

## Physical Map of Chromosome of the Phytopathogenic *Pseudomonas syringae* pv. *maculicola* M2

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*Pseudomonas syringae* pv. *maculicola* M2 (*Psm*) is a phytopathogenic bacteria and one of about 50 pathovars reported in the *Pseudomonas syringae* group. The host range of *Psm* included collard, cauliflower, broccoli and others cruciferae plants, producing blight, and among other cruciferae host plants, *Arabidopsis thaliana* as model study in plant-pathogen interactions. To understand the traits that could regulate the pathogenicity of *Psm*, the physical map was constructed employing transposable elements as pTn5*scat1* and pTn5*Spcat* (endowed with restriction sites for the rare cutting restriction endonucleases *PacI*, *PmeI* and *SwaI*) derived from transposon Tn5 to obtain insertional mutants. The chromosome from the wild type *Psm* was digested with the rare-cutting endonucleases *PacI*, *PmeI* and *SwaI*, producing 14, 15 and 16 fragments, respectively. All fragments were separated using pulsed field gel electrophoresis (PFGE) and the size of the chromosome was determinate to be around 6.53 Mb. To assemble all fragments caused by single restriction, random insertional mutants of *Psm* carrying an extra site for *PacI*, *PmeI* and *SwaI* were evaluated. Around 11 different insertional mutants were selected after screening using PFGE analysis and confirmed by Southern-blot hybridization, partial digestions were done in DNA of wild type *Psm* to complete the physical map.

**Key words:** *Pseudomonas syringae* pv. *maculicola*, PFGE,  
Chromosome size, transposon tagging, physical map.

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*Pseudomonas syringae* is a Gram-negative rod-shaped bacterium and it represents a

model for the study of plant-pathogen interactions<sup>1</sup>. *P. syringae* includes approximately 50 pathovars. They were classified mainly on the original host of isolation and pathogenicity traits in diseased plants. The plant pathogenic *P.*

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*syringae* pv. *maculicola* M2 (*Psm*) infect mainly plants of family Brassicaceae (*Arabidopsis*, broccoli, cauliflower, collard) producing leaf spot<sup>2,3,4</sup>.

For the past decade, many efforts were made for use reverse genetic approaches to investigate important factor/genes in the pathogenesis of *P. syringae*<sup>5</sup>. Ritter and Dangl<sup>3</sup> demonstrated that the avirulence gene *avrRpm1*, isolated from *Psm* in interaction with the *Arabidopsis* resistance gene *RPM1*, is required for the maximal virulence on this host. In others models, *Psm* can induce defensive responses in tobacco plants (*Nicotiana tabacum* cv. Xanthi); following *Psm* infiltration developed a hypersensitive response (HR) after 4.5 h post-infiltration (hpi), at this time, the symptoms and reactive oxygen species (ROS) were evoked for non-host *Psm-Arabidopsis* interactions<sup>6</sup>. As mainly pathogenicity factor, *Psm* has a Type III Secretion System (TTSS)<sup>7</sup>. The TTSS is encoded by *hrp* genes (*hipersensitive response* and *pathogenicity*) and is crucial in the infection process, and it is high conserved in almost Gram-negative phytopathogen bacteria<sup>8</sup>. All pathovars of *P. syringae* use the TTSS to inject proteins into plant cells, this is essential to multiply in apoplast and to promote the development of disease symptoms<sup>9</sup>.

To understand the process of *Psm* pathogenicity, it is important to know the genes related in the pathogenesis process, where they are located and clustered into the genome, and how the genes related to pathogenesis are regulated. Although very little is known about the size and organization of the genome, the constructing of a physical map of the *Psm* chromosome made by macrorestriction analysis and PFGE could help to determine them. The physical map of the *Psm* chromosome was elaborated using mutants obtained by transposable elements similar to and derived from pTn5*cat*<sup>10</sup>: pTn5*cat*1 and pTn5*Spcat* (Fig. 1; unpublished). This map would reveal the plasticity of bacterial genomes on its comparison against other physical maps reported for different *P. syringae* strains and that will help to understand the gene order in the chromosome of *Psm*.

