The Dual Function in Virulence and Host Range Restriction of a Gene Isolated from the pPATH_{Ehg} Plasmid of *Erwinia herbicola* pv. *gypsophila*

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The host range of the gall-forming bacterium *Erwinia herbicola* pv. *gypsophila* (*Ehg*) is restricted to *gypsophila* whereas *Erwinia herbicola* pv. *betae* (*Ehb*) attacks beet as well as *gypsophila*. Both pathovars contain an indigenous plasmid (pPATH_{Ehg} or pPATH_{Ehb}) that harbors pathogenicity genes, including the *hrp* gene cluster. A cosmid library of *Ehg*824-1 plasmid DNA was mobilized into *Ehb*4188 and the transconjugants were screened for pathogenicity on beet. One *Ehb* transconjugant harboring the cosmid pLA173 of pPATH_{Ehg} induced a hypersensitive-like response and abolished pathogenicity on beet. Transposon mutagenesis of an open reading frame (ORF) located on this cosmid eliminated its effect on pathogenicity. Marker exchange of this mutation into *Ehg*824-1 caused a substantial reduction in gall size on *gypsophila* and caused *Ehg*824-1 to extend its host range and incite galls on beet. The ORF (1.5 kb) was designated as *pthG* (pathogenicity gene on *gypsophila*). DNA sequence analysis of *pthG* revealed no significant homology to known genes in the data bank. Only remnants of the *pthG* sequences were identified on the pPATH of *Ehb*4188. The deduced protein lacked an N-terminal signal peptide but contained a short trans-membrane helix in its C terminus. The gene product, as determined by expression in *Escherichia coli* and Western blots (immunoblots), was a 56-kDa protein.

Additional keywords: avirulence, host specificity, *Pantoea agglomerans*.

Erwinia herbicola (*Pantoea agglomerans*) is an epiphyte on many different plants. However, some strains of this bacterium have evolved into plant pathogens that incite gall formation in *gypsophila* (*Gypsophila paniculata* L.) (Cooksey 1986) and table 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 beet as well as *gypsophila*. The basic question that we are concerned with is what causes *E. herbicola* to switch from an epiphyte on nu-

merous different plants to a host-specific pathogen. An initial answer has been provided by demonstrating that pathogenicity of both pathovars is determined by a common indigenous plasmid (designated as pPATH_{Ehg} in *Ehg* and pPATH_{Ehb} in *Ehb*) of approximately 150 kb that is present exclusively in all pathogenic strains (Manulis et al. 1991; I. Barash and S. Manulis, *unpublished*). The insertion element, IS1327, that has been found in several copies on the pPATH of both pathovars (Lichter et al. 1996) was presumably instrumental in the evolution of this plasmid. Further studies revealed a functional *hrp* cluster on pPATH_{Ehg} with high homology and collinearity to the *hrp* genes of *E. amylovora* (Nizan et al. 1997; H. Mor, S. Manulis, and I. Barash, *unpublished*). Since gall formation by phytopathogenic bacteria has been attributed to indole-3-acetic acid (IAA) and cytokinins (Morris 1986), elucidation of their role in the pathogenicity of *Ehg* has been extensively studied (Clark et al. 1993; Lichter et al. 1995a, 1995b; Manulis et al. 1998). Inactivation of IAA and cytokinin biosynthesis, either separately or simultaneously, substantially reduced gall size but did not prevent gall initiation (Manulis et al. 1998). The latter results lead to the conclusion that gall initiation is triggered by factors other than IAA and cytokinins secreted by the pathogen.

Host specificity in gram-negative, phytopathogenic bacteria has been attributed either to negative-acting determinants, which induce incompatible interactions between the host and the pathogen, or to positive-acting determinants, which induce compatible interactions. Most investigations have focused on negative-acting factors, i.e., avirulence (*avr*) genes, that restrict host range by provoking the hypersensitive response (HR) (Long and Staskawicz 1993; Dangl 1994; Leach and White 1996). In contrast, a few reports have suggested that host range is governed by the presence of positive-acting factors encoded by host-specific virulence (*hsv*) genes (Ma et al. 1988; Salch and Shaw 1988; Gabriel 1989; Waney et al. 1991). We have previously isolated and partially characterized from pPATH_{Ehg} a positive-acting, host-species specificity gene, designated as *hsvG*, that determines the ability of either *Ehg* or *Ehb* to incite galls on *gypsophila* (Valinsky et al. 1998). Inactivation of *hsvG* narrowed the host range of *Ehb* by one host plant (i.e., *gypsophila*) but did not affect its virulence on the other host plant (i.e., beet) or HR elicitation on tobacco leaves. Since the host range of *Ehg* is restricted to *gypsophila*, inactivation of this gene rendered *Ehg* nonpathogenic.

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Nucleotide and/or amino acid sequence data have been submitted to the GenBank data base as accession number AF071231.

The differential host range of *Ehg* and *Ehb* can be examined in the light of two hypotheses: (i) host specificity is determined exclusively by positive-acting *hsv* genes, in which case *Ehg* should carry only *hsvG* whereas *Ehb* should carry both *hsvG* and a presumed gene that determines specificity on beet, namely, *hsvB* (*hsv* gene on beet); or (ii) host specificity is determined by interaction between positive-acting and negative-acting genes, in which case both pathovars should carry both *hsvG* and *hsvB*, with *Ehg* also carrying a gene encoding an HR-like elicitor on beet that prevents the effect of *hsvB* and pathogenicity. The present paper supports the second hypothesis and describes the isolation and characterization of a gene from pPATH_{Ehg} that encodes a protein that acts simultaneously as a virulence factor on gypsophila and as an HR-like elicitor on beet.

