A Pathogenicity Gene Isolated from the pPATH Plasmid of *Erwinia herbicola* pv. *gypsophila* Determines Host Specificity

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The host range of the gall-forming bacterium *Erwinia herbicola* pv. *gypsophila* (*Ehg*) is restricted to the gypsophila plant whereas *E. herbicola* pv. *betae* (*Ehb*) incites galls on beet as well as gypsophila. The pathogenicity of *Ehg* and *Ehb* was previously shown to be dependent on a plasmid (pPATH). Transposon mutagenesis was used to generate mutants on the cosmid pLA150 of the pPATH from *Ehg*824-1. A cluster of nonpathogenic mutations flanked by two IS1327 elements was identified on a 3.2-kb *Nde*I DNA fragment. All mutants were restored to pathogenicity by complementation in *trans* with the wild-type *Ehg* DNA. DNA sequence analysis of the 3.2-kb *Nde*I fragment revealed a single open reading frame (ORF) of 2 kb as well as a potential ribosome binding site and a putative *hrp* box upstream to the ORF. The ORF had no significant homology to known genes. Southern analysis also revealed the presence of DNA sequences that hybridized to the ORF in the beet pathovar *Ehb*4188. This gene was isolated and sequenced. Marker exchange mutants generated in the ORF of *Ehb* eliminated the pathogenicity of *Ehb* on gypsophila but fully retained its pathogenicity on beet. Since the putative gene appeared to encode a host-specific virulence factor for gypsophila it was designated as *hsvG*.

Additional keywords: type III secretion system.

Erwinia herbicola is widespread in nature as an epiphyte on many plants (Starr 1981). However, some strains have evolved into plant pathogens that incite gall formation in gypsophila (*Gypsophila paniculata* L.), an ornamental used in commercial cut-flower production (Cooksey 1986), and beet (*Beta vulgaris* L.) (Burr et al. 1991). Infection by *Erwinia herbicola* pv. *gypsophila* (*Ehg*) is initiated at wound sites, mainly in the crown region of the stem. The host range of *Ehg* is restricted to gypsophila (Volcani 1985) whereas, in contrast to *Ehg*, the beet pathovar *Erwinia herbicola* pv. *betae* (*Ehb*) generates galls on beet as well as on gypsophila (Burr et al. 1991). The pathogenicity of both pathovars is associated with a similar indigenous plasmid (pPATH) of approximately 150 kb that is exclusively present in all the

pathogenic strains (Manulis et al. 1991a; S. Manulis and I. Barash, *unpublished*).

What causes *E. herbicola* to switch from being an epiphyte on many different plants to being a pathogen, and what determinants are responsible for host range? An initial answer to the first question was recently obtained by demonstrating the presence of a functional *hrp* cluster on the pPATH, with high homology and collinearity to the *hrp* genes of *E. amylovora* (Nizan et al. 1997; R. Nizan, *unpublished*). Superimposed on the *hrp* system were the genes encoding phytohormone synthesis. *Ehg* possesses two major pathways for indole-3-acetic acid (IAA) biosynthesis: the indole-3-acetamide (IAM) pathway, which is present only in pathogenic strains, and the indole-3-pyruvate pathway, which is present in both pathogenic and nonpathogenic strains (Manulis et al. 1991b, 1997). In addition, only the pathogenic strains produced cytokinins (Lichter et al. 1995a; Manulis et al. 1997). Inactivation of IAA production through the IAM pathway (Clark et al. 1993) or of cytokinin biosynthesis (Lichter et al. 1995a), or simultaneous inactivation of the two IAA pathways and cytokinin production (Manulis et al. 1998), caused a substantial reduction in gall size but did not eliminate gall initiation. These findings led to the assumption that gall initiation is triggered by virulence factors other than IAA or cytokinins, secreted by the pathogen.

The molecular basis for host specificity in gram-negative, phytopathogenic bacteria has been a subject of intensive investigation. Most investigations have focused on negative-acting factors that restrict host range by provoking the hypersensitive response (HR) (Long and Staskawicz 1993; Dangel 1994; Leach and White 1996). These negative determinants include the products of the avirulence (*avr*) genes and harpins (Alfano and Collmer 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; Waney et al. 1991; Gabriel 1989). The present paper describes the isolation and characterization of a host-specific virulence gene from pPATH, which determines pathogenicity on gypsophila.

RESULTS

Isolation, sequence analysis, and expression of the pathogenicity gene *hsvG*.

The transposon-reporter Tn3-Spice was used to generate nonpathogenic mutants on the cosmid clone pLA150 derived

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Nucleotide and/or amino acid sequence data have been submitted to GenBank as accession number U61275.

from the pPATH of *Ehg* 824-1 (Lichter et al. 1995a). A cluster of six marker exchange, nonpathogenic mutants designated as *EhgMx* (# = B45; A30; 14; B35; 24; B10) was identified on a 3.2-kb *NdeI* DNA fragment located immediately downstream from the phytohormone biosynthetic genes (Fig. 1). The maximal extent of the putative gene on the right end of the fragment was defined by the Tn3-Spice insertion *Ehg*824-1Mx4, which retained full pathogenicity. The location of these mutants was determined by mapping with multiple restriction enzymes, hybridization, and polymerase chain reaction (PCR), as described in Materials and Methods. Two additional, nonpathogenic mutants (i.e., *EhgMx*PI and PII) were obtained by inserting a kanamycin-resistance cassette into the two *PstI* sites of the 3.2-kb *NdeI* fragment (Fig. 1). The nonpathogenic mutants spanned through an approximately 2-kb fragment that was flanked by two *IS1327* elements (Lichter et al. 1996). All the nonpathogenic mutants were restored to full pathogenicity *trans* by either pLA150 or pLA-Nd3.2 (Fig. 1).

The 3.2-kb *NdeI* DNA fragment was subcloned into pBS (KS⁺) to yield pMBV-Nd3.2, and subjected to sequence analysis of both strands. A single open reading frame (ORF) of 2 kb was found 340 bp downstream from the termination codon of *IS1327* and predicted a protein with 671 amino acids and a molecular mass of 71.3 kDa (Fig. 2). A potential ribosome binding site, 5'-CGAGA-3', was found 7 bp upstream from the translation start codon, whereas a putative *hrp* box, 5'-GGAACC-N₁₅-TTACA-3', was identified 65 bp upstream from the start codon. The predicted ORF protein lacked an N-

terminal signal peptide and any likely membrane-spanning domains. The ORF had no significant homology to known genes. The nucleotide sequence of the 3.2-kb *NdeI* fragment has been submitted to GenBank as accession number U61275. Since, as will be further described, this putative gene was found to encode a host-specific virulence factor for gypsophila it was designated as *hsvG*.

