Brazilian Journal of Microbiology 46, 3, 929-936 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246320140655

Research Paper

Characterization of the *hrpZ* gene from *Pseudomonas syringae* pv. *maculicola* M2

César Álvarez-Mejía[1], Dalia Rodríguez-Ríos[2], Gustavo Hernández-Guzmán[3], Varinia López-Ramírez[4], Humberto Valenzuela-Soto[5], Rodolfo Marsch[6]

[1]Instituto Tecnológico Superior de Irapuato Plantel Abasolo, Guanajuato, México.
[2]Departamento de Ingeniería Genética de Plantas, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Guanajuato, México.
[3]División de Ciencias de la Vida, Universidad de Guanajuato, Guanajuato, México.
[4]Instituto Tecnológico Superior de Irapuato, Guanajuato, México.
[5]Departamento de Plásticos en Agricultura, Centro de Investigación en Química Aplicada, Coahuila, México
[6]Departamento de Biotecnología y Bioingeniería, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, D.F. México, México.

Submitted: August 2, 2014; Approved: November 16, 2014.

Abstract

Pseudomonas syringae pv. *maculicola* is a natural pathogen of members of the Brassicaceae plant family. Using a transposon-based mutagenesis strategy in *Pseudomonas syringae* pv. *maculicola* M2 (PsmM2), we conducted a genetic screen to identify mutants that were capable of growing in M9 medium supplemented with a crude extract from the leaves of *Arabidopsis thaliana*. A mutant containing a transposon insertion in the *hrpZ* gene (PsmMut8) was unable to infect adult plants from *Arabidopsis thaliana* or *Brassica oleracea*, suggesting a loss of pathogenicity. The promotorless *cat* reporter present in the gene trap was expressed if PsmMut8 was grown in minimal medium (M9) supplemented with the leaf extract but not if grown in normal rich medium (KB). We conducted phylogenetic analysis using *hrpAZB* genes, showing the classical 5-clade distribution, and nucleotide diversity analysis, showing the putative position for selective pressure in this operon. Our results indicate that the *hrpAZB* operon from *Pseudomonas syringae* pv. *maculicola* M2 is necessary for its pathogenicity and that its diversity would be under host-mediated diversifying selection.

Key words: *hrpZ*, mutant non-pathogenic, transmid, Tn*5*, phylogenetic.

Introduction

The majority of Gram-negative pathogenic bacteria are endowed with the type III secretion system, which is a highly conserved apparatus that exports proteins that are essential to induce disease (Deane *et al.*, 2006; Tang *et al.*, 2006; Mansfield, 2009). Exported proteins play an important role in disease development at the cellular level. In phytopathogenic bacteria, the apparatus is called the Hrp system and is encoded by the *hrp* gene cluster (*h*ypersensitivity *r*esponse and *p*athogenicity) (Alfano and Collmer, 2004; Block and Alfano, 2011), which is usually included in a pathogenicity island (Gropp and Guttman, 2004). The product of these genes is a structure resembling a straight flagellum (Jin *et al.*, 2001; Arnold *et al.*, 2011), of which the Hrp pilus contacts the plant cell surface during infection (Büttner, 2012). Two types of proteins are exported through the Hrp pili: the *avr* (*av*i*r*ulence) gene products and the "harpins," which are products of the *hrpZ* and *hrpW* genes (Reboutier and Bouteau, 2008; Schumacher *et al.*, 2014). Avr proteins appear to be injected into plant cells (Jin *et al.*, 2001; Fu *et al.*, 2006), where they modulate the cell metabolism to export nutrients to the apoplast (van Dijk *et al.*, 1999). In an incompatible interaction, the Avr protein is recognized by the product of a gene for resistance, *R*, which triggers the hypersensitive response and results in disease abortion (Mansfield, 2009). The harpins are

Send correspondence to R. Marsch. Departamento de Biotecnología y Bioingeniería, Cinvestav. Avenida I.P.N 2508, Colonia San Pedro Zacatenco, 07360. México, D.F. México. E-mail: rmarsch@cinvestav.mx.

encoded by *hrp* genes but are not included in the *hrp* pilus structure; instead, they are secreted into the medium or the apoplast, where they perform their activity. The function of harpins is not fully known (Choi *et al.*, 2013). There are contradictory reports regarding HrpZ being essential (He *et al.*, 1993) or not (Preston, 2000) for pathogenesis.

In this work, the function of the *hrpZ* gene from *P. syringae* pv. *maculicola* strain M2 (PsmM2) was interrupted using a transposable element promoter probe. The mutant strain was unable to infect adult plants from *Arabidopsis thaliana* or *Brassica oleracea*, indicating a complete loss of bacterial pathogenicity. The PsmM2 *hrpZ* gene is almost identical to its homolog in *Pseudomonas syringae* pv. *tomato* DC3000, suggesting that pathovars are conserved among distinct susceptible plant species. Our results suggest that *hrpZ* is an essential gene that is necessary for bacterial infection in plants.