RESULTS

Isolation and expression of a gene encoding an HR-like elicitor on beet from pPATH_{Ehg}

To screen for a positive-acting, host-species specificity gene on beet (i.e., *hsvB*), a cosmid library of total plasmids from the beet pathovar *Ehb*4188 was mobilized by triparental mating into the gypsophila pathovar *Ehg*824-1, and 500 transconju-

gants obtained were tested for pathogenicity on beet. However, it was found that none of the *Ehg* transconjugants could generate galls on beet. We then attempted to screen for a gene encoding an HR-like elicitor on beet by mobilizing a cosmid library of total plasmids from *Ehg*824-1 into *Ehb*4188. Five hundred transconjugants were tested for pathogenicity on beet. An *Ehb*4188 transconjugant, harboring the cosmid pLA173 of pPATH_{Ehg} (Fig. 1), induced necrosis and was nonpathogenic on beet while retaining full pathogenicity on gypsophila (Fig. 2, Table 1). Transposon mini-Tn10 was used to generate mutations in cosmid pLA173. Three insertional mutants in pLA173, designated as E1, E13, and E27 (Fig. 1), were pathogenic on beet when conjugated into *Ehb*4188 (Fig. 2, Table 1). The location of these mutations on pLA173 (Fig. 1) was determined by mapping with multiple restriction enzymes and hybridization, as described in Materials and Methods.

A 2.8-kb *Bam*HI/*Sac*II DNA fragment, in which the mini-Tn10 mutations were located, was subcloned from pMBE-R/K9 (Fig. 1, Table 2) into pBS (KS⁺) to yield pKMBE-B/S2.8 and subjected to sequence analysis of both strands. A single open reading frame (ORF) of 1.5 kb was identified, with a predicted protein of 506 amino acids and a molecular mass of 56 kDa (Fig. 3). The ORF had no significant homology to known genes. A potential ribosome binding site, 5'-

Fig. 1. Physical map of the cosmid clone pLA173, a derivative of the pPATH from *Erwinia herbicola* pv. *gypsophila* (*Ehg*), and the *pthG* locus. A 9-kb *Eco*RI/*Kpn*I DNA fragment from pLA173 was subcloned into pLAFR3 to yield pMBE-R/K9. A 2.8-kb *Bam*HI/*Sac*II DNA fragment was subcloned from pMBE-R/K9 into pVSP61 to yield pMBE-B/S2.8. Apparent open reading frame (ORF) of *pthG* indicated by arrow. Mutants obtained by insertion of mini-Tn10 into the ORF indicated by triangles, and marked as E1, E13, and E27. Abbreviations: R, *Eco*RI; H, *Hind*III; B, *Bam*HI; K, *Kpn*I; S, *Sac*II; C, *Cla*I.

AGGAG-3', was found 10 bp upstream from the translation start codon, whereas a putative *hrp* box, 5'-GGAAGT-N16-CCACAGATT-3', was identified 49 bp upstream from the start codon. The predicted protein lacked an N-terminal signal peptide. Analysis for predicting *trans*-membrane helices (TMHs) (L. Burla and N. Ben Tal, *unpublished*) revealed that overall the protein was very polar. However, a putative short TMH, namely IPGTLPCIAIVIKLCI, was identified in its C terminus (Fig. 3). The nucleotide sequence of the 2.8-kb fragment (Fig. 3) has been submitted to GenBank as accession number AF071231. Since the isolated ORF appeared to induce a hypersensitive-like response, which is attributed to activation of plant defense reactions, it may be considered a negative-acting host virulence gene on beet. However, as will be described further, mutation in this putative gene also reduced pathogenicity of *Ehg824-1* on gypsophila. Therefore, it was named *pthG* (pathogenicity gene on gypsophila).

The complete ORF was cloned into the T7 promoter-driven vector pET-11a to yield pET-N/B1.5 and expressed as described in Materials and Methods. Proteins of lysed *Escherichia coli* cells containing pET-N/B1.5 in the presence or absence of isopropyl-D-thiogalactopyranoside (IPTG) were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Results shown in Figure 4 demonstrate the appearance of a strong protein band with an apparent molecular mass of about 56 kDa only in the presence of pET-N/B1.5 induced by IPTG. The expressed protein corresponded in size to the expected deduced protein of the ORF.

Identification of an ORF from *Ehb4188* with partial homology to the *pthG* of *Ehg*.

Screening for the presence of homologous sequences in a cosmid (pLAFR3) library of plasmids from *Ehb4188*, with pMBE-B/S2.8 as a probe, resulted in the isolation of the cosmid clone pLE204. This cosmid was digested with *EcoRI*/*HindIII* and subjected to Southern blot analysis. A single 2-kb band hybridized with pMBE-B/S2.8 (results not shown). This band was subcloned into pBS (KS⁺) to yield pMBE-E/H2 and subjected to sequence analysis of both strands. A 0.68-kb ORF was present as opposed to the ORF of 1.5-kb in *Ehg* (Fig. 5).

Fig. 2. Effect of *pthG* on pathogenicity of *Erwinia herbicola* pv. *betae* (*Ehb*) and *Erwinia herbicola* pv. *gypsophila* (*Ehg*) on beet. *Ehb*: **A**, *Ehb4188*; **B**, *Ehb4188*/pLA173 (in *trans*); **C**, *Ehb4188*/pLA173-E27 (in *trans*). *Ehg*: **A**, *Ehg824-1*; **B**, *Ehg824-1*MxE27.