The complete *hsvG* ORF was cloned into the T7 promoter-driven vector pET-11a to yield pET2K and expressed as described in Materials and Methods. Proteins of lysed cells containing either pET2K or pET-11a in the presence or absence of isopropyl-β-D-thiogalactopyranoside (IPTG) were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Results shown in Figure 3 demonstrate the appearance of a strong protein band with an apparent molecular mass of about 72 kDa only in the presence of pET2K with IPTG. This band was absent in identical cells that were grown without IPTG and in cells containing the vector alone in the presence of IPTG. The expressed protein corresponded in size to the expected deduced protein of the *hsvG* sequence.

Phenotypic expression of *hsvG* in gypsophila.

Insertional mutants in *hsvG* eliminated gall formation on either gypsophila cuttings or whole seedlings that were inoculated with 10⁷ CFU/ml. However, inoculation with a higher bacterial concentration of 10¹⁰ CFU/ml resulted in the appearance of a crown around the cutting edge (Fig. 4). The size of the crown was directly related to the distance of the

Fig. 1. Physical map of the cosmid clone pLA150, a derivative of pPATH, and the *hsvG* locus. pLA-Nd3.2 contains a 3.2-kb *NdeI* fragment of pLA150 cloned into pLAFR3. pLA-Nc1.7 contains a 1.7-kb *NcoI* fragment of pLA-Nd3.2 cloned into pLAFR3. The apparent open reading frame of *hsvG* is indicated by bold line. Marker exchange mutants obtained by Tn3-Spice are indicated by open triangles and marked as *Ehg*824-1Mx(#); # = B45, A30, 14, B35, B10, and 4. Black triangles (*Ehg*824-1MxPI and PII) are marker exchange mutants obtained by insertion of kanamycin cassette. *iaaM* and *iaaH* are indole-3-acetic acid (IAA) biosynthetic genes (Clark et al. 1993), *pre-etz* and *etz* are cytokinin biosynthetic genes (Lichter et al. 1995a), *IS* is the insertion element *IS1327*, L-left, R-right (Lichter et al. 1996), and *hrp* genes (Nizan et al. 1997). Abbreviations: R, *EcoRI*; H, *HindIII*; K, *KpnI*; Nd, *NdeI*; Nc, *NcoI*; B, *BamHI*; P, *PstI*.