Materials and Methods

Bacterial strains, plants and plasmids

Pseudomonas syringae pv. *maculicola* strain M2 (Rif^R) was a kind gift from Dr. Jeffrey L. Dangl (Ritter and Dangl, 1995), and PsmMut8 was obtained in this work. *E. coli* S17-1 λpir (*thi pro hsdR hsdM* Δ*recA* RP4-2*tra*Tc::Mu Km::Tn*7*) (de Lorenzo *et al.*, 1990) was obtained from Dr. Kate J. Wilson. *E. coli* DH5α competent cells (*supE44* Δ*lacU169* (f80 *lacZ* DM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) (Sambrook and Russell, 2001) were used for cloning experiments. A *Swa*I restriction site was added into the *Sma*I site on pUIRM504 (Marsch-Moreno *et al.*, 1998) to form the plasmid pMDC505 (unpublished results); with this change, the transposable element pTn*5cat* (Marsch-Moreno *et al.*, 1998) was modified into pTn*5cat*1. King's B medium (King *et al.*, 1954), minimal medium M9 (Sambrook and Russell, 2001) or M9CA (Difco) was used to culture *P. s. maculicola* strains and in the assay to determine the conditions for *cat* expression, with or without the additions described below. LB medium was used to culture the *E. coli* strains. Chloramphenicol, rifampicin and kanamycin were purchased from Serva or Sigma-Aldrich Chemicals.

Mutagenesis and mutant selection

Mutants of PsmM2 were generated using the transmid element pTn*5cat*1 according a published protocol (Marsch-Moreno *et al.*, 1998). *E. coli* S17-1 (pMDC505) was used to mobilize pTn*5cat*1 to PsmM2 by conjugation, and the bacteria were then spread onto M9 Rif_{50} Km_{50} plates. Mutants were screened for their ability to growth on M9 Cm_{50} with plant extract. To obtain crude plant extract, mature rosette leaves from 3-week-old *Arabidopsis thaliana* plants were frozen in liquid nitrogen and ground into a powder, which was then centrifuged at 13,000 rpm for 10 to 20 min. The liquid phase was recovered and added to the growth medium as an effector of pathogenesis.

Assay for promoter strength

The promoter strength was evaluated as the cell density after the bacteria were grown in a medium containing chloramphenicol (Alvarez-Mejia *et al.*, 2013). The assays were performed in sterile 96-well polystyrene plates. First, 50 μL of a 0.04-OD_{620} culture of mutant PsmMut8 in KB Km_{50} was added to wells containing 200 μL of M9, M9Ca or KB medium supplemented or not with plant extract (2 μL/mL) or sucrose (5%); all media contained kanamycin (50 μg/mL) and chloramphenicol (150 μg/mL). The plates were incubated at 28 °C, and the cell density was measured at 0, 24 and 48 h using a Titertek Multiskan Plus (EFLAB, Joint Venture Company of Lab System and Flow Laboratories) with a 492-nm filter.

Pathogenesis assays

To test the ability of the mutants to induce disease in *A. thaliana*, 3-week-old plants were inoculated by infiltration with mutant or wild type PsmM2 cell suspensions (~20 μL per leaf). The cell suspensions were prepared by growing PsmMut8 or PsmM2 in 5 mL KB, incubated at 28 °C overnight with strong shaking to reach an 0.4 of OD_{600}. Then, 3 mL were centrifuged at 14,000 rpm for 2 min at 4 °C (rotor: Sorvall SS34). The pellet was washed two times with sterile water, and the cells were resuspended in 3 mL of sterile distilled water. Leaves were inoculated with the undiluted cell suspension or with a 1:10 dilution.

Cloning and sequencing

Total PsmMut8 DNA was purified using a previously described method (Chen and Kuo, 1993). First, ten μg of DNA were completely digested using the restriction endonuclease *Eco*RI in a reaction volume of 50 μL. The enzyme was then inactivated at 65 °C for 20 min. Next, 1 μg of cut DNA was religated with T4 DNA ligase in a reaction volume of 50 μL at 28 °C for 4 h. The ligated DNA was then used to transform competent *E. coli* DH5α cells to become kanamycin resistant. To sequence the cloned chromosomal fragments, oligonucleotides 1212 (5'-GTGCCTGACTGC GTTA-3'; from the mob end), 1213 (5'-CCTTAGCTCCTGAAA-3': from the *cat* end), 1658 (5'-GTTGACCTACGTCAACGCTGGC-3'), 2176 (5-GTGTCGAACACCGAAAG-3 to sequence *hrpB*), and 2149 (5-TCTGAAGAGTGGCGTTGGAAGC-3 to sequence *hrpA*) were used. Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs, Inc. or Invitrogen. Enzymes were used following the suppliers' recommendations.