Comparison of the deduced amino acid sequences of the ORF from *Ehb* with a partial ORF of *pthG* (Fig. 5A) revealed that the first 39 amino acids of both ORFs were almost identical, but no further homology could be observed in the deduced proteins. Alignment of the nucleotide sequences of *pthG* and pMBE-E/H2 revealed the presence of two frame shifts in the latter sequence between base pairs 120 to 126 and 483 to 494, but the homology between the two sequences up to 1023 bp was 92% (Fig. 5B). The C-terminal sequence of *pthG* (1023 to 1500 bp) was absent from the beet pathovar. These results imply that, although remnants of *pthG* were present in *Ehb4188*, the gene was nonfunctional in the beet pathovar. Whether the ORF detected in *Ehb* possesses any function in the pathogenicity of *Ehb* remains to be investigated.

Distribution of *pthG* among gram-negative, phytopathogenic bacteria.

The total DNA of the tested strains was digested with *EcoRI* and *HindIII* and subjected to Southern blot analysis with pMBE-B/H1.8 (which contains *pthG*) as a probe. Results shown in Figure 6 indicate that *pthG* sequences were present in other strains of the gypsophila pathovar (i.e., *Ehg824-1*, *Ehg350*, and *Ehg3-1a*), and the beet pathovar (i.e., *Ehb4188* and *Ehb2188*), but not in nonpathogenic strains of *E. herbicola* (i.e., *Eh3-1*, *Eh717-2*, and *Eh24-8*), which lack pPATH (Manulis et al. 1991). In the gypsophila pathovar the size of the fragment was 9 kb whereas in the beet pathovar two bands, of 6.4 and 2 kb, were obtained (Fig. 6). These differences could result from polymorphism between *Ehg* and *Ehb*. No hybridization with *pthG* sequences could be detected in *E. herbicola* pv. *visteriae*, *E. amylovora*, *E. stewartii*, *Xanthomonas campestris* pv. *campestris*, or *Pseudomonas syringae* pv. *savastanoi*.

Marker exchange mutants of *Ehg824-1* in *pthG* exhibit reduced virulence on gypsophila and acquired pathogenicity on beet.

Mini-Tn10 insertional mutations in *pthG* (Fig. 1) were marker exchanged into *Ehg824-1*, as described in Materials and Methods. Results shown in Figure 2 and Table 1 demonstrate that the marker-exchange mutant of *Ehg824-1* in *pthG* caused the gypsophila pathovar to extend its host range and elicit gall formation on beet. The pathogenicity of *EhgMxE27* and *EhgMxE1* on gypsophila cuttings was substantially reduced, by 50 to 90%, respectively (Fig. 7), and could be restored to its full level by complementation with pMBE-B/S2.8. Overexpression of *pthG* in *Ehg824-1* did not have any

Table 1. Effect of mutations in *pthG* on pathogenicity of *Erwinia herbicola* pv. *gypsophila* (*Ehg*) and of *Erwinia herbicola* pv. *betae* (*Ehb*) on gypsophila and beet

Pathogen strain/mutant	Pathogenicity ^a	
	Beet	Gypsophila
<i>Ehb4188</i> (wild type)	+	+
<i>Ehb4188</i> /pMBE-B/S2.8 (in <i>trans</i>)	HR-like	+
<i>Ehb4188</i> /pMBE27 (in <i>trans</i>)	+	+
<i>Ehg824-1</i> (wild type)	HR-like	+
<i>Ehg824-1</i> MxE27	+	+—

^a Pathogenicity was carried out on beet cubes or gypsophila cuttings as described in Materials and Methods. +, gall formation; HR-like, necrosis without gall; +—, very small gall.

effect on gall size. It may be postulated that the difference in gall size between these mutants results from their differing proximity to the C terminus of the ORF (Fig. 1).

DISCUSSION

We have isolated and characterized from the pPATH of *Ehg* a pleiotropic gene designated as *pthG*. This gene encodes a protein that acts in *Ehg* as a virulence factor in gypsophila and as an elicitor of a hypersensitive-like response in beet. Mobilization of *pthG* into the beet pathovar in *trans* caused the *Ehb* transconjugant to induce a hypersensitive-like response, which prevents gall formation in beet, while completely retaining pathogenicity on its second host, gypsophila. Most interest-

ingly, marker exchange of the *pthG* mutation into *Ehg* caused the gypsophila pathovar to extend its host range and induce galls on beet, while it substantially reduced gall formation on gypsophila.

As indicated earlier, two explanations have been proposed for host range determination in gram-negative, plant-pathogenic bacteria. The first is based on host range restriction by determinants that confer "induced resistance" (Whalen et al. 1988; Kobayashi et al. 1989; Alfano and Collmer 1996; Leach and White 1996; Keen 1990). The negative interactions result from recognition of Avr proteins by the plant defense gene surveillance system (*R* genes product) and elicitation of the HR (Keen 1990). An alternative explanation for host range determination is based on positive-acting *hsv* genes that confer