NdeI
1 CCATATGATACGCAAGGGCAGTATAAACATCCGACGGGGGAACGGGTTGTCACCGGCAGAACATTCCTATCTTTGATTGACTGAAACAGATTACGCA
NcoI
101 GCTTTTGCTGACTTACTGCCGTTAATGCGGCAGAGCCGTAAGTTTAGTGCTGATTATTCAACCACTACAAAGTGTCCATGGCAGAAACCGGGGCAAAA
201 ATAAAGACGGTCTGTATGTGAGGCTTTTCGCTGTTCTGCGTCTGCCGGCGGATATGACTCAGCCTTTCCAGTAGCCGTCGATTTTGTACCTCCGTTGA
hrp box?
301 TGCGCATGAACACATAACGGGGTTTTATGCCGAACCGCGGGCGGTTTTCCTTACAAAAGAGGGAGCATTAGCGCCGACTTTAGCGGCTGCCATTAAAC
SD?
401 CTGACTCCTGACGAGACACCGCATGTGGAACAACATTAAACCGTACGGCAGGCTTCCCGTAAACAGCCGGGAGCTGATGAACTCCACCGCGGCAGGA
M W N N I N P Y G R L P V N S R G A D E T P P A A D
501 CCCGCATCCTCAGGGTACCGCGGGCGGCACGCCACCGCTCCGGGACGACCCGGTGCCGCGCCCTATCCTGAGGATGAGGTGCTCAGGAGTGGTTTTGT
P H P Q G T A G G T P P L R D D P G A A P Y P E D E V L T E W F C
601 GACTGCGCCACCGCTGGCGGCACCGCCAGAGTGGCAGTGATTACGATGAGGCGCTTCCGCGAGGTTGAGCGGCTTCTCAGCCTGAACGGGCTGCCCG
D C A T A G R T R Q Q V A S D Y G W A L R R F S G F L S L N G L P A
701 CCCTCTCCGCCCCCGGAGGCTGACGAAACCGCAGGCTGAGGCGCGACGCCATGTCTTATCGAACGTTTTCATGAGTCGGGGCGCCAAAAAACCT
PstI
L S A P G R L Q E P D R L R R D A H V F I E R F H E S G R Q K N L
801 CATACCGGCGCTGAACCGCTCACCAGCTGGCGGCGTGGGAGAGGACAGCATCACGATACCGGGTCGAGAGACCCCGGCTGAAGTTCCCGACGG
I P A L N R L T E L A A S G E D S I T I R G S Q R P R L K V P D A
901 GACGGGCGCTGATTAAGGGGCGTTTCCGGGTCTGCCCGGGCCAGGCGAGGACATTATAGCGTGAACCTCAGAAATGCCGCGCGGCTCCCTCAGCG
SacII
D G A L I K G A F P G L P A G P G R D I Y S V N L R N A A R S L S A
1001 CCTGGCTTACGAAACCGCAGGCGCGGCTGAGCGATACCGCTTACCTGTTCTCGGAGCAGGCGCGGAGCGCGGTCGCTTCCCGGGCTCATCC
W L H E N G R P G L S D T A Y L F S E Q A G A D A G A F S R A H P
1101 GGCCATGCGCGGAGGTAAACATGATACTGGCCACCTCGCGCCCGACCGCGGGGGAGGTGCCGACGTCGGAAGACTCCAGAACACCGGACGATA
A N A G R V N M I L A H L R A H A R G E V P H V G R L Q N T R T I
1201 CCGTGGCAGACACGCGGCTGGCCCTGGCGTACCTGGGTATCGGAAAGACCTCGCACGGCAGGCGCGCACAGTGATAAAAAAACTCAACGGCCATG
P L A D Q R L A L A Y L G I A K D L A R Q A G T V Y K K N S N G H D
1301 ACTCCATTGACAAAAAGCTCTCAGTTAAGTTCATTACGCGCTGGCGCACGGCCAGGGGCTCCCGCGCTGACCGACACCTTCATGACCCGGGGCT
S I D K K A S Q L R S F S A W R T A R G L P A L T D H L H D P G L
1401 GCAGATGGACATGGACATGTACACCGAGGATAAATATCAGAGAAGTCAGAACACGGGCGGTCCGCTGGCAGCAGCAGCGCAGGTGGCAGCATCCAGG
Q M D M D M Y T E D K Y Q R S Q T N G R S A G S S T A R S A R S R
1501 CTGAGCTCCGCACTGAAACACTTCAGACCCGCTTTCCGCGGGAACCCGGTCAACGCTCCGAGGACCGCGGAGTGGTTCGGGCTCCCGGCTCAG
SacI
L S S A L K T L Q T R F P P G T P V T V P E A P A E S F G L P G S D
1601 ACTGGTCAGGCTGGGACATGAGCCTGTCCGTCGCCGCGCGGACGCGGCGCTCGCGCGGACAGACGATTCCTGTTTCGGGGGCTGGCGCGCTGGA
W S G W D M S L S V P G A D G G P A P A D S D S V F G G L A P L D
1701 CTCCCGCAGCGCTTTTCTCTGACGGACTTTCCGGTGCCTGCGCCCTGCGCTTCCGAGACCGCGAGGTGAACCGTCCCGTCCCGAGGACCGGGG
S R E R F S S D G L S G A L A P G L P E T G E V N R P V P E A P A
1801 GAATCGTTCGCGCTCCCGGCTCAGACTGGTCAGGCTGGGGCATGAGCCTGTCCCTCGGTGGCCATGGTCCAGCAGACAGCATTCCTGTTCGGGGGCC
E S F A L P G S D W S G W G M S L S L G G H G P A D S D S V F G G L
1901 TGGCGCGCTGGACTCCCGCAGCGCTTTCTCCGCGGGCTCTCCGGTACACCGGCTCCGGCTTCCGGAGACCGCGAGGTGAACCGTCCCGGCCC
A P L D S R E R F S S G G L S G T P A S G L P E T G E V N R P G
2001 GGACGGGCTCCTTTCTGTGAGCATGCGGACCGGTCCCGCTTCCGTCGCCGAGGGTGGGTTTCGCGCAGCGCGAGGGTTACGCTGCTGCTGGAC
D G A V P P F R D G V G F A S G E G F S C L L D
2101 AGCATCTCCAGGGCTACCAACATCCGCGCGGCGATGGCGGCCAGGGGCTGACGGACTGGCTGGACGGAGGTCGGCGGGTCCGCGAGGCGC
S I L Q G Y H N I R R G H G A P R G L T D W L D G E V R R V R E A L
2201 TGTGGCGCGAGGGGTTGACCTGCTGCCGCCCGGGGAAATTGATATTTACGGCGGTCCAGGACGCTACCTTCCCGGGCAATGGGCTCCGGCTGCA
PstI
S A Q G V D L V P A R G E I D I Y G G P G S Y L A G A M G L R L Q
2301 GGTGATTACGGCGAGTTTTCAGGAGGAGCCGACGGGACGTACGTGAGGTACACGGCACACCGGAAATCGGCGAGGCGGGCGGGGCTGTACGGCTG
V I Q A E F Q E E P D G T Y V R Y T A H P E I G E A G G R A V R L
2401 CTGCACACGCGGGCCACTTTACGCGCTCTGGGGGTGAAGGCGGGCAACGTCATCATCTTGGGGTGCCCGCATTACCGCTGAAAGTGAACGGCTCTGT
L H T P G H F Q P L W G *
2501 CGCATTAAGGAGTCGTACGGTAACATAACTGTTTTTTTTTTCGATGTCCCTGCCGATGTCTTATCCGAAAAGCCTTCAACGCCCTGCATTATCCCG
2601 TCGATATTATTGCCAGTGCCTTCGCTGGTATCTGGCTTACTCACTCCAGCTTGCGAAATCCTTGAGAAGAGATAATTGCTTGGAGCGGGGGATTGTG
2701 GTTTGATCAATTCCAACGCTTCAACGCTGGCTCAATCCGCTGGTACCAACGCTTAGATAAGGCGTTCCGCGGGGTATAAACGTAATCTGCGCGACGGG
2801 CGGCGAATGGACGAAACCTACATCCAAATCAGGGGGCAGTGAAATACCTGTACCGGGCAGTTGACAGTACGGGCGAGCCATCGACTTCTGCTGG
PvuII
2901 TCGCTAAGCGTGATGCGGCGAGCTCTGCTGCTTCTCCGCAAGGCTATCCGCAATAACGGGGAACCGGAGGTGGTAACCATTTGATAAAGCGCGCGAA
SacII
3001 TACCGCGGCTCTGGATACACTTAACGCGGTAAGGAGATGAAGGAATATTATCGTCAGCCAAAGCAGGTATCTGAATAATTGATTGAGCAGGATCAC
3101 CGGAACATCAACGCGGATACGACTGATGCTGGGGTCAATCATTTNCGCGGGCGCAGACGATACTGGCGGCTAGTGAATTAATCCATAT
NdeI

Fig. 2. Nucleotide sequence of the 3.2-kb *NdeI* fragment and deduced amino acid sequence of *hsvG*. The beginning of the open reading frame and its stop codon are indicated (M and *, respectively). The following features are underlined: putative *hrp* box, proposed Shine-Delgarno (SD) ribosome binding site, and relevant restriction sites. Molecular mass of the predicted peptide is 71.3 kDa, containing 671 amino acids.

insertion from the start codon. Thus, mutants *EhgMxB45* and *EhgMxA30*, which were inserted within the initial 350 bp of the ORF, exhibited only a slight swelling whereas mutants *EhgMxB35* and *EhgMx24*, which were located near the second *Pst*I (700 bp from the *N* terminus) showed a larger crown (Fig. 4). The largest crown was observed with mutant *EhgMxB10*, which was located near the C terminus of the *hsvG*. Pathogenicity of all the mutants was fully restored in *trans* by either pLA150 or pLA-Nd3.2 (Fig. 5). pLA-Nc1.7, which contained only 70.6% of the *hsvG* ORF, also restored gall formation in these mutants. However, the restored gall

size obtained by pLA-Nc1.7 was considerably lower than that of pLA-Nd3.2 (Fig. 5). The former and latter results may support the notion that the C terminus of the protein is not mandatory for gene expression but that the whole ORF is required for full activity.