Bioinformatics analysis and alignment

hrpAZB operons from diverse *Pseudomonas* strains were retrieved from the GenBank database and used in our analysis (Table 1). Most of them had been used in a previous work (Inoue and Takikawa, 2006). Nucleotide polymorphism analysis was conducted using DnaSP (Rozas *et al.*, 2003), and the sliding window analysis for *hrpAZB* operon was conducted using 25 nt in a window of 50 nt only for unique *P. syringae* strains. Bioinformatics analysis was performed using the BLASTn program (Altschul *et al.*, 1990; Worley *et al.*, 1998), and alignments were performed using Clustal W and edited with BioEdit (Hall, 1999); *Pseudomonas viridiflava* and *Pseudomonas cichorii* were included as outgroups.

Table 1 - Strains used in the phylogenetic analysis. All of the data were retrieved from GenBank.

Bacteria	Strain	Accession
Pseudomonas syringae		
sesami	PSES-1	AB112563
lachrymans	cucum-1	AB112561
? "kiwi"	KW741	AB112559
eriobotryae	PERB8031	AB112557
oryzae	1-1.1	AB112580
coronafaciens	AVPCO8101	AB112578
aceris	kaede1-1	AB112576
japonica	BPST802	AB112574
striafaciens	avena2	AB112579
magnoliae	PMG8101	AB112570
theae	tea632	AB112568
mori	mori1	AB112562
morsprunorum	U7805	AB112560
myricae	yamamomo801	AB112558
dendropanacis	kakuremino-1	AB112556
pisi	Pisum-1	AB112577
phaseolicola	NPS3121	AB112552
tomato	DC3000	AF232004
	ICMP2844	AB112567
tagetis	LMG5090	DQ246442
aptata	SB8601	AB112575
tabaci	ATCC11528	FJ946987
lapsa	NCPPB2096	AB112573
actinidiae	KW11	AB112571
delphinii	PDDCC529	AB112569
maculicola	R1	AB112565
	M2	AY325899
	PMC8301	AB112566
glycinea	r0	AB112554
	race4	L41862
syringae	61	EF514224
	LOB2-1	AB112572
	ICMP3414	AB112581
Pseudomonas savastanoi		
savastanoi	5	FR717896
Pseudomonas		
viridiflava	RMX23.1a	AY597282
cichorii	SPC9018	AB433910
ficuserectae	L-7	AB112564

Results

Selection of PsmMut8

A collection of PsmM2 mutants harboring the pTn*5cat*1 transposon-based construct was screened for the induction of *cat* expression in M9 medium containing a plant extract (see the Materials and Methods section for details). A total of 14 candidates were identified by their ability to grow in M9 Km_{50} Cm_{150} because the reporter gene *cat* was induced by the plant extract. All of these mutants were tested in pathogenesis assays by inoculating *Arabidopsis* plants. Mutant number 8 (PsmMut8) was selected because it was unable to infect and cause disease symptoms or hypersensitivity reaction (HR) in either *A. thaliana* or *Brassica oleracea* (Figure 1).

Promoter expression detected in PsmMut8

The *cat* reporter gene in pTn*5cat*1 allows for the estimation of promoter expression under conditions that resemble those in the apoplast. The cell density in liquid media in the presence of chloramphenicol is associated with the resistance level to the antibiotic, suggesting that the measurement of cell density in the presence of chloramphenicol in different media (M9, M9Ca or KB) with or without the addition of plant extract or sucrose reflects the expression level of the detected promoter under these conditions (Alvarez-Mejia *et al.*, 2013). The cell density values of PsmMut8 growing in different media at 28 °C after 48 h are shown in Figure 2. The cell density was higher in M9 than in KB medium, suggesting that chloramphenicol resistance in response to the plant extract was increased in M9 but that casamino acids preclude the stimulatory effect of the plant extract. No different effects were observed in the assay with sucrose.

pTn*5cat*1 is inserted into a gene homologous to the *hrpZ gene of Pseudomonas syringae*

A 14-kb chromosomal fragment corresponding to the pTn*5cat*1 borders and their flanking genomic sequences were cloned, sequenced, and compared to the genomic information contained in GenBank. Both flanking sequences are homologous to the *hrpZ* gene from *Pseudomonas syringae* pv. *tomato* DC3000 (PstDC3000) (99% identity, six nucleotide substitutions over 1,110 bp, Figure 3A).