Table 2. Bacterial strains, cosmids, and plasmids used in this study

Strain/plasmid	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) Φ80 <i>dlacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>endA1 recA1 deoR</i> Δ(<i>ara⁺ leu</i>)7697 <i>araD139 galU galK nupG rpsL λ⁻</i>	GIBCO BRL, Gaithersburg, MD
HMS174(DE3)	F ⁻ <i>recA hsdR</i> (rK12 ⁻ mK12 ⁺) Rif ^r (DE3).	Novagen, Madison, WI
<i>Erwinia stewartii</i>	nal ^r , wild-type pathogenic strain DC283	D. Coplin, Ohio State University
<i>Erwinia amylovora</i>	Wild-type pathogenic strain 209	S. Manulis, The Volcani Center, Israel
<i>Xanthomonas campestris</i> pv. <i>campestris</i>	Wild-type pathogenic strain 418	S. Manulis, The Volcani Center, Israel
<i>Erwinia herbicola</i> pv. <i>millettiae</i>	Wild-type pathogenic strain 800	M. Hanson, Sacramento, California
<i>Pseudomonas syringae</i> pv. <i>savastanoi</i>	Wild-type pathogenic strain ITM318	N. S. Iacobellis, University of Bari, Italy
<i>E. herbicola</i> pv. <i>gypsophilae</i> (<i>Ehg</i>)		
<i>Ehg</i> 824-1	Wild-type pathogenic strain with Rif ^r , gypsophila pathovar, serotype I	Manulis et al. 1991
<i>Ehg</i> 3-1a	Wild-type pathogenic strain, gypsophila pathovar, serotype I	Manulis et al. 1991
<i>Ehg</i> 350	Wild-type pathogenic strain with Rif ^r , gypsophila pathovar, serotype II	Manulis et al. 1991
<i>Eh</i> 3-1, <i>Eh</i> 717-2, <i>Eh</i> 24-8	Wild-type nonpathogenic strains, serotype I	Manulis et al. 1991
<i>Ehg</i> 824-1/pMBE# (E1, E13, E27)	Km ^r , pathogenic, in <i>trans</i> carried pMBE	This study
<i>Ehg</i> Mx# (E1,E13,E27)	Km ^r , marker-exchange mutants in <i>pthG</i>	This study
<i>Erwinia herbicola</i> pv. <i>betae</i> (<i>Ehb</i>)		
<i>Ehb</i> 2188	Wild-type pathogenic strain, beet pathovar	Burr et al. 1991
<i>Ehb</i> 4188	Wild-type pathogenic strain with Rif ^r , beet pathovar	Burr et al. 1991
<i>Ehb</i> 4188/pLA173	Tc ^r , pathogenic on gypsophila, nonpathogenic on beet, in <i>trans</i> carried pLA173	This study
Cosmids		
pLAFR3	Tc ^r , broad host range vector <i>IncP-1 rixRK2⁺ lacZa Tra⁻ Mob⁺</i> cos	Staskawicz et al. 1987
pLA173	Tc ^r , a 25-kb cosmid clone from the pPATH of <i>Ehg</i> in pLAFR3	Lichter et al. 1995b
pMBE-R/K9	Tc ^r , a 9-kb <i>EcoRI/KpnI</i> fragment from PLA173 cloned into pLAFR3	This study
pLMBE-B/H1.8	Tc ^r , a 1.8-kb <i>BamHI/HindIII</i> fragment cloned from pMBE-B/S2.8 into pLAFR3	This study
pLE204	Tc ^r , a 27-kb cosmid of pPATH from <i>Ehb</i> in pLAFR3	This study
Plasmids		
pKMBE-B/S2.8	Amp ^r , 2.8-kb <i>BamHI/SacII</i> subcloned from pMBE-R/K9 in KS ⁺ (pBS)	This study
pMBE-B/S2.8	Km ^r , a 2.8-kb <i>BamHI/SacII</i> fragment cloned from pMBE-R/K9 into pVSP61	This study
pVMBE-B/H1.8	Km ^r , a 1.8-kb <i>BamHI/HindIII</i> PCR fragment cloned from pMBE-B/S2.8 into pVSP61	This study
pMBE-E/H2.0	Amp ^r , 2.0-kb <i>EcoRI/HindIII</i> subcloned from pLE204 in KS ⁺ (pBS)	This study
pBluescript	Amp ^r , subcloning and sequencing vector, KS ⁺ (pBS)	Stratagene, La Jolla, CA
pRK2073	Sp ^r , Sm ^r , helper plasmid for triparental matings	Ditta et al. 1980
Mini Tn10-Km	Km ^r	Way et al. 1984
pET11a	Amp ^r , plasmid for protein overexpression	Novagen, Madison, WI
pETN/B 1.5	Amp ^r , a 1.5-kb <i>NdeI/BamHI</i> fragment cloned from pVMBE-B/H1.8 into pET11a	This study

^a Abbreviations: Amp = ampicillin; Km = kanamycin; Rif = rifampicin; Sm = streptomycin; Sp = spectinomycin; Tc = tetracycline; r = resistance.

“induced susceptibility” (Salch and Shaw 1988; Gabriel 1989; Waney et al. 1991). It is quite likely that host specificity of phytopathogenic bacteria is determined by interactions between positive- and negative-acting virulence genes, although the negative-acting determinants are dependent on the presence of positive-acting factors.

Our previous demonstration that *hsvG* is a positive-acting gene that determines host specificity of *Ehg* and *Ehb* for gypsophila (Valinsky et al. 1998) could suggest the presence of a postulated parallel gene, *hsvB*, that confers host specificity on beet. Results obtained imply that *hsvB* is present in both pathovars, since marker-exchange mutants of *Ehg* in *pthG* became pathogenic on beet. Thus, the differential host ranges of *Ehg* and *Ehb* can be attributed only to host restriction con-

ferred by *pthG* rather than to the lack of a positive-acting gene that determines host specificity for beet. This fact may explain our failure to obtain beet-pathogenic *Ehg* transconjugants following the mobilization of a cosmid library from the beet pathovar into the gypsophila pathovar. Interestingly, although remnants of *pthG* sequences were detected on pPATH_{Ehb}, this gene appears to be nonfunctional in the beet pathovar. Since *Ehb* is pathogenic on gypsophila as well, and *pthG* in *Ehg* encodes a virulence determinant for the latter host, it can be assumed that *pthG* may have been replaced in *Ehb* by an alternative gene for virulence on gypsophila that fulfills a similar function. Although both pathovars elicit galls on gypsophila, they may not necessarily carry identical genes for pathogenicity on this host. This supposition is supported by the differing