## MATERIALS AND METHODS

### Biological materials, bacterial strains and culture conditions

*Pseudomonas syringae* pv. *maculicola* strain M2 (*Psm*) was kindly provided by Dr. Jeffery L. Dangl<sup>3</sup>. *Psm* and insertional mutants were routinely grown in King's B (KB) medium<sup>11</sup> on a rotary shaker (200 rpm) at 28°C. *Escherichia coli* S17-1 lpir (pMC505) was constructed in the laboratory and was grown in LB broth supplemented with chloramphenicol and kanamycin (both 50 µg/ml) at 37°C. Plasmids pMC505 (Fig. 1; including pTn5*cat*1, derived from pTn5*cat*<sup>10</sup> with a *Swa*I restriction site) and pMDR1234 (including pTn5*Spcat*) were constructed in the laboratory (Fig. 1; unpublished data). Ez-Tn5 Transposase was purchased from Epicentre. Restriction endonucleases *Pac*I, *Pme*I and *Swa*I were purchased from New England Biolabs (Ipswich, MA, USA). PCR Supermix High Fidelity was purchased from Invitrogen Life Technologies (Carlsbad, CA).

### Mutation of *Psm*

Mutants of *Psm* using pTn5*cat*1 were obtained through conjugal transfer of pMC505 containing the transposable element from the auxotrophic strain *E. coli* S17-1 lpir harboring the plasmid. An early log culture of *Psm* was concentrated 1:25 by centrifugation and suspended in KB broth, onto a 0.22 µm Millipore® membrane on a KB plate 25 µl of the suspension was placed, and incubated at 28°C during 12 h. An early log culture the *E. coli* strain in KB medium was concentrated as above, and 20 µl of this suspension was placed over the growth of *Psm* and mixed well. The Millipore® membranes with the grown *Psm* were transferred to a fresh KB plate before mixing with the *E. coli* suspension. The plates were incubated at 28°C for 12 h and the membranes were transferred to an assay tube with 5 ml of sterile distilled water. The cells were suspended vortexing the tubes and the suspension was use to be seed on M9 minimal medium supplemented with chloramphenicol and kanamycin (both 50 µg/ml). The plates were incubated at 28°C during 7 d, and the insertional mutants were collected. Mutants M1, M2, M7 and M8 were obtained in this way.

The transposable element pTn5Spcat was used to mutate *Psm* mutants. The EZ-Tn5 Transposase was conjugated to lineal transposable elements according the manufacturer indications to obtain the corresponding transposomes. pTn5Spcat was amplified using a primer with the sequence of the "mosaic" external ends<sup>12</sup> and PCR SuperMix High Fidelity. The amplicon obtained was cleaned with phenol-chloroform, precipitated with isopropanol<sup>13</sup>, and was suspended in distilled sterile water before to be mixed with EZ-Tn5 Transposase.

*Psm* electrocompetent cells were prepared according the user manual of Bio-Rad Gene Pulser, cells were grown to OD<sub>620</sub> = 0.6, harvested by centrifugation at 7,000 rpm in a Sorvall SL50T rotor at 4°C during 10 min, then the cells were suspended in the same volume of ice cold 10% glycerol prepared in deionized water, centrifuged again as above, suspended in half volume of 10% glycerol, centrifuged again and suspended in 1:10 initial volume with the same ice cold 10% glycerol. Two microliters of the linear transposable element-EZ Transposase were mixed with 25 µl of the electrocompetent cells and poured into an electroporator cell (0.1 cm) and the electrotransformation was fulfilled (2.5 Kv, 200 W and 25 mF). Mutants were selected in KB agar supplemented with spectinomycin (25 µg/ml). The colonies were picked after incubating at 28°C during 48 h to seed them in fresh medium for preparing agar-blocks with chromosomal DNA, and maintenance. The mutants 1i, 2i, 6i, 7i, 9, 16, 18, 21, 118, 869 and X2 were obtained by this way.