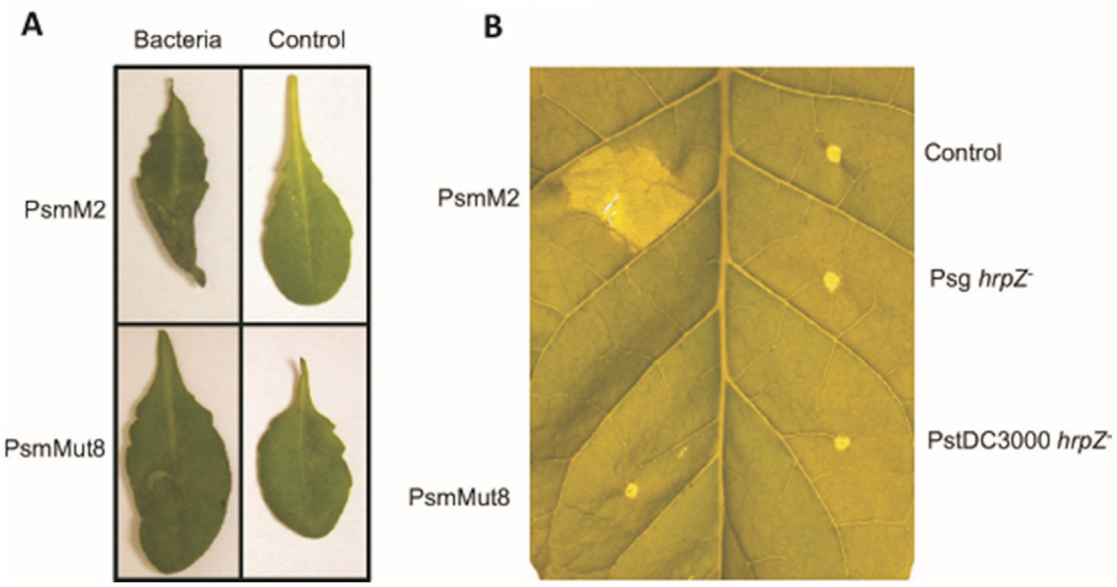

Figure 1 - Pathogenesis and HR assays for PsmM2 and PsmMut8. A. Arabidopsis leaves were infected by PsmM2 but not by PsmMut8. B. HR assay in collard leaves; PsmM2 but not PsmMut8 was able to produce HR, similarly to *hrpZ* from *Pseudomonas syringae* pv. *glycinea* (Psg) and *Pseudomonas syringae* pv. *tomato* DC3000 (PstDC3000). Phosphate buffer was used as a control.

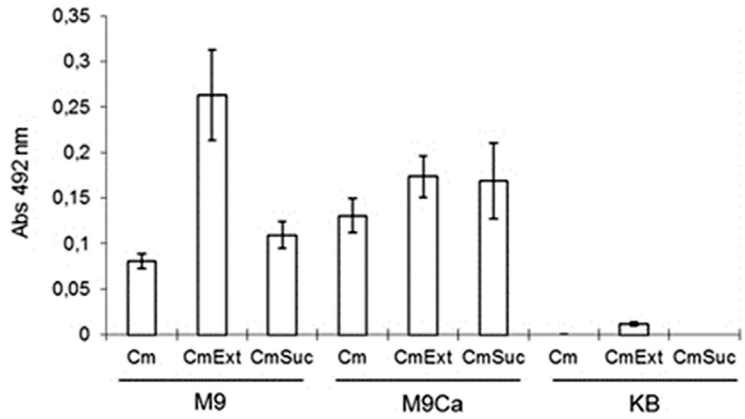

Figure 2 - PsmMut8 was cultured at 28 °C for 48 h in M9, M9Ca or KB medium. All of the media contained chloramphenicol (Cm, 150 μg/mL), and some of the media were supplemented with plant extract (Ext) or sucrose (Suc).

Alignment with other sequences reported in GenBank for HrpZ proteins revealed two shared regions between PsmM2 and PstDC3000, including genomic locations 102-125, IGAGGGGGGIGGAGSGSGVGGGLS, and 229-244, SGVTSGGGLGSPVSDS.