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1      TGAACCTGATCTGCAGACCCAGATGAGCGTCTTTTCATAAGTAAGCATAACTTCCTGCTCAAGATCTCCATTAAATTGCAGGAACTCCGCTGTAATA
101    CATCAACAGAGCGGTTTCCGGCTGATTTTGTCTATCCGACACAGATATTCGCTGATGTTTGGCTGTTCAATGATGCCGTTTTCGCTTATAAAATCCCG
201    ATTCTGAGGCGACCATGACAAATGGGAAGGTGCGTCCACACATGAGTCTCAGGAGAAATGACCTGCATTAATGCAGGTTTATAGTGATTTCCACGTTT
301
401    CGAAAAAATTTGGCGTTGGCCTACGTTTTCAGGAACCTGAATCCATCCAGATGCCACAGATTAAAGTCATTTAAATTAACCTTGGTGCTGCATAAC
501    TCCATCGTTAGGGTTTAAAACTTTAAAGCAGCAGTGAAGCAAGCAGACCGAGAGCTTATCGCTGACGTTGGATGCAGACCAATATCATGTAAG
SD?
GAGAACTAAGCTATGGGATGTTTCAACGTTACGGGTGCCAGTGGCAGAGCGAACAATTATGTTGTCGAGCACCACGACGATCGGAGTTCGATAGGGCAG
M G C F N V T G A S G R A N N Y V V E H H D D R S S I G Q
601    GAAAGCCCCAGGCAAGAGATTGCTGATAGTCCGCTGAGTCGATCAGGCCGTTTCATCCAATAGAACCTCTCCCGCGTATCGCTGGACAGGCTCAGAGCT
E S P R Q E I A D S P L S R S G R S S N R T S P R V S L D R L R A
701    TTCAATCCCCAAGCGCGCTCAGTTGGCGCTTTGAGAAATGTTTCTGGCAAGGACAGGCTGCTCAACATCTGCAGCAGCTCGAAGCAGAGCTCAAC
F S N P P S G A V G A L R N W F W Q G Q A A Q H L Q Q L E A E L N
801    AACGGTTCGAACATTCGCGAGCTGTTGGACGGCCCTGGATTGGCGGATGCTGACCCATGTGATGGAGGATATCGCCTCGATTCAAACGCGAAAAAT
N G P S N I R E L L D G G F G G L L T H V M E D I A S I S N A E N
901    TTGTGCGCGATCGAGGTCGACAGGAGCTAAGGCAAGCTATTAGCACCGGGCGCGGGCGAACAATTTGGATATATCTTTGAAAAACGTTCTACAATTC
L S P I E V A E E L R Q A I S T G R G A N N L D I S L K N V L Q F
1001    GGACTTTATCGGTCAGGAAATCTGCAAGGAGAAGCCGAGATCGCCGGCTCGATGCAAAACACACCCTGCTTTTCAGTCTTTCCCGGTGGCCAGGCC
G L Y A M V R E I L Q G E A E I A G S Q T H P A F Q S F P V A Q A
1101    TGGAAATTTGCAATTGAGCAAGAGCAATTTGGGCTGATTACGGCGCCGATCCGGCATCGATTTGACAAACGAGCCCGGCTACATGGGTAGTATGTTTCGC
W K F C I N E Q E H W G D Y G A A S G M R F D E P G Y M G S M F R
1201    GGCCTAAATTCGTCATCGACTCGAAGCGGGTCTCTGCTCCGAAAAAAGGCGAAATCGATGCTACTTGGTTGGAGAAATTTGCACGACATCGCTGTC
G L K F V I D S E A G S S R K K G E I D A T W L E N L H D I A V
1301    AATGGCGTGTTCAGCGCAGAGTCATGCATGATTGGCAGTTGGCGCCCAATGTCGAAGAAGCTGTGAATGCAGTCGCGGCCCTTACCGCGCTTGGCAGC
N G V F Q R A R V M H D W Q F G A N V E E A V N A V A L T P G S
1401    AATATTAGCGATAAGGTGAGGAGCGCAGCTCGGCAATCTTCTATTGCGGAAACACCTATGSCCAATTGGAGGTGGGTATCGACAAGGCCAGTTGGTC
N I S D K V R S A A S A I F Y L P E H L W P L E G G Y R Q G Q L V
1501    CAGTTCAAACGTTTATCCCAAGAATCTGACTCCGGTGGGCTCGGCGATCTGCAAAAGTTCCAGTCCCGGTTCCGCGGGTGGGCTGAGTTTCGAAGT
F K L V Y P K Q N L T P A G L G D L Q K F Q S R F A G W A E F R S
1601    CTGGGTAATCGTCCGATCGACTGGGATGAGGTAGAACCGGCAACGCCAGGTTATGTGGGCGTGCACGGGAAAAAGCCGAAAAGAGGTTATGAAGCTG
V G N R P I D W D E V E R G N A Q V M W A C T G K S R K E V M K L
1701    GTGAAAAATGTCTCAAAACGATTATAACGATATAAAAAAGGACTAACCAACGAGAGGCGCACTGCATGTGTTGGGCGCAACGTCGCGTACCTGG
V K N V L Q T H Y N D I K R T N Q T Q R P H C M W W H E R A L P W
1801    AGCGCGCGACGTGTTTCATGGATGGCAACGCAGCAATTTTCGGTTGCCTGCTCCTCAATAAACTTTTGGCTTTCCGAGGCTTATCACCATGCATGGCCC
S A R T C S W M A T H E L S V A C S S I N F C F P Q A Y H H A W P
1901    CTGATGCCAACGAATTCCTGCAGGAATTCGACGGTACTCGAACGAAGAATGGTGGCGCGGATTGAGGAAGGACAGAATATATCCGGGCACATTGCCA
L M P T N S C R N S T V T R T K N G G A D S G R T E Y I P G T L P
2001    TGCATAGCGATTGTTATAAAATGCTTATGCAATTAAGCTGAGGCTATAAAGGAGGCGTTATGCCGATCATGGAGCAGAGTTCCTTGTCTCTCTGTTGT
C I A I V I K C L C I K L R L *
2101    GCCATATCGCTGCGAGATTGCGAGCGCATATCGTAATGACCAACAAAACATCCGCAATGTTTCAACTTAAGGGTGTACAAATAGAGTTTATGTATA
NdeI
2201    CGGCGCCTTATCGAGATAAGGCCATTGGAAGTTTGTCTGACCTCGACCTTACCGTGATTATTATCGCGAACCAGATACAGATGCCACTGCAAAAAT
BamHI
2301    CGACGTTGTTAAGACTGCGCTTGAAGGATCTGTGCTTCTGAAATTCAGCCGCTGAAATGCCGCGATTTCGGTATTACCTGTTTCGACCACCAGGAAC
2401    TGGTGTGCAATATGATTATCGGCGCGACCTTCTCTGA

