Characterization of the \textit{hrpZ} gene from \textit{Pseudomonas syringae} pv. \textit{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.

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Abstract

\textit{Pseudomonas syringae} pv. \textit{maculicola} is a natural pathogen of members of the Brassicaceae plant family. Using a transposon-based mutagenesis strategy in \textit{Pseudomonas syringae} pv. \textit{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 \textit{Arabidopsis thaliana}. A mutant containing a transposon insertion in the \textit{hrpZ} gene (PsmMut8) was unable to infect adult plants from \textit{Arabidopsis thaliana} or \textit{Brassica oleracea}, suggesting a loss of pathogenicity. The promotorless \textit{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 \textit{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 \textit{hrpAZB} operon from \textit{Pseudomonas syringae} pv. \textit{maculicola} M2 is necessary for its pathogenicity and that its diversity would be under host-mediated diversifying selection.

Key words: \textit{hrpZ}, mutant non-pathogenic, transmid, Tn5, 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 \textit{et al.}, 2006; Tang \textit{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 \textit{hrp} gene cluster (\textit{hyper-sensitivity response and pathogenicity}) (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 \textit{et al.}, 2001; Arnold \textit{et al.}, 2011), of which the Hrp pilus contacts the plant cell surface during infection (Böttner, 2012). Two types of proteins are exported through the Hrp pili: the \textit{avr} (\textit{avirulence}) gene products and the “harpins,” which are products of the \textit{hrpZ} and \textit{hrpW} genes (Reboutier and Bouteau, 2008; Schumacher \textit{et al.}, 2014). Avr proteins appear to be injected into plant cells (Jin \textit{et al.}, 2001; Fu \textit{et al.}, 2006), where they modulate the cell metabolism to export nutrients to the apoplast (van Dijk \textit{et al.}, 1999). In an incompatible interaction, the Avr protein is recognized by the product of a gene for resistance, \textit{R}, which triggers the hypersensitive response and results in disease abortion (Mansfield, 2009). The harpins are
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 (RifR) 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 ΔrecA RP4-2traTc::Mu Km::Tn7) (de Lorenzo et al., 1990) was obtained from Dr. Kate J. Wilson. *E. coli* DH5α competent cells (supE44 ΔlacU169 (80 lacZ DM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) (Sambrook and Russell, 2001) were used for cloning experiments. A *SalI* restriction site was added into the *Smal* site on pUIRM504 (Marsch-Moreno et al., 1998) to form the plasmid pMDC505 (unpublished results); with this change, the transposable element *pTn5cat* (Marsch-Moreno et al., 1998) was modified into *pTn5cat1*. King’s B medium (King et al., 1954), minimal medium M9 (Sambrook and Russell, 2001) or M9CA (Difco) was used to transform competent *E. coli* strains. Chloramphenicol, rifampicin and kanamycin (50 μg/mL) and chloramphenicol (150 μg/mL) were 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.

#### Mutagenesis and mutant selection

Mutants of PsmM2 were generated using the transposon element *pTn5cat1* according a published protocol (Marsch-Moreno et al., 1998). *E. coli* S17-1 (pMDC505) was used to mobilize *pTn5cat1* to PsmM2 by conjugation, and the bacteria were then spread onto M9 Rif50 Km50 plates. Mutants were screened for their ability to growth on M9 Cm50 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 effecter 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-OD620 culture of mutant PsmMut8 in KB Km50 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 OD600. 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 EcoRI 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 ligated 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'-CCCTAGCTCCTGAAA-3'; from the cat end), 1658 (5'-GGTACCATCGTCAACGGTCG-3'), 2176 (5-GTTGCGAAAACCGAAAG-3 to sequence hrpB), and 2149 (5-TCTGAGAGTGGCCTGGAAGC-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             | PERBB8031 | AB112557 |
| oryzae                  | 1-1.1  | AB112580    |
| coronafaciens           | AVPC08101 | AB112578 |
| aceris                  | kaede-1-1 | AB112576 |
| japonica                | BPST802 | AB112574    |
| striafaciens            | avera2 | AB112579    |
| magniolae               | PMG8101 | AB112570    |
| theae                   | tea632 | AB112568    |
| mori                    | moli1  | AB112562    |
| morsprunorum            | U7805  | AB112560    |
| myricae                 | yamamomo801 | AB112558 |
| dendropanacis           | kakuremimo-1 | AB112556 |
| pisi                    | Pisum-1 | AB112577    |
| phaseolicola            | NPS3121 | AB112552    |
| tomato                  | DC3000 | AF232004    |
| tagetis                 | ICMP2844 | AB112567 |
| aptata                  | SB8601 | AB112575    |
| tabaci                  | ATCC11528 | FJ946987 |
| lapsa                   | NCPPB2096 | AB112573    |
| actinidiae              | KW11   | AB112571    |
| delphinii               | PDDCC529 | AB112569 |
| maculicola              | R1     | AB112565    |
| M2                      | M2     | AY325899    |
| PMC8301                 | AB112566   |
| glycinea                | r0     | AB112554    |
| race4                   | race4  | L41862      |
| syringae                | 61     | EFS14224    |
| ICMP3414                | ICMB2-1 | AB112572 |
| Pseudomonas savastanoi  | 5      | FR717896    |
| savastanoi              |        |             |
| Pseudomonas viridiflava  | RMX23.1.a | AY597282 |
| cichorii                | SPC0918 | AB433910    |
| ficuserectae            | L-7    | AB112564    |

Results

Selection of PsmMut8

A collection of PsmM2 mutants harboring the pTn5cat1 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 Km50 Cm150 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 pTn5cat1 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.

pTn5cat1 is inserted into a gene homologous to the hrpZ gene of Pseudomonas syringae

A 14-kb chromosomal fragment corresponding to the pTn5cat1 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).
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 pTn5cat1 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 CGCAGGCTGCTGACCCACCTA) 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 (TGAAATCTCT and GCCCCCTCATCAGAGGGGC, 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-
different *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).
Discussion

The use of a Tn5 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 (Chatjerjee 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 Guttmann and Inoue in five phylogroups (Inoue and Takikawa, 2006; Guttmann 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 Guttmann (Guttmann 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).

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