Additional sequences flanking pTn*5cat*1 are similar to the *hrp* genes of *Pseudomonas syringae* DC3000

To further investigate the location of the interrupted gene in PsMut8, we sequenced the regions upstream and downstream of *hrpZ*. All of the generated sequences corresponded to previously identified genes encoding Hrp proteins: *hrpS, hrpA, hrpZ, hrpB* and *hrpC* (Figure 3B). The first and the last open reading frames (ORFs) were only partially sequenced. A putative hrp box (GGAACCGATT CGCAGGCTGCTGCCACCTA) was identified in the 5' region of *hrpA* (Zwiesler-Vollick *et al.*, 2002), and a putative ribosome binding site (RBS) was identified within the *hrpA* gene. The 3'-UTRs of *hrpA* and *hrpZ* are predicted to fold into hairpin structures reminiscent of bacterial transcription terminators (TGAGTACCAAGCAATCACGCT GGTAAATCTTA and GCCCCCTCATCAGAGGGGGC, respectively). The presence of a putative RBS within the terminator suggests that the transcription of *hrpZ* proceeds independently of *hrpA*. To explore a possible conservation of the *hrpAZB* operon in different pathovars, including PsmM2 and PstDC3000, we conducted a phylogenetic analysis with 35 *Pseudomonas syringae* sequences; 2 dif-

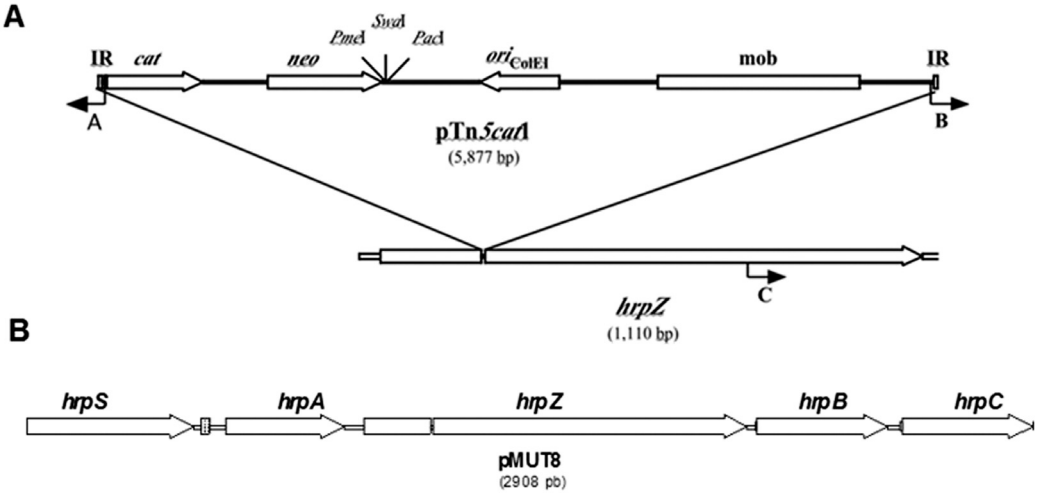

Figure 3 - A. The insertion of pTn*5cat*1 into the *hrpZ* gene. IR, inverted repeated; *cat*, chloramphenicol acetyltransferase; *neo*, neomycin phosphotransferase; ori_{ColE1}, replication origin type ColE1; mob, mobilization region from RP4. The *Pac*I, *Pme*I and *Swa*I restriction sites are shown. The angled arrows indicate the position of the oligonucleotides for the following sequences: A. 1213, 5'-TTTCAGGAGCTAAGG-3'; B. 1212, 5'-GTGCCTGACTGCGTTA-3'; and C. 1638, 5'-CGTGGTTTGCAGTCGGTTT-3'. B. The genes detected around pTn*5cat*1 are similar to *hrpS*, *hrpA*, *hrpB* and *hrpC* (GenBank accession number AY325899).

ferent *Pseudomonas* species were included as outgroups. Our analysis was based on maximum likelihood estimations and the Kimura two-parameter substitution model with 1000 bootstraps. Our results showed that PsmM2 belongs to phylogroup II, as described by Inoue (Inoue and Takikawa 2006), or group 5, as described by Guttman (Guttman *et al.*, 2006), and is closely related to the *tomato* pathovar, as well as to other *maculicola* strains (Figure 4). They also showed that nucleotide polymorphisms within the operon are particularly abundant in the *hrpA* gene and the 5 region of *hrpZ*, whereas polymorphisms are less abundant in the intergenic regions (Figure 5).