The nonpathogenic, marker exchange mutants in *hsvG* were tested for HR elicitation on tobacco leaves (*Nicotiana tabacum* L. 'Hicks') as described previously (Nizan et al. 1997). All the mutants caused the HR, as did the wild-type strain (results not shown).

The prevalence of *hsvG* among gram-negative, phytopathogenic bacteria.

The total DNA of the tested strains was digested with *Eco*RI and subjected to Southern blot analysis with the 1.7-kb *Nco*I fragment as a probe. Results shown in Figure 6 indicate that in addition to the two strains of the gypsophila pathovar, i.e., *Ehg824-1* and *Ehg350*, *hsvG* sequences are also present in the beet pathovar *Ehb4188*, but not in the nonpathogenic strain of *E. herbicola* *Eh3-1*, which lacks pPATH (Manulis et al. 1991a). Additional pathogenic strains, e.g., *Ehg3-1a* and *Ehb1188*, were also found by Southern hybridization to contain *hsvG*, whereas two other nonpathogenic strains, i.e., *Eh717-2* and *Eh24-8*, did not contain this gene (results not shown). It is noteworthy that the band obtained with the two strains of the gypsophila pathovar differed in size from that of the beet pathovar, which may reflect an existing polymorphism between *Ehg* and *Ehb*. No hybridization with the *hsvG* sequence could be detected in *Erwinia stewartii*, *Erwinia amylovora*, *Ralstonia solanacearum*, *Xanthomonas campestris* pv. *campestris* (Fig. 6), or *Pseudomonas syringae* pv. *savastanoi* (data not shown).

hsvG determines host specificity of *E. herbicola* on gypsophila.

Since the host range of *Ehb* includes beet and gypsophila whereas *Ehg* is restricted to gypsophila, it was of special interest to investigate the function of *hsvG* in *Ehb*. In order to examine the effect of *hsvG* on the pathogenicity of *Ehb*, marker exchange mutants (*EhbMx14*, *EhbMxPI*, and *EhbMxPII*) were generated in *Ehb4188*. The presence of the

Fig. 3. Expression of *hsvG* in a T7 expression system. After growth and induction, as described in Materials and Methods, proteins in 20- μ l amounts of cell lysates were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 40 mA until the dye front reached the bottom of the gel. Lane M, prestained broad range protein markers (New England Biolabs, Beverly, MA); Lane 1, *Escherichia coli* (pET2K); lane 2, *E. coli* (pET2K) with isopropyl- β -D-thiogalactopyranoside (IPTG); lane 3, *E. coli* (pET11a) with IPTG; lane 4, *E. coli* with IPTG.

Fig. 4. Phenotypic expression of insertional Tn3-Spice mutants in *hsvG* following inoculation of gypsophila cuttings. Inoculation was performed with 10^{10} CFU/ml. Location of each mutant on *hsvG* is given in Figure 1. C: control, cutting not inoculated. Mutants: 1, *EhgMxB45*; 2, *EhgMx14*; 3, *EhgMxB35*; 4, *EhgMx24*; and 5, *EhgMxB10*. A crown appears around the cutting edges. Size of the crown was proportional to distance of mutant site from *N* terminus of the open reading frame.

insertions in the homologous region of the 3.2-kb *NdeI* fragment in *Ehb4188* was confirmed by hybridization with the 1.7-kb *NcoI* fragment (results not shown). The obtained mutants were then tested for pathogenicity on beet and gypsophila. Results given in Table 1 clearly demonstrate that these mutants specifically eliminated the pathogenicity of *Ehb* on gypsophila but fully retained its pathogenicity on beet. On the other hand, the corresponding marker exchange mutants in *Ehg* were nonpathogenic on both host plants. Consequently, and as already indicated, *hsvG* could be defined as an *hsv* gene that determines specificity on gypsophila.

Further studies were aimed at isolating and characterizing the *hsvG* from *Ehb*. A cosmid (pLAFR3) library of *Ehb4188* plasmids was screened for the presence of *hsvG* sequence with the 1.7-kb *NcoI* fragment as a probe. The cosmid pLE564 was isolated and the corresponding 3.2-kb *NdeI* fragment was subcloned into pBS (KS⁺) and sequenced. Comparison of the *Ehg* and *Ehb* *hsvG* sequences revealed 98.1 and 98.8% identity in nucleotides and deduced amino acids, respectively (Fig. 7). It is noteworthy that the eight sites in which differences in base pairs were observed did not interfere with the continuity of the ORF of *hsvG* in *Ehb* and most of the base displacements were found in the C-terminal region.

Complementation experiments were carried out in order to prove the identical function of *hsvG* in both pathovars. Thus, pathogenicity of the marker exchange mutants of the gypsophila pathovar (*EhgMx14* and *EhgMxB45*) was restored by either the cosmid clone pLE564 or pLE-Nd3.2 from *Ehb*. Similarly, the pathogenicity of the marker exchange mutants of the beet pathovar (*EhbMxPI* and *EhbMxPII*) was restored by either the cosmid clone pLA150 or its subclone pLA-Nd3.2 from *Ehg*.

The *hsvG* in *Ehg824-1* is located between two *IS1327* elements on pLA150, a derivative of pPATH in *Ehg 824-1* (Fig. 1). In order to prove that *hsvG* of *Ehb4188* also resides on pPATH of the beet pathovar, an insert of 450 bp containing the left-end portion of the 3.2-kb *NdeI* fragment was obtained by digesting pMBV-Nd3.2 with *KpnI* (Fig. 1). The 450-bp fragment, which contained a segment of *IS1327*, was used as a probe for detecting the insertion (IS) element. Results (not shown) demonstrated the presence of *IS1327* in pLE564 from which *hsvG* of *Ehb* was isolated. Since it has been previously shown that *IS1327* elements are exclusively present on the pPATHs of the gypsophila

and beet pathovars (Lichter et al. 1996), it can be concluded that the *hsvG* of *Ehb* is also present on this plasmid.