#### Chromosomal DNA preparation of *Psm*

Chromosomal DNA of *Psm* was prepared by embedding whole cells in low-melting agarose blocks as described previously<sup>14</sup>, with some modifications. The cell were grown at 28°C overnight on a rotary shaker in 5 ml KB, from this culture 500 µl was inoculated into 10 ml fresh KB. Cells were grown at 28°C in KB to the late exponential phase (60 Klett units). The cells were chilled to 4°C and harvested by centrifugation at 8,000 rpm for 15 min and washed in 10 ml Pett IV buffer (0.01 M Tris/HCl, pH 7.6, 1 M NaCl) followed by centrifugation at 8,000 rpm for 10 min. Cells were suspended in the same buffer (1.6 ml) and mixed with an equal volume of 1.2% (w/v) of low-melting-temperature agarose (SeaPlaque GTG

Agarose; Lonza Rockland, Inc., Rockland, ME, USA) prepared with sterile water at 42°C. The cells-containing agarose blocks were chilled at 4°C for 10 min and were submerged in EC-lysis solution (6 mM EDTA, pH 7.6, 0.5% Brij 58, 0.2% sodium deoxicholate, 0.5% sodium lauryl sarcosine, 1 mg/ml lysozyme, 10 mg/ml DNase-free RNase) and incubated at 37°C for 18 h. The EC-lysis was replaced with ESP solution (0.5 M EDTA, pH 8.0, 1% sodium lauryl sarcosine, 1 mg/ml proteinase K) and incubated for 24 h at 50°C. The agarose blocks were washed several times with TE buffer (50 mM Tris-HCl, 20 mM EDTA, pH 8.0) and were stored in TE buffer (10 mM Tris-HCl, 50 mM EDTA, pH 8.0) at 4°C until use.

#### Restriction endonuclease and partial digestion

For restriction endonuclease digestion, the blocks were washed three times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) prior to digestion. Each block was subjected to single digestion with the rare-cutting endonucleases (*PacI*, *PmeI* and *SwaI*) with the digestion buffer recommended by the manufacturer. The blocks were incubated overnight at 4°C and then incubated at 37°C with *PacI* or *PmeI*, while with *SwaI* at 25°C, all for 2 h. Partial digestions were made at same conditions except for incubation during 30 min.

#### PFGE analysis

PFGE electrophoresis was performed with a Bio-Rad CHEF Mapper XA System and CHEF-DR III Variable Angle System. The digested DNA was separated on 1% agarose-1X TAE (40 mM Tris-Acetate, 1 mM EDTA) and was run in 1X TAE buffer at 14°C for 24 h at 6 V/cm with switch time ramping from 60 to 90 s.  $\lambda$  DNA ladder (monomer 48.5 kb) and *Saccharomyces cerevisiae* YPH80 chromosome (size range 225-1900 kb) from New England Biolabs (Ipswich, MA, USA) were used for size marker. DNA was stained with ethidium bromide (0.025 mg/ml 1X TAE) for 20 min and visualized with a Bio-Rad UV Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories Hercules, CA, USA). All size fragments were calculated at least 15-times and compared with both size markers in Quantity One Software from Bio-Rad.

#### Southern-blot hybridization and probe labeling

All insertional mutants were used to prepare and digest their chromosomal DNA as indicated above. After the screening by PFGE the

collection of insertional mutants of *Psm*, 11 strains were chosen. Later other PFGEs were prepared, DNA in gels was transferred to a Hybond-N<sup>+</sup> nylon membranes (Amersham Biosciences, UK) according to Sambrook *et al.*<sup>13</sup>. Plasmid was used as probe for non-radioactive random prime labeling (*Gene Images random prime labeling module*; Amersham Pharmacia Biotech, UK). Hybridization was done with gently agitation at 60°C overnight in a bag sealed, membranes were washed, first with SSC 1X, SDS 0.1% (w/v) at 60°C for 15 min, and finally, with SSC 0.5X, SDS 0.1% (w/v) at 60°C during 15 min with gently agitation. After washes, the membranes were detected with *Gene Images CDP-Star* detection module (Amersham Pharmacia Biotech, UK) following the manufacturer's instructions.

## RESULTS

### *Pseudomonas syringae* pv. *maculicola* M2 macrorestriction pattern of DNA chromosome with *PacI*, *PmeI* and *SwaI*

To construct the physical map of the chromosome of *Psm*, three rare-cutting restriction endonucleases were chosen based on G+C content of *P. syringae* strains, several enzymes with A+T rich recognition sequences were tested, among these enzymes *PacI* (TTAATTA), *PmeI* (GTTTAAAC) and *SwaI* (ATTTAAAT) were selected which originated 14, 15 and 16 fragments, respectively. The macrorestriction pattern of chromosomal DNA with endonucleases mentioned above is showed in Figure 2. All DNA fragments produced by digestion were separated by PFGE, two running conditions were assayed for separate large-size fragments ranging from 1000 to 2500 kb (Fig. 3) and small-size fragments from 20 to 800 kb (Figs. 4 and 5).