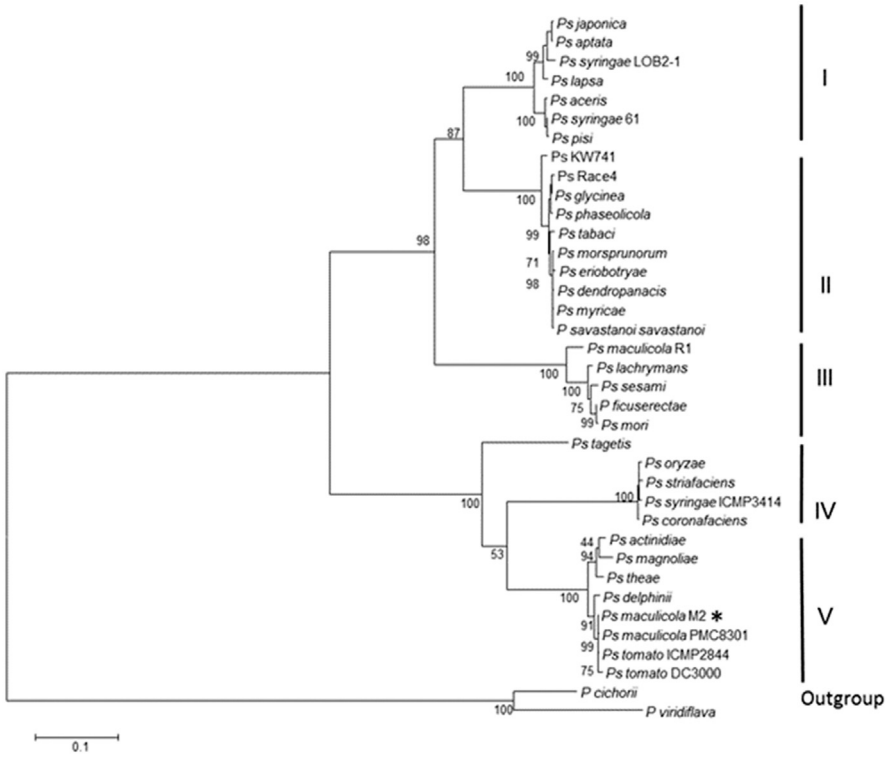

Figure 4 - Phylogenetic distribution of the *hrpABZ* operon by the maximum likelihood method. PsmM2 is located in clade V, and *P. s. tagetis* is basal to clades IV and V.

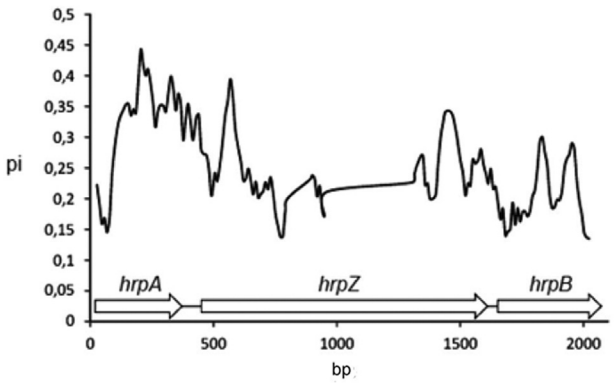

Figure 5 - Nucleotide polymorphism analysis (pi) for the *hrpAZB* operon. Only sequences from *Pseudomonas syringae* pathovars were used. bp, base pair.

Discussion

The use of a Tn*5* derivative carrying suitable reporter genes has allowed for the isolation of bacterial genes that are responsive to a variety of environmental conditions (Haapalainen *et al.*, 2012). As a means to simulate conditions prevalent in the apoplast (low osmotic pressure, low pH, and the absence of amino acids, polysaccharides and phenolic compounds), we used a transposon-based element to isolate mutants showing high expression levels of the *cat* gene in the presence of plant extract or minimal medium (Marsch-Moreno *et al.*, 1998). In selected mutants, rich medium partially blocked *cat* expression. Analogous to this observation, rich medium containing a nitrogen source has been shown to negatively regulate *hrpL*, a transcriptional regulator of *hrpRS*, indicating a possible regulatory role mediated by operons with an *hrp* box in their promoter sequence (Jovanovic *et al.*, 2011). This regulation is antagonistic to those mutants prevailing in minimal medium enriched with plant extracts, which was shown to induce the expression of *gacS*, a positive regulator of *hrpL* (Chatterjee *et al.*, 2003). Our conditions are similar to those that induce the activity of other pathogenicity genes such as *avr*, *hrp*, and *argK*, as well as the expression of genes involved in the synthesis of coronatine, syringomycin and phaseolotoxin (Rahme *et al.*, 1992; Palmer and Bender, 1993; Budde *et al.*, 1998; Zwiesler-Vollick *et al.*, 2002; Ortiz-Martin *et al.*, 2010). The incubation of *P. syringae* at a low temperature and low pH can also induce the activity of *hrp* genes, suggesting that the global activity of genes involved in pathogenesis is correlated with the activity of genes involved in the stress response (Hauser, 2009). The natural conditions that are necessary for the expression of the promoter detected in PsmMut8 resemble those described above. The expression of a detected regulatory sequence was also stimulated in M9 medium. Casamino acids have two effects: on one hand, they facilitate growth and partially circumvent the necessity of synthesizing amino acids; on the other, they inhibit the stimulation of transcription by plant metabolites (Schumacher *et al.*, 2014). On the basis of our results, the regulation of the *hrpZ* promoter can be predicted to respond to environmental conditions and to diverse metabolites that depend on the presence of amino acids (Schumacher *et al.*, 2014). Our results also show that our assay could serve as a probe to searching for specific plant metabolites capable of inducing the expression of genes related to pathogenesis in *P. syringae*.