DISCUSSION

We have isolated and characterized a host-species specificity gene, designated as *hsvG*, that determines the ability of either *Ehg* or *Ehb* to incite galls on *Gypsophila paniculata*. Inactivation of *hsvG* narrowed the host range of *Ehb* by one host plant (i.e., gypsophila) but did not affect the 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 the latter pathovar nonpathogenic. Analysis of the amino acid sequence data bases revealed that, so far, HsvG homologues have not been identified either in other gram-negative, phytopathogenic bacteria or in any other organisms. The predicted protein, which was also

Fig. 6. Southern blot showing distribution of *hsvG* sequences among the major genera of gram-negative, phytopathogenic bacteria. Total DNA from indicated strains was digested with *EcoRI* and resolved in 1.2% agarose gel before blotting and hybridization at moderate stringency with the 1.7-kb *NcoI* fragment as a probe. Lane 1, λ -*PstI* DNA digest; lane 2, *Ehg824-1* (gypsophila pathovar); lane 3, *Ehg350* (gypsophila pathovar); lane 4, *Ehb4188* (beet pathovar); lane 5, *Erwinia herbicola* 3-1 (a nonpathogenic strain); lane 6, *E. amylovora* 209; lane 7, *E. stewartii* DC283; lane 8, *Xanthomonas campestris* pv. *campestris* 418; lane 9, *Ralstonia solanacearum* 1455.

Fig. 5. Restoration in *trans* of insertional mutant *EhgMx14* with pLA-Nc1.7 and pLA-Nd3.2. 1, *EhgMx14*; 2, *EhgMx14*+pLA-Nc1.7; 3, *EhgMx14*+pLA-Nd3.2; and 4, *Ehg824-1*.

synthesized by an expression vector, has a molecular mass of about 72 kDa. The observations that HsvG is predominantly hydrophilic, lacks any membrane-spanning domains, and lacks a cleavable signal peptide, preclude the possibility that it constitutes a membrane protein or that it is being secreted through the type II *sec*-dependent secretion system (Salmond

and Reeves 1993). An interesting finding is the detection of a conserved motif (“*hrp* box”) upstream of *hsvG* that may be recognized by HrpL (Fig.2). *hrpL* functions as a positive regulatory gene in directing the expression of *hrp* genes (Xiao et al. 1994; Wei and Beer 1995). It is a member of the ECF (extra-cytoplasmic functions) subfamily of eubacterial RNA polymerase σ factors that recognize consensus promoter sequences. A comparison of the ECF subfamily σ -dependent putative promoter sequences of *hrp* genes revealed a striking similarity at the motif corresponding to the -35 region (5'-GGAACC-3') but an absence of clear similarity at the corresponding -10 recognition region (Wei and Beer 1995). Similar results were obtained with *hsvG* (Fig. 2). Whether the conserved motif identified upstream of *hsvG* truly forms an HrpL responsive promoter sequence should await further studies. All the above-mentioned features of *hsvG* are reminiscent of the *avr* genes that have been linked to the activities of the *hrp* cluster (Leach and White 1996) and that have recently been

Table 1. Effect of insertional mutations on pathogenicity of *Erwinia herbicola* pv. *gypsophila* and *E. herbicola* pv. *betae* on gypsophila and beet

Bacterial strains and mutants	Pathogenicity on gypsophila	Pathogenicity on beet
<i>Ehg</i> 824-1 (wild type)	+	–
<i>Ehg</i> Mx(#) ^a	–	–
<i>Ehb</i> 4188 (wild type)	+	+
<i>Ehb</i> Mx(#)	–	+

^a *Ehg*Mx(#) (# = 14, PI, PII) and *Ehb*Mx(#) (# = 14, PI, PII) are insertional Tn3-Spice mutants of *Ehg*824-1 and *Ehb*4188, respectively.

<i>Ehg</i>	MWNNINPYGRLPVNSRGADETPPAADPHPQGTAGGTPPLRDDPGAAPYPEDEVLTWEVFC	60
<i>Ehb</i>	MWNNINPYGRLPVNSRGADETPPAADPHPQGTAGGTPPLRDDPGAAPYPEDEVLTWEVFC	60
<i>Ehg</i>	CATAGRTRQQVASDYGWALRRFSGFLSLNGLPALSAPGRLQEPDRLRRDAHVFIERFHES	120
<i>Ehb</i>	CATAGRTRQQVASDYGWALRRFSGFLSLNGLPALSAPGRLQEPDRLRRDAHVFIERFHES	120
<i>Ehg</i>	GRQKNLI PALNRLTELAASGEDSITIRGSQRPRCLKVPDADGALIKGAFFPGLPAGPGRDIY	180
<i>Ehb</i>	GRQKNLI PALNRLTELAASGEDSITIRGSQRPRCLKVPDADGALIKGAFFPGLPAGPGRDIY	180
<i>Ehg</i>	SVNLRNAARSLSAWLHENGRLPGLSDTAYLFSEQAGADAGAFSRAHPANAGRNVNMLAHLR	240
<i>Ehb</i>	SVNLRNAARSLSAWLHENGRLPGLSDTAYLFSEQAGADAGAFSRAHPANAGRNVNMLAHLR	240
<i>Ehg</i>	AHARGEVPHVGRLQNTRTIPLADQRLALAYLGIKDLARQAGTVYKKNSNGHDSIDKKAS	300
<i>Ehb</i>	AHARGEVPHVGRLQNTRTIPLADQRLALAYLGIKDLARQAGTVYKKNSNGHDSIDKKAS	300
<i>Ehg</i>	QLRSFSAWRTARGLPALTDHLHDPGLQMDMDMYTEDKYQRSQTNGRSAGSSTARSARSRL	360
<i>Ehb</i>	QLRSFSAWRTARGLPALTDHLHDPGLQMDMDMYTEDKYQRSQTNGRSAGSSTARSARSRL	360
<i>Ehg</i>	SSALKTLQTRFPFGTPVTVPPEAPAESFGLPGSDWSGWMSLSVPGADGGPAPADSDSVFG	420
<i>Ehb</i>	SSALKTLQTRFPFGTPVTVPPEAPAESFGLPGSDWSGWMSLSVPGADGGPAPADSDSVFG	420
<i>Ehg</i>	GLAPLDSRERFSSDGLSGALAPGLPETGEVNRVPPEAPAESFALPGSDWSGWGMSLSLGG	480
<i>Ehb</i>	GLAPLDSRERFSSDGLSGALAPGLPETGEVNRVPPEAPAESFALPGSDWSGWGMSLSLGG	480
<i>Ehg</i>	HGPADSDSVFGGLAPLDSRERFSSGGLSGTPASGLPETGEVNRPGPDGAPFVSMRDAVPP	540
<i>Ehb</i>	YGPADSDSVFGGLAPLDSRERFSSGGLSGTPASGLPETGEVNRPGPDGAPFVSMRDAVPP	540
<i>Ehg</i>	FRPDGVGFASGEGLSCLLDSILQGYHNIRRGHGAPRGLTDWLDGEVRRVREALSAQGVDL	600
<i>Ehb</i>	FRPDGVGFASGEGLSCLLDSILQGYHNIRRGHGAPRGLTDWLDGEVRRVREALSAQGVDL	600
<i>Ehg</i>	VPARGEIDYGGPGSYLAGAMGLRLQVIOAEFQEEP DGTYVRYTAHPEIGEAGGRAVRL	660
<i>Ehb</i>	VPARGEIDYGGAGSYLAGAMNVRQLQVIOAEFQEEP DGTYVRYTSHPEMGRSDAPMVR	660
<i>Ehg</i>	HTPGHFQPLWG*	672
<i>Ehb</i>	HTPGHFQPLWG*	672