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Fig. 3. Nucleotide sequence of pKMBE-B/S2.8 and deduced amino acid sequence of *pthG*. Start and stop codons are indicated (M and *, respectively). The following features are underlined: proposed Shine-Delgarno (SD) ribosome binding site, a putative *hrp* box, amino acids that may form short putative *trans*-membrane helix (TMH), and relevant restriction sites.

morphology of galls induced by the two pathovars on gypsophila: galls induced by *Ehg* are yellow and spherical, whereas those induced by *Ehb* are white and ring shaped (D. Ezra, S. Manulis, and I. Barash, *unpublished*).

The phenomenon of dual functionality observed with *pthG* has been reported for several Avr-like effector proteins in plant-pathogenic bacteria (e.g., Swarup et al. 1992; Leach and White 1996; Gaudriault et al. 1997; Bogdanove et al. 1998). For example, the DspE protein of *E. amylovora*, which is essential for pathogenicity on pears but not for elicitation of the HR on a nonhost, is highly homologous with AvrE of *Pseudomonas syringae* pv. *tomato*, which elicits the HR on certain tomato cultivars (Gaudriault et al. 1997). The *avrE* gene could partially substitute for the *dspE* gene in mutant strains of *E. amylovora* (Bogdanove et al. 1997). Similarly, the gene *pthA*, which in *Xanthomonas citri* is essential for elicitation of Asiatic citrus canker, confers cultivar-specific avirulence in *Xanthomonas campestris* pv. *malvacearum* (Swarup et al. 1992). Structurally, *pthA* is closely similar to *avrBs3* from *X. campestris* pv. *vesicatoria*. Collectively, these data indicate that the relative contributions of virulence and *avr* genes to disease depend on the host background in which they are expressed. These results are in accordance with the assumption that the primary function of Avr proteins is to promote parasitism, and, in fact several of the *avr* genes that have been cloned from *P. syringae* and *X. campestris* make a quantitative contribution to virulence in permissive hosts (Baker et al. 1997). It remains to be determined whether the *pthG* gene of *Ehg* can be considered a potential *avr* gene and whether the hypersensitive-like response caused by this pathogen has the essential characteristics of an HR. Preliminary results suggest that the hypersen-

sitive-like response caused by *Ehg* requires living bacteria and may depend on the type III protein secretion system (D. Ezra, S. Manulis, and I. Barash, *unpublished*).

The findings that many pathogenicity or virulence genes as well as insertion elements reside on pPATH_{Ehg}, and that the presence of this plasmid is mandatory for transformation of the epiphytic *E. herbicola* into a gall-forming bacterium, support the hypothesis that *Ehg* and *Ehb* are recently evolved pathogens. Consequently, it is tempting to postulate that *pthG* could be viewed as an *avr* gene in its earliest stage of evolution. In this connection it is noteworthy that *pthG* contains an *hrp* box, which is known to be transcriptionally activated by HrpL, as reported for other known *avr* genes (Alfano and Collmer 1996). Also similar to Avr proteins is the predicted protein of *pthG*, which lacks an N-terminal signal peptide and is very polar with the exception of the short TMH in its C terminus.

Host range determination by *Ehb* and *Ehg* deserves further comments. On the one hand, as expected from biotrophic, phytopathogenic bacteria, they are highly restricted in their host specificity. On the other hand, both *Ehb* and the marker-exchanged mutant of *Ehg* in *pthG* elicit galls on both beet and gypsophila, which belong to taxonomically different families (i.e., Chenopodiaceae and Caryophyllaceae). Such a diverse host spectrum could spring from the epiphytic nature of *E. herbicola*, which implies intimate interaction of the bacterial population with many taxonomically different plants. Thus, the pathogenicity of these bacteria was presumably developed from horizontal transfer of the *hrp* gene cluster as well as effector virulence proteins that are also dependent on the *hrp* cluster for their regulation and secretion. Genes that appear to meet these criteria are *hsvG* (Valinsky et al. 1998) and the herein described *pthG*.

The present findings could support a hypothesis that *Ehg* and *Ehb* originated from a common ancestor that exhibited pathogenicity on both beet and gypsophila. Recognition of one of the virulence genes (i.e., *pthG*) by the beet defense gene surveillance system caused a hypersensitive-like response that prevented pathogenicity on beet and resulted in the evolution of the current gypsophila pathovar. The beet pathovar may have evolved from the gypsophila pathovar by evading the beet defense response. Presumably, this was achieved by deleting and modifying *pthG* sequences and replacing the role of the gene in pathogenicity of *Ehb* on gypsophila by an alternative gene.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains, cosmids, and plasmids used in this study are presented in Table 2. Bacteria were grown in Luria-Bertani (LB) broth or LB agar plates with appropriate antibiotics, at 28°C for *E. herbicola* and at 37°C for *E. coli*. Genetic manipulation were carried out in *E. coli* DH10B (GIBCO BRL, Gaithersburg, MD). Antibiotics were used in the following concentrations (µg per ml): ampicillin (Amp), 100; kanamycin (Km), 50; spectinomycin (Spec), 50; rifampicin (Rif), 100; and tetracycline (Tc), 15.