The transposon insertion in PsmMut8 interrupts the function of a gene homologous to *hrpZ* from *P. s. tomato* DC3000. Its sequence is distinct from other reported HrpZ proteins by 28 glycine-rich peptide residues that are absent in most family members; however, the percentage of similarity among family members is high (99.5%), and the divergence is small (0.5%). Figure 4 shows the phylogenetic structure of the *hrpAZB* operon between *Pseudomonas* species. The distribution from 35 pathovars is similar to that reported by Guttman and Inoue in five phylogroups (Inoue and Takikawa, 2006; Guttman *et al.*, 2006). The operon *hrpAZB* belongs to phylogroup II, sharing features with the *tomato* and *maculicola* pathovars. The nucleotide polymorphism analysis shows that *hrpA* is the most diverse gene (Figure 5), as was reported by Guttman (Guttman *et al.*, 2006). This gene appears to be under positive selection compared with *hrpZ* and *hrpB*, suggesting a possible role of this gene during the fast co-evolution of host-pathogen interactions (Gropp and Guttman 2004; Mansfield 2009). Additionally, a possible interaction of the HrpZ hairpin and the N-terminal region of HrpA could be related to the nucleotide sequence of the 5 *hrpZ* region. Regions with low numbers of nucleotide polymorphisms include the hrp box, the RBS region, and the putative translational signal regions of each gene detected in this work. It is not surprising that PstDC3000 has been included as a member of the *maculicola* pathovar, and, similarly to PsmM2, PstDC3000 is capable of infecting *A. thaliana* (Bao *et al.*, 2014). It has been previously described that *hrpZ* is not essential for pathogenesis in *P. s. tomato* or *syringae*. Although our results indicate that PsmMut8 is non-pathogenic, based on the location of transcription termination sequences around the replication origin and the *pas* sites, it is possible that the insertion of the transposable element resulted in a polar mutation (Balbas *et al.*, 1986). Additional experiments will be necessary to explore the function of *hrpB* or the importance of *hrpZ* in the control of pathogenesis (Accession number AY325899).

Acknowledgments

We would like to acknowledge Dr. Jeff Dangl for providing the PsmM2 strain, as well as Dr. Carol Bender at Oklahoma State University for her support with the pathogenesis assays and plants. This work was supported by grants from Consejo Nacional de Ciencia y Tecnología

(Conacyt No. 28539-N) and Consejo de Ciencia y Tecnología del Estado de Guanajuato (Concyteg).

References

Alvarez-Mejia C, Hernandez-Guzman G, Curiel-Quesada E *et al.* (2013) The use of cell density to estimate the bacterial promoter strength. J Pure Appl Microbiol 7:1743-1748.

Alfano JR, Collmer A (2004) Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu Rev Phytopathol 42:385-414.

Altschul SF, Gish W, Miller W *et al.* (1990) Basic local alignment search tool. J Mol Biol 215:403-410.

Arnold DL, Lovell HC, Jackson RW *et al.* (2011) *Pseudomonas syringae* pv. *phaseolicola*: from 'has bean' to supermodel. Mol Plant Pathol 12:617-627.

Balbas P, Soberon X, Merino E *et al.* (1986) Plasmid vector pBR322 and its special-purpose derivatives—a review. Gene 50:3-40.

Bao Z, Stodghill PV, Myers CR *et al.* (2014) Genomic Plasticity Enables Phenotypic Variation of *Pseudomonas syringae* pv. *tomato* DC3000. PLos One 9:e86628.

Block A, Alfano JR (2011) Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? Curr Opin Microbiol 14:39-46.

Büttner D (2012) Protein export according to schedule: architecture, assembly, and regulation of type III secretion systems from plant- and animal-pathogenic bacteria. Microbiol Mol Biol Rev 76:262-310.

Chatterjee A, Cui Y, Yang H *et al.* (2003) GacA, the response regulator of a two-component system, acts as a master regulator in *Pseudomonas syringae* pv. *tomato* DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. Mol Plant Microbe Interact 16:1106-1117.

Chen WP, Kuo TT (1993) A simple and rapid method for the preparation of Gram-negative bacterial genomic DNA. Nucl Acids Res 21:2260.

Choi MS, Kim W, Lee C *et al.* (2013) Harpins, multifunctional proteins secreted by Gram-negative plant-pathogenic bacteria. Mol Plant Microbe Interact 26:1115-1122.

Deane JE, Roversi P, Cordes FS *et al.* (2006) Molecular model of a type III secretion system needle: Implications for host-cell sensing. Proc Natl Acad Sci USA 103:12529-12533.

de Lorenzo V, Herrero M, Jakubzik U *et al.* (1990) Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. J Bacteriol 172:6568-6572.