Fig. 7. Alignment of deduced amino acid sequences of *hsvG* from *Ehg*824-1 and *Ehb*4188. Different amino acid residues are boxed.

shown to deliver the Avr proteins directly into the host plant cell through the type III secretion system (Gopalan et al. 1996; Leister et al. 1996; Van den Ackerveken et al. 1996).

Evidence obtained from restoration of pathogenicity to insertional mutants in *hsvG* as well as from the phenotypic expression of these mutants in gypsophila cuttings suggests that, although the entire HsvG protein may be needed for full virulence, its C-terminal region is not essential for expressing partial virulence. Thus, restoration of mutants with pLA-Nc1.7, which contained only 70% of the *hsvG* ORF (starting from the translation start codon), resulted in formation of galls, albeit smaller galls than resulted from restoration of mutants with the full ORF (Fig. 5). Additional confirmation of the dispensability of the C-terminal region could be gained from studies on crown appearance at the foot base of gypsophila cuttings following inoculation with an excessive concentration of the nonpathogenic mutants (Fig. 4). The size of the crown was correlated with the distance of the mutant's site from the start of the ORF. It is also noteworthy that most of the sequence alterations in the deduced proteins of *hsvG* in *Ehg*, compared with *Ehb*, were detected in the C-terminal region, whereas the N-terminal region remained almost identical in the two pathovars (Fig. 7).

The crown phenomenon observed in gypsophila cuttings during the phenotypic expression of mutants is intriguing and deserves further consideration. At the outset it should be emphasized that the conditions used for crown formation were highly artificial, namely, inoculation of gypsophila cuttings with bacterial concentration at least three orders of magnitude higher than that required for gall formation by the wild-type strain. Although the mechanism that triggers crown development is not understood, it might be speculated that it is incited by the disrupted HsvG, which acts as an elicitor. Accordingly, we suggest that a rudimentary binding activity to a hypothetical plant receptor is still retained by the modified elicitor and is capable of eliciting a plant morphogenic response, which leads to crown rather than gall formation. The extent of the elicitation activity appears to be influenced by the site of the insertional mutant on the ORF as described above.

Biotrophic microbial pathogens generally appear to be limited in their interactions to a particular plant species, genus, or family, or, occasionally, to members of different families. Two concepts, which are not necessarily mutually exclusive, have been proposed to determine the host range of plant-pathogenic bacteria. The first is based on host range restriction by determinants that confer incompatibility. This view has mainly evolved from numerous studies on the function and extent of the role(s) that *avr* genes play in plant-microbe interactions (Dangl 1994; Leach and White 1996). Dominant *avr* genes were originally defined in the context of race- and cultivar-specific interactions and were shown to require the presence of specific resistance (R) genes in the host. These negative (incompatible) interactions were termed gene-for-gene interactions (Keen 1990). However, it has also been demonstrated that single cloned *avr* genes from a pathogen of one host species can cause an otherwise virulent pathogen of another host species to become avirulent on its own host (Whalen et al. 1988; Kobayashi et al. 1989; Whalen et al. 1991; Dangl 1992; Fillingham et al. 1992; Swarup et al. 1992). These data indicated that *avr* genes not only limit bacterial host range within a host plant species but many are also genetically dominant

determinants in limiting the host range of phytopathogenic bacteria on multiple plant species (Keen 1990; Dangl 1994). Interestingly, although the *hrp*-encoded harpins that elicit HR on nonhost plants could be considered candidates for host restriction, their contribution to host range determination is not clear (Alfano and Collmer 1996). It should also be pointed out that the negative-acting factors involved in host range determination have been demonstrated to be superimposed on *hrp* genes that are responsible for "basic pathogenicity" (Leach and White 1996).

An alternative view of host range determination, based on positive-functioning *hsv* genes, has been proposed (Gabriel 1989). This notion was inferred from the primary function of positive-acting, host-specific *nod* genes of *Rhizobium* 1996) and from the argument that the role of *avr* genes in nonhost incompatibility is generally gratuitous, so that they may not be suitable to evolve as primary determinants of host specificity (Gabriel 1989; Waney et al. 1991). However, evidence supporting the role of positive-acting virulence genes in host range determination of phytopathogenic bacteria has been very scanty. Such genes have so far been demonstrated only in *Pseudomonas syringae* pv. *tabaci* for host specificity on tobacco (Salch and Shaw 1988), in *Ralstonia solanacearum* for host specificity on peanut (Ma et al. 1988), and in genes conferring host specificity in *Xanthomonas campestris* pv. *translucens* for various graminaceous host genera (Waney et al. 1991). Although all the above-mentioned reports clearly demonstrated the presence of positive-acting, *hsv* genes, in none of these studies had any of these putative *hsv* genes been characterized.

We hypothesize that host specificity of *Ehg* and *Ehb* may be determined either by *hsv* genes only or by interaction between *hsv* and *avr* genes. Thus, the differing host ranges of the beet and gypsophila pathovars can be assigned to the presence of two different *hsv* genes, specific for beet and gypsophila, respectively, in *Ehb* and the absence of the beet-specific *hsv* in *Ehg*. Alternatively, each pathovar may contain both the gypsophila-specific and beet-specific *hsv* genes, while *Ehg* possesses a heterologous *avr* that causes this pathovar to become incompatible with beet, so that the expression of the beet-specific *hsv* is suppressed. Experiments are now in progress to distinguish between these two alternatives.

