-H6. Sequence analysis confirmed the presence of *hrpN* in the cloned fragment. PMBZ-H6 was subjected to mutagenesis with Tn3-Spice (Lindgren et al. 1989). Two mutations in *hrpN* were marker exchanged into *Ehg*824-1 to obtain *Ehg*Mx14 and *Ehg*Mx41 (Fig. 1). To confirm that the resulting mutants could not produce harpin, protein extracts from cells of *Ehg*824-1, *Pnss*DC283, and the two marker-exchanged mutants were subjected to Western blotting (Fig. 2), with *P. stewartii* subsp. *stewartii* antiharpin polyclonal antibodies (M. Ahmad and D. Coplin, unpublished). The results clearly indi-

cated that *Ehg*Mx41 and *Ehg*Mx14 were deficient in harpin production (Fig. 2). The two mutants were then tested for pathogenicity on gypsophila cuttings and for HR elicitation in tobacco leaves. Both mutants retained full pathogenicity, and a slight reduction in the necrotic area caused by the HR response was observed compared with the wild type. These results are similar to the phenotypes exhibited by harpin mutants of other bacterial plant pathogens. For example, *hrpN* or *hrpZ* mutations abolish HR elicitation in *E. amylovora* Ea321 (Wei et al. 1992) greatly reduce the HR produced by *E. amylovora* CFBP1430 (Barny 1995) or *E. chrysanthemi* (Bauer et al. 1995) but have little or no effect on HR elicitation by *P. syringae* (Alfano et al. 1996). Similarly, mutations in *HrpN*_{Ehg} and *HrpN*_{Pnss} did not exhibit any effect on pathogenicity of *E. herbicola* pv. *gypsophila* or *P. stewartii* subsp. *stewartii* (D. Coplin, unpublished), respectively. Unlike *E. herbicola* pv. *gypsophila*, however, loss of *hrpN* of *P. stewartii* subsp. *stewartii* greatly reduced HR elicitation.

It is noteworthy that the apparent molecular mass of *HrpN*_{Ehg} was slightly lower than that of *HrpN*_{Pnss}, but both were 41 to 44 kDa (Fig. 2). Despite common physical and chemical properties, e.g., heat stable, glycine rich, and acidic, the harpins from different genera seem to be quite distinct from each other in regard to their amino acid composition (Lindgren 1997). The percent identities of *HrpN*_{Ehg} with harpins of *P. stewartii* subsp. *stewartii*, *E. amylovora*, *E. chry-*

Table 2. Comparison of *hrp* gene sequences from *Erwinia herbicola* pv. *gypsophila* with corresponding genes of *Erwinia amylovora* and *Pantoea stewartii* subsp. *stewartii*

Gene ^a	Percentage of identity			
	<i>E. amylovora</i>		<i>P. stewartii</i>	
	Nucleotides	Amino acids	Nucleotides	Amino acids
<i>hrpJ</i>	70	66.9		
<i>hrcV</i>	78	84		
<i>hrpQ</i>	69	65		
<i>hrcN</i>	79	80		
<i>hrpO</i>	69.3	60.8		
<i>hrpP</i>	65.1	64.3		
<i>hrcQ</i>	66.7	73.4		
<i>hrcR</i>	80.1	90.4		
<i>HrcS</i>	80.3	96		
<i>HrcT</i>	76.3	81.5		
<i>HrcU</i>	89 (150 bp) ^b	94.4 (50 aa)		
<i>HrpL</i>	70.2	77.4	77.8	80.8
<i>hrpX</i>	71.7	71.7	78	79.6
<i>hrpY</i>	78.2	75.3	82.2	88.7
<i>hrpS</i>	75.3	83.6	77	79.5
<i>hrpA</i>	73	66.7	78	73
<i>hrpB</i>	81.5	100 (24 aa)	86 (130 bp)	95 (22 aa)
	(136 bp)			
<i>hrcJ</i>	83 (263 bp)	85 (87 aa)	81.2	80 (87 aa)
			(263 bp)	
<i>hrpD</i>	66.6	63.2	72.2	65
<i>hrpE</i>	64	62.6	70	62.2
<i>hrpF</i>	75	75	80	82
<i>hrpG</i>	64	47	67.4	56
<i>hrcC</i>	73	85 (200 aa)	74.4	86 (207aa)
	(1,529 bp)		(1,550 bp)	
<i>hrpT</i>	67	62.5	75	72.3
<i>hrpV</i>	68	68	70	65
<i>hrpN</i>	64	60	73.8	69.7
<i>DspA/E</i>	65	61	73.5	75
<i>DspB/F</i>	72	65.3	73.8	65.3

^a Open reading frame (ORF) sequences of *E. herbicola* pv. *gypsophila* were named according to their homology with *E. amylovora* *hrp* genes. Sequences of *hrp* genes from *P. stewartii* subsp. *stewartii* were obtained from GenBank, accession no. AF282857.