Pathogenicity tests.

Pathogenicity tests on cuttings and seedlings of *Gypsophila paniculata* cv. *perfecta* were carried out as described by Valin-

Fig. 4. A Western blot (immunoblot) of PthG in a T7 expression system. After growth and induction, as described in Materials and Methods, proteins in 20-µl amounts of cell lysates were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, *Escherichia coli* (pET-N/B1.5) with isopropyl-D-thiogalactopyranoside (IPTG); lane 2, without IPTG.

sky et al. (1998). Pathogenicity tests on table beet cubes and beet seedlings were performed according to Burr et al. (1991). Whole, mature beets were soaked in 1% sodium hypochloride for 10 min and then washed twice in sterile water. They were then cut into $0.5 \times 0.7 \times 0.7$ cm cubes, each of which was placed on sterile 1.5 % water agar in a petri dish. Inoculation was carried out with a culture grown overnight on LB agar, by puncturing the top of the cube and inserting the bacteria with a sterile toothpick. The dishes were incubated for 5 days at 28°C and then tested for gall formation. Three beet cubes were used for each strain, and the experiment was performed at least three

times. Pathogenicity tests were also carried out on 3-week-old beet seedlings (*Beta vulgaris* cv. Red Egyptian) grown on vermiculite. Inoculation was carried out by puncturing the stem with a sterile needle and applying the bacteria to the wound with a toothpick. Gall formation was evaluated after 2 weeks.

DNA manipulations.

Isolation of plasmid DNA from *E. coli*, restriction enzyme digestions, subcloning, transformations, Southern blot analysis, and polymerase chain reaction (PCR) were carried out by standard procedures (Sambrook et al. 1989; Ausubel et al.

A

Ehg	MGCFNVTGASGRANNYVVEHHDDRSSIGQESPRQEIADSPLSRSGRSSNRTSPRVSLDR
Ehb	MGCLNVTGASGRTNNYVVEHHDDRSSIGQESPSQEIADSSAHPIEPPCVSLDRLRTFNP
Ehg	LRAFNPPSGASVGALRNWFWQGQAAQHLQQLEAELNNGSNIRELLDGPFGGLLTHVME
Ehb	PGGSSVGALRNWFWQGQAAQHLQQLEAKLDNGSNIRELLDGPFGGALLTHVIDDISSIS
Ehg	DIASISNAENLSPIEVAEELRQAISTGRGANLDISLKNVLQFGLYAVREILQGEAEIAG
Ehb	NAENLSPSEVAEALRQAENLSPSEVAEALRQANVLQFGLYAVRMQSGKNCKVKPSSPAR
Ehg	SNMQTHPAFQSFVPAQAWKFCIEQEHWDYGCAASGMRFDPNEPGYMGSMFRGLKFVIDS
Ehb	CKHIRLFSLSRWPTRGNFALSKTIGMITVPYPACDLTTS PATWVCAA*
Ehg	EAGSSSRKKGEIDATWLENLHDIANGVFQRRVMHDWQFGANVEEAVNAVAALTAPGSN
Ehg	ISDKVRSAAIAFYLP EHLWPLEGGYRQGLVQFKLVYPKNLTPAGLGDLQKFQSRFAG
Ehg	WAEFRSVGNRPIDWDEVERGNAQVMWACTGKSRKEVMKLVKNVLQTHYNDIKRTNQTOR
Ehg	PHCMWWHERALPWSARTCSWMATHELSVACSSINFCFPQAYHHAWPLMPTNSCRNSTVT
Ehg	RTKNGGADSGRTEYIPGTLPCIAIVIKCLCIKLRL*

B

Fig. 5. Comparison of the open reading frame (ORF) of *pthG* with homologue detected in the beet pathovar. **A**, Alignment of deduced amino acids of the N terminus of *pthG* with homologous ORF obtained from *Ehb4118*. **B**, A schematic comparison between ORF isolated from *Ehb4118* and ORF of *pthG*. Identical nucleotide and deduced amino acid sequence (white); sequences homologous only at nucleotide level (vertical lines); a sequence that exists exclusively in *pthG* (black). Frame shifts occur between 120 to 136 bp and 483 to 494 bp of *pthG*.

Fig. 6. Southern blot showing distribution of *pthG* sequence among major genera of gram-negative, phytopathogenic bacteria. Total DNA from indicated strains was digested with *EcoRI* and *HindIII* and resolved in a 1.2% agarose gel before blotting and hybridization at moderate stringency with the 1.8-kb *BamHI/HindIII* fragment from pVMBE-B/H1.8 as a probe. Lanes: 1, λ -*HindIII* DNA digest; 2, *Pseudomonas syringae* pv. *savastanoi*; 3, *Xanthomonas campestris* pv. *campestris*; 4, *E. amylovora*; 5, *E. herbicola* pv. *milletiae* (visteria pathovar); 6, *E. stewartii*; 7, *Ehg824-1* (gypsophila pathovar); 8, *Ehb4188* (beet pathovar); 9, *Ehb2188* (beet pathovar); 10, *Ehg350* (gypsophila pathovar); 11, *Ehg3-1a* (gypsophila pathovar); 12, *E. herbicola* 3-1 (a nonpathogenic strain); 13, *E. herbicola* 717-2 (a nonpathogenic strain); 14, *E. herbicola* 24-8 (a nonpathogenic strain) 15, λ -*PstI* DNA digest.