As indicated above, gall initiation by *Ehg* has been shown to be independent of IAA and cytokinin production by the pathogen. It has been reported that host-specific Nod factors of *Rhizobium* spp. act as species-specific morphogens of plants and cause initiation of host root cortical cell division and root hair cell growth (Long 1996). It is tempting to speculate that the binding of HsvG to a host-plant receptor leads to production of morphogens that cause hypertrophy and gall initiation. Finding the presumed receptor of HsvG in gypsophila would be most useful in understanding the mechanism of gall formation.

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 *Escherichia coli*. Genetic manipulations 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 (Sp), 50; rifampicin (Rif), 100; and tetracycline (Tc), 15.

Pathogenicity tests.

Pathogenicity tests on cuttings and seedlings of *Gypsophila paniculata* var. *perfecta* were essentially as described previously (Manulis et al. 1991a; 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 10^7 CFU/ml and placed in vermiculite-filled trays. The greenhouse temperature was maintained at 26 to 29°C and high humidity was generated by computer-controlled mist sprinklers as described elsewhere (Lichter et al. 1995a). Gall formation was scored 14 days after inoculation on three or four replicas per strain, in at least three independent experiments. Pathogenicity was also performed on 5-week-old gypsophila seedlings grown under a shed net. Inoculation was carried out by injecting 0.05 ml of bacterial

suspension with a fine hypodermic needle, through the apex of the seedling and into the stem. Three seedlings per strain were inoculated, and results were obtained from two independent experiments. Symptoms were evaluated according to an arbitrary scale 1 month after inoculation (Lichter et al. 1995a).

Pathogenicity tests on table beet and beet seedlings were performed similarly 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 blocks, each of which was placed on sterile 1.5% water agar in a petri dish. Inoculation was carried out with an overnight culture grown on LB agar by puncturing the bacteria into the top of the cube with a sterile toothpick. The dishes were incubated for 5 days at 28°C and then tested for gall formation. Three beet blocks 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* var. Red Egyptian) grown on vermiculite. Inoculation was carried out by puncturing the stem with a sterile nee-

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>mrcA</i> Δ (<i>mrr hsdRMS-mcrBC</i>) ϕ 80 <i>dlacZ</i> Δ M15 <i>lacX74 deoR recA1 araD139</i> Δ (<i>ara leu</i>)7697 <i>galU</i> <i>galK</i> λ^- <i>rpsL endA1 nupG</i>	GIBCO BRL, Gaithersburg, MD
HMS174(DE3) <i>Erwinia stewartii</i>	F ⁻ <i>recA hsdR</i> (rK12 ⁻ mK12 ⁺) Rif ^r (DE3). nal ^r , wild-type pathogenic strain DC283	Novagen, Madison, WI D. Coplin, Ohio State University, U.S.A.
<i>Erwinia amylovora</i> <i>Xanthomonas campestris</i> pv. <i>campestris</i> <i>Ralstonia solanacearum</i>	Wild-type pathogenic strain 209 Wild-type pathogenic strain 418 Wild-type pathogenic strain 1455	S. Manulis, Volcani Center, Israel S. Manulis, Volcani Center, Israel E. Levi, Ministry of Agriculture, Israel
<i>Pseudomonas syringe</i> pv. <i>savastanoi</i>	Wild-type pathogenic strain ITM318	N. S. Iacobellis, University of Bari, Italy
<i>Erwinia herbicola</i> pv. <i>gypsophila</i> (Ehg) Ehg824-1 Ehg3-1a Ehg350	Wild-type pathogenic strain with Rif ^r , gypsophila pathovar, serotype I Wild-type pathogenic strain, gypsophila pathovar, serotype I Wild-type pathogenic strain with Rif ^r , gypsophila pathovar, serotype II	Manulis et al. 1991a Manulis et al. 1991a Manulis et al. 1991a
Eh3-1, Eh717-2, Eh24-8 EhgMx(#)(# = 4, 14, 24, A30, B10, B35, B45, PI, PII) EhgMx19	Wild-type nonpathogenic strains, serotype I Amp ^r , Sp ^r , Sm ^r , marker exchange mutants on the pathogenicity-associated plasmid pPATH of Ehg Amp ^r , Sp ^r , Sm ^r , marker exchange mutant of insertional Tn3-Spice in the <i>hrp</i> cluster	Manulis et al. 1991a This study Nitzan et al. 1997
<i>Erwinia herbicola</i> pv. <i>betae</i> (Ehb) Ehb1188 Ehb4188 EhbMx(#)(# = 14, PI, PII)	Wild-type pathogenic strain, beet pathovar Wild-type pathogenic strain with Rif ^r , beet pathovar Amp ^r , Sp ^r , Sm ^r , marker exchange mutants on the pathogenicity-associated plasmid pPATH of Ehb	Burr et al. 1991 Burr et al. 1991 This study
Cosmids pLAFR3 pLA150 pLA-Nc1.7 pLA-Nd3.2 pLE564 pLE-Nd3.2	Tc ^r , broad-host-range vector <i>IncP-1 rixRK2⁺ lacZa Tra⁻ Mob⁺ cos</i> Tc ^r , a 28-kb cosmid of pPATH in pLAFR3 Tc ^r , a 1.7-kb <i>NcoI</i> fragment of Ehg cloned into pLAFR3 Tc ^r , a 3.2-kb <i>NdeI</i> fragment of Ehg cloned into pLAFR3 Tc ^r , a 27-kb cosmid of pPATH of Ehb in pLAFR3 Tc ^r , a 3.2-kb <i>NdeI</i> fragment of Ehb cloned into pLAFR3	Staskawicz et al. 1987 Lichter et al. 199b This study This study D. Ezra, Volcani Center, Israel This study
Plasmids pBluescript pRK2073 pTn3-Spice pUC4K pET11a pMBV-Nc1.7 pMBV-Nd3.2 pMBV-Nd3.2b pET2K	Amp ^r , subcloning and sequencing vector, KS ⁺ (pBS) Sp ^r , Sm ^r , helper plasmid for triparental matings Amp ^r , Sp ^r , Sm ^r , clone containing the <i>inaZ</i> reporter gene in Tn3 Km ^r , Amp ^r , Km gene cassette used for gene disruption Amp ^r , plasmid for protein over expression Amp ^r , a 3.2-kb <i>NdeI</i> fragment cloned into pBS Amp ^r , a 1.7-kb <i>NcoI</i> fragment cloned into pBS Amp ^r , a 3.2-kb <i>NdeI</i> fragment of Ehb cloned into pBS Amp ^r , a 2-kb fragment from Ehg pPATH in pET11a	Stratagene, La Jolla, CA Ditta et al. 1980 Lindgren et al. 1989 Pharmacia, Uppsala, Sweden Novagen This study This study This study This study

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

dle and applying the bacteria to the wound with a toothpick. The appearance of galls was evaluated after 10 days.