Gropp SJ, Guttman DS (2004) The PCR amplification and characterization of entire *Pseudomonas syringae hrp*/*hrc* clusters. Mol Plant Pathol 5:137-140.

Guttman DS, Gropp SJ, Morgan RL *et al.* (2006) Diversifying selection drives the evolution of the type III secretion system pilus of *Pseudomonas syringae*. Mol Biol Evol 23:2342-2354.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95-98.

Haapalainen M, Dauphin A, Li CM *et al.* (2012) HrpZ harpins from different *Pseudomonas syringae* pathovars differ in molecular interactions and in induction of anion channel responses in *Arabidopsis thaliana* suspension cells. Plant Physiol Biochem 51:168-174.

Hauser AR (2009) The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat Rev Microbiol 7:654-665.

Inoue Y, Takikawa Y (2006) The hrpZ and hrpA genes are variable, and useful for grouping *Pseudomonas syringae* bacteria. J Gen Plant Pathol 72:26-33.

Jin Q, Hu W, Brown I *et al.* (2001) Visualization of secreted Hrp and Avr proteins along the Hrp pilus during type III secretion in *Erwinia amylovora* and *Pseudomonas syringae*. Mol Microbiol 40:1129-1139.

Jovanovic M, James EH, Burrows PC *et al.* (2011) Regulation of the co-evolved HrpR and HrpS AAA+ proteins required for *Pseudomonas syringae* pathogenicity. Nat Commun 2:177.

Mansfield JW (2009) From bacterial avirulence genes to effector functions via the hrp delivery system: an overview of 25 years of progress in our understanding of plant innate immunity. Mol Plant Pathol 10:721-734.

Marsch-Moreno R, Hernández-Guzmán G, Alvarez-Morales A (1998) pTn*5cat*: a Tn*5*-derived genetic element to facilitate insertion mutagenesis, promoter probing, physical mapping, cloning, and marker exchange in phytopathogenic and other Gram-negative bacteria. Plasmid 39:205-214.

Ortiz-Martín I, Thwaites R, Macho AP *et al.* (2010) Positive regulation of the Hrp type III secretion system in *Pseudomonas syringae* pv. *phaseolicola*. Mol Plant Microbe Interact 23:665-681.

Palmer DA, Bender CL (1993) Effects of environmental and nutritional factors on production of the polyketide phytotoxin coronatine by *Pseudomonas syringae* pv. *glycinea*. Appl Environ Microbiol 59: 1619-1626.

Preston GM (2000) *Pseudomonas syringae* pv. *tomato*: the right pathogen, of the right plant, at the right time. Mol Plant Pathol 1:263-275.

Rahme LG, Mindrinos MN, Panopoulos NJ (1992) Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. J Bacteriol 174:3499-3507.

Reboutier D, Bouteau F (2008) Harpins and ion channels modulations: Many ways to die. Plant Signal Behav 3:314-316.

Ritter C, Dangl JL (1995) The *avrRpm1* gene of *Pseudomonas syringae* pv. *maculicola* is required for virulence on *Arabidopsis*. Mol Plant Microbe Interact 8:444-453.

Rozas J, Sanchez-Del Barrio JC, Messeguer X *et al.* (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 19: 2496-2497.

Sambrook J, Russell DW (2001) Molecular Cloning: A laboratory manual. (3rd ed) Cold Spring Harbor Laboratory Press. USA.

Schumacher J, Waite CJ, Bennett MH *et al.* (2014) Differential secretome analysis of *Pseudomonas syringae* pv tomato using gel-free MS proteomics. Front Plant Sci 5:242.

Tang X, Xiao Y, Zhou JM (2006) Regulation of the type III secretion system in phytopathogenic bacteria. Mol Plant Microbe Interact 19:1159-1166.

van Dijk K, Fouts DE, Rehm AH *et al.* (1999) The Avr (effector) proteins HrmA (HopPsyA) and AvrPto are secreted in culture from *Pseudomonas syringae* pathovars via the Hrp (type III) protein secretion system in a temperature- and pH-sensitive manner. J Bacteriol 181:4790-4797.

Worley KC, Culpepper P, Wiese BA *et al.* (1998) BEAUTY-X: enhanced BLAST searches for DNA queries. Bioinformatics 14:890-891.

Zwiesler-Vollick J, Plovanich-Jones AE, Nomura K *et al.* (2002) Identification of novel hrp-regulated genes through func-

tional genomic analysis of the *Pseudomonas syringae* pv. *tomato* DC3000 genome. Mol Microbiol 45:1207-1218.

Associate Editor: Fernando Dini Andreote