^b Number of base pairs or deduced amino acids of partially sequenced ORFs is indicated by parentheses.

Fig. 2. Immunoblot analysis of harpin_{Ehg} production in *Ehg*824-1 and its *hrpN* mutants. To induce harpin production, plasmid pVSP61 containing the regulatory gene *hrpS* under a *lac* promoter (*P*_{lac}::*hrpS*) (R. Nizan, unpublished) was mobilized into *Ehg*824-1, *Ehg*Mx14, and *Ehg*Mx41 by triparental mating. The transconjugants were then grown in 3 ml of Luria-Bertani medium for 10 h at 28°C in the presence of 1.5 mM isopropyl-β-D-thiogalactoside. *Pantoea stewartii* DC283 was similarly grown in inducing medium (Nizan et al. 1997). Bacterial cells were centrifuged, resuspended in loading buffer, and heated at 100°C for 3 min and then loaded onto a sodium dodecyl sulfate–10% polyacrylamide gel. Western blotting was carried out with antiharpin antibodies prepared against *HrpN*_{Pnss} from DC283 (D. L. Coplin, unpublished). Lane A: *Pnss*DC283; B: *Ehg*824-1(*P*_{lac}::*hrpS*); C: *Ehg*Mx14(*P*_{lac}::*hrpS*); and D: *Ehg*Mx41(*P*_{lac}::*hrpS*). Numbers indicate protein mass in kilodaltons.

santhemi, and *E. carotovora* were 69, 60, 52, and 48% respectively.

In order to test the functionality of the type III protein secretion system in *E. herbicola* pv. *gypsophila*, the secretion of harpin into the medium was compared between *Ehg824-1* and its marker-exchanged mutant no. 19 (Nizan et al. 1997). In the latter strain, the mutation resides in *hrpQ* of the *hrpJ* operon encoding for proteins of the type III system (Table 2). Following introduction of $P_{lac}::hrpS$, the wild type and the mutant were grown in LB. Harpin could be detected by immunoblotting in concentrated medium of the wild-type trans-conjugant but not of the mutant, suggesting a type III-dependent secretion.

As described above, the *dspA/EB/F* operon of *E. herbicola* pv. *gypsophila* was completely sequenced. The *dspA/E* and *dspB/F* ORFs were 5.5 and 0.38 kb, respectively, encoding predicted proteins of 201.2 and 13.7 kDa. A potential "hrp box" promoter was identified 109 bp upstream of the operon (GGAACC-N₁₅-CCCAC). The percentage of amino acid identities of DspA/E with that of *E. amylovora* and of *P. stewartii* subsp. *stewartii* were 61 and 75%, respectively, whereas DspB/F exhibited 65.3% identity with both bacteria (Table 2). Three marker-exchanged mutants, *EhgMx23*, *EhgMx25*, and *EhgMx39* (Fig. 1), were generated by mutagenesis of pMBZ-H6 with the *inaZ* reporter transposon Tn3-Spice. Mutations in *dspA/E* completely abolished pathogenicity but did not affect HR elicitation on tobacco. Transcription of *dspA/E* was determined by Tn3-Spice *inaZ* gene fusions (Lindgren et al. 1989). To demonstrate whether *dspA/E* is Hrp-regulated, a kanamycin cassette was inserted into *hrpL* of *EhgMx39* (Table 1) to yield the double mutant *EhgMx39(MxhrpL::aphA3)*. For *hrp*-induction in planta, three gypsophila cuttings were inoculated by dipping them into cell suspensions (approximately 10⁹ CFU/ml) of *EhgMx39*-(*MxhrpL::aphA3*) and *EhgMx39*. Following incubation overnight under humid conditions at 25°C, the cuttings were washed with water and 1 cm of the lower part of the stem was removed and ground in water for determination of ice nucleation activity. The log of ice nuclei per cell for the HrpL⁻ mutant *EhgMx39(MxhrpL::aphA3)* was -5.55 compared with -2.86 for *EhgMx39*. These results indicate that inactivation of *hrpL* reduced the transcriptional activity of *dspA/E* by almost three orders. Thus, the transcription of *dspA/E* in *Ehg824-1* is under Hrp regulation, as reported for *E. amylovora* (Bogdanove et al. 1998b; Gaudriault et al. 1997).

The results of this study clearly demonstrate that the pPATH plasmid of *E. herbicola* pv. *gypsophila* carries a fully functional *hrp* gene cluster and *dspA/EB/F* operon, very similar to *E. amylovora* and *P. stewartii*. Because the *hrp* gene cluster of *E. herbicola* pv. *gypsophila* resides on a plasmid instead of the chromosome, as it does in other *erwinias*, we speculate it was mobilized by horizontal transfer from other bacteria, presumably *Erwinia* spp. into *E. herbicola* pv. *gypsophila*.

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