DNA manipulations.

Isolation of plasmid DNA from *E. coli*, restriction enzyme digestions, subcloning, transformations, Southern blot analysis, and PCR were carried out by standard procedures (Sambrook et al. 1989) or as recommended by the suppliers. Isolation of plasmid and genomic DNA from *E. herbicola* was performed as described previously.

DNA sequencing.

A 3.2-kb *NdeI* fragment of pLA150 (Fig. 1) was subcloned into pBS (KS⁺) to yield pMBV-Nde3.2. Generation of unidirectional deletions of the fragment was obtained with the "Erase a Base" kit (Promega, Madison, WI). Screening for clones with 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 the dideoxy chain termination method with the Sequenase 2.0 kit (U.S. Biochemical, Cleveland, OH) and α -³⁵S-dATP (Du Pont, NEN, Boston). The sequence was completed by constructing extra subclones with the restriction enzymes *NcoI*, *PstI*, *KpnI*, and *SacII* for *Ehg* (Fig. 2) and *PstI*, *SacI*, *SacII*, and *PvuII* for *Ehb*. This was done by automated sequencing with *Taq* DNA polymerase (Department for Biological Services, Weizmann Institute, Rehovot, Israel). DNA for sequencing was prepared with Wizard Minicolumns (Promega, Madison, WI). Analysis of sequence data for the DNA and deduced protein sequences was done according to the Program Manual for the Wisconsin Package (Version 9, Dec. 1996, Genetic Computer Group, Madison, WI).

Construction of mutants and marker exchange.

Cosmid libraries of plasmid DNA from *Ehg*824-1 and *Ehb*4188 in pLAFR3 were available from previous studies (Lichter et al. 1995a; D. Ezra, *unpublished*). Insertional mutagenesis was carried out on cosmid clones with the transposon reporter pTn3-Spice as described elsewhere (Lindgren et al. 1989). 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 as described above.

The location and orientation of the Tn3-Spice in the non-pathogenic mutants was determined by mapping with multiple restriction enzymes and hybridization with the following two probes: a 1.5-kb *BamHI* fragment derived from Tn3-Spice, and a 1.7-kb *NcoI* fragment obtained from pLA150 (Fig. 1). Additional confirmation of the insert location in the mutants was obtained by PCR, with primers based on pLAFR3 and Tn3-Spice for mutated inserts in *E. coli* and primers based on Tn3-Spice and the 3.2-kb *NdeI* fragment for *E. herbicola* (Table 3). Primers were chosen according to the expected location and orientation of the Tn3-Spice insertion in the examined fragment, which were determined by Southern hybridization with the above-mentioned probes.

Site-directed insertional mutagenesis was performed by introducing the kanamycin-resistance gene cassette (KM cassette) into the two *PstI* sites of the 1.7-kb *NcoI* fragment in pMBV-Nc1.7 (Fig. 1). Mobilization of the constructs to the wild-type strain and plasmid curing were carried out as described above. These two marker exchange mutants were designated as *Ehg*MxPI and *Ehg*MxPII (Fig. 1). Verification of the insert location in these mutants was performed by digesting the DNA with *NcoI* followed by Southern hybridization, with the 1.7-kb *NcoI* fragment used as a probe.

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

The T7 promoter-driven system pET (Novagene, Madison, WI) was employed to express the HsvG protein. The ORF of *hsvG* was amplified by PCR with primers 222 and 1252 (Table 3), which were designed on the basis of the 5' and 3' ends, respectively, of this gene (Fig. 2), with an appropriate restriction enzyme sites for cloning. The "Expand High-Fidelity PCR system" (Boehringer, Mannheim, Germany) was used to amplify the predicted ORF (2 kb) of *hsvG*. The amplified fragment was digested with *NdeI* and *BamHI* and cloned into pET11a through the same restriction sites to form pET2K. The recombinant plasmid and the vector were transformed into *E. coli* HMS174 (DE3). For protein expression, a fresh culture of the transformed *E. coli* (pET2K) 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 800 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

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

Primer	Sequence	Source	Position	Direction
500	5'-AGAGGCGTCAGAGGCAG-3'	pTn3-Spice	64 to 48 ^b	Upstream
367	5'-TCTCATGACCAATCCC-3'	pTn3-Spice	10677 to 10694	Downstream
53	5'-CAAGGCGATTAAGTTGGG-3'	pLAFR3	333 to 316 ^c	Upstream
54	5'-GAATTGTGAGCGGATAAC-3'	pLAFR3	177 to 194	Downstream
lea1	5'-GGGAATTCAACCACTACAAAGTGTCC-3'	pLA-Nd3.2	160 to 180 ^d	Downstream
651	5'-CTTTCTCTGACGGACTTCC-3'	pLA-Nd3.2	1713 to 1734	Downstream
769	5'-GAGTGAGTAAGCCAGATACC-3'	pLA-Nd3.2	2648 to 2628	Upstream
4148	5'-TTAACTGAGAAGCTTTTAGTCAATGGAGTCATGGCCGTTT-3'	pLA-Nd3.2	1340 to 1290	Upstream
35107	5'-GTGGCCAGTGAATTCGGATGGGCCCTGCGCAGGTT-3'	pLA-Nd3.2	634 to 668	Downstream
1072	5'-GCCGTCGACTCCGCCGTTGCCTCC-3'	pLA-Nd3.2	1582 to 1563	Upstream
222	5'-GCGAATTCATATGGTGGAAACAACATTAACCCG-3'	pLA-Nd3.2	424 to 444	Downstream
252	5'-CGGGATCTCACCCAGAGCGGC-3'	pLA-Nd3.2	2439 to 2424	Upstream

^a The primers were synthesized by Biotechnology General, Nes Ziona, Israel.

^b The position is from the transcription initiation of pTn3-Spice.

^c The position is from the *lac*-operon of pLAFR3.

^d The position is from the beginning of the 3.2 *NdeI* fragment of *Ehg*.

E. coli (pET2K) without IPTG, *E. coli* (pET11a) in the presence and absence of IPTG, and nontransformed *E. coli* were used as controls. The samples were heated at 100°C for 3 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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