Fig. 7. Pathogenicity on gypsophila of marker-exchange mutants of *Ehg824-1* in *pthG*. **A**, *Ehg824-1*; **B**, *EhgMxE27*; **C**, *EhgMxE1*; **D**, Control (H_2O).

1995) or as recommended by the suppliers. Isolation of plasmid DNA from *E. herbicola* was performed as described previously (Lichter et al. 1995b). The primers in this study were synthesized by Gibco, BRL (Palsley, U.K.).

DNA sequencing.

A 9-kb *EcoRI/KpnI* fragment of pLA173 was subcloned into pLAFR3 to yield pMBE-R/K9 (Fig. 1). A 2.8-kb *BamHI/SacII* fragment of pMBE-R/K9 was subcloned into pBS (KS⁺)

Table 3. Sequence and location of primers used in this study

Primer	Sequence	Source	Position	Direction
D1B	5'-GCACAGGATCCTGCTCAAGCGC-3'	pMBE-B/S2.8	2810 to 2789	Downstream
D2H	5'-CGCAACTGATACAGAAGCTTC-3'	pMBE-B/S2.8	98 to 120	Upstream
D3H	5'-CGCAACTGATACAGAAGCATC-3'	pMBE-B/S2.8	98 to 120	Upstream
D4Nde1	5'-GGAGAAGCTTAAGCATATGGGATG-3'	pMBE-B/S2.8	571 to 595	Upstream
204T71	5'-CGGGTGCCAGTGGCAGAACGAACAA-3'	pMBE-E/H3.5	1979 to 1202	Upstream
204T31	5'-CCGGCAAATAGTAGATTGCCGACGC-3'	pMBE-E/H3.5	822 to 800	Downstream
204T72	5'-GCATGCGATTGACAACGAGCCCCG-3'	pMBE-E/H3.5	1014 to 1031	Downstream
204T31	5'-GCCGTCGACTCCGCCGGTGCCTCC-3'	pMBE-E/H3.5	71 to 90	Upstream

for sequencing. Generation of unidirectional deletions of the latter fragment was obtained with the Erase a Base kit (Promega, Madison, WI). Screening for clones of an appropriate size was carried out by PCR with T3 and T7 primers and verified with restriction enzymes. The entire fragment was sequenced in two directions by automated sequencing with *Taq* DNA polymerase (Department for Biological Services, Weizmann Institute, Rehovot, Israel). The sequence was completed by constructing extra subclones with the restriction enzymes *EcoRI*, *SacI*, and *SacII* (Fig. 1). DNA for sequencing was prepared with the High Pure Plasmid Isolation Kit (Boehringer Mannheim, Indianapolis, IN). Analysis of sequence data for the DNA and deduced protein sequences were according to the program manual for the Wisconsin Package (version 9, Dec. 1996; Genetic Computer Group, Madison, WI).

Construction of mutants and marker exchange.

A cosmid library of plasmid DNA from *Ehg824-1* in pLAFR3 had been prepared by Lichter et al. (1995a) and a cosmid library of plasmid DNA from *Ehb4188* in pLAFR3 was constructed as previously described (Lichter et al. 1995a). Insertional mutagenesis was carried out on cosmid clones in *E. coli* DH10B with mini-Tn10 as described elsewhere (de Lorenzo et al. 1990). The mutated cosmids were mobilized to the wild-type strains of *Ehg* or *Ehb* by triparental mating with pRK2073 as a helper plasmid (Ditta et al. 1980). Plasmid curing was performed as previously described (Lichter et al. 1995b). The marker-exchange mutants were subjected to pathogenicity tests on beet and gypsophila as described above. The location of the mini-Tn10 in the mutants was initially determined in *E. coli* DH10B by mapping with multiple restriction enzymes. The mutated plasmids were then mobilized into *Ehb4188* or *Ehg824-1* by triparental mating and their location was verified by digesting the DNA with *EcoRI* and *HindIII* followed by Southern hybridization with a 1.8-kb *BamHI/HindIII* fragment from pVMBE-B/H1.8 as a probe.

Expression of *pthG* in a bacteriophage T7 RNA polymerase-promoter expression system.

The T7 promoter-driven system pET (Novagene, Madison, WI) was employed to express the PthG protein. The ORF of this gene was amplified by PCR with primers D4NdeI and D1b (Table 3), which were designed on the basis of the 5' and 3' ends, respectively, of this gene (Fig. 2), with appropriate restriction enzyme sites for cloning. The Expand High Fidelity PCR system (Boehringer, Mannheim, Germany) was used to amplify the predicted ORF (1.5 kb) of *pthG*. The amplified fragment was digested with *NdeI* and *BamHI* and cloned into pET11a through the same restriction sites to form pET-

N/B1.5. The recombinant plasmid and the vector were transformed into *E. coli* HMS174 (DE3). For protein expression, a fresh culture of the transformed *E. coli* (pET-N/B1.5) was grown in LB at 37°C up to an OD₆₀₀ = 0.5, prior to the addition of 0.04 mM IPTG. The culture was then grown for an additional 3 h at 37°C and 300 µl of cells was centrifuged, resuspended in 50 µl of distilled water, and mixed with 25 µl of sample buffer, three times (New England Biolabs, Beverly, MA). Cultures of *E. coli* (pET-N/B1.5) without IPTG and *E. coli* (pET11a) in the presence and absence of IPTG were used as controls. The samples were heated at 100°C for 5 min and then loaded onto an SDS 10% polyacrylamide gel. The procedure for SDS-PAGE was performed in a single-concentration minigel as described elsewhere (Ausubel et al. 1995).

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