### Chromosome-size estimation of *Pseudomonas syringae* pv. *maculicola* M2

The size of the chromosome of *Psm* was the result of the summation of the estimated size of individual DNA restriction fragments generated with *PacI*, *PmeI* and *SwaI* and separated by PFGE. All fragments were compared with both a  $\lambda$  DNA ladder and *Saccharomyces cerevisiae* YPH80 chromosome markers that facilitated estimate the size of fragments. The *PacI* digestion produced

fragments in size from 34 to 1873 kb, *PmeI* produced fragments from 61 to 1721 kb and the *SwaI* digestion from 22 to 1728 kb in size (Table 1). The chromosome size was determinate at approximately 6.53 Mb.

### Screening for insertional mutants of *Pseudomonas syringae* pv. *maculicola* M2

To evaluate all random insertional mutants of *Psm*, the chromosomal DNA of many mutants was subjected to complete endonuclease restriction, and then analyzed through PFGE. Eleven insertional mutants were selected for further analysis. An example for the transposable element insertion in large-size fragments is showed in Figure 3, mutant 21 was tagged in the largest *SwaI* fragment (1W; Fig. 2), while mutant 16 was tagged in the second fragment 2W (Fig. 2), both mutants were compared with the wild type *Psm* strain, all digested with *SwaI* (Fig. 3). Mutants M2, M7 and 9 were digested with *PacI* and *PmeI* (Fig. 4) and the insertions tagged in *PacI* fragments 12A, 1A and 3A, respectively, and in *PmeI* fragments 2E, 2E and 3E, respectively, these mutants were compared with the wild type *Psm* strain (Fig. 3). The digestion with the three restriction enzymes of mutants 6i and 7i is shown in Figure 5, the insertions tagged in *PacI* fragments 6A and 10A, respectively; with *PmeI* in fragments 5E and 6E, respectively, and with *SwaI* both insertions tagged in fragment 3W. Mutant 18 has the insertion in *PmeI* fragment 11E (Fig. 5). All fragments produced by an additional site in the mutants by *PacI*, *PmeI* and *SwaI* digestion were identified and showed in Table 2. To corroborate the position of inserts in the restriction fragments, Southern blot hybridization was fulfilled using pMC505 as probe (data not shown). As an example, Figure 6 shows the restricted DNA from mutant M2 with *PmeI* and *SwaI* (insertion in fragments 2E and 5W), and from mutants 7i and M1 with *SwaI* (insertion in fragments 3W and 1W, respectively), both the gel after PFGE and its Southern blot hybridization using pTn5*catI* as probe are showed. Analysis for four insertional mutants involved in pathogenicity (data not shown) of *Psm* were selected for its physical map position. Mutants 118 and M8 were tagged in both fragments 7E and 2W; while mutant 869 was tagged in 6E and 3W; mutant X2 was tagged in fragments 5E and 3W (Fig. 7). Analysis of sequences of

insertional mutants 869, M8, X2 and 118 corresponding to *hrpA*, *hrpZ*, *hrpR* and *gacS*, respectively (unpublished data).

#### Partial digestions of chromosome of *Psm* with *SwaI*

Many efforts for identify and detect small *SwaI* fragments tagging with the transposable elements were done without result, to solve this problem, partial digestions in wild type *Psm* chromosome were assayed. All the partial digestion were done as mentioned above, the short time for

incubating endonuclease *SwaI* produced fragment which are the sum of two, three, four or more fragments, the size of joined fragments 10W-12W was 332.86 kb, and 13W-15W joined showed a 157.8 kb band, all fragments were resolved by PFGE (data not shown). Partial digestions allowed construct the physical map of chromosome from *Psm*, although the correct position of fragments 15W and 16W could not be exactly determined (Fig. 7).

**Table 1.** Sizes of individual restriction fragments from DNA chromosome of the *Pseudomonas syringae* pv. *maculicola* M2 digested with *PacI*, *PmeI* and *SwaI*.

Fragment	<i>PacI</i> Size (kb±SD)	<i>PmeI</i> Size (kb±SD)	<i>SwaI</i> Size (kb±SD)
1	1873.98±65	1721.68±38	1728.90±42
2	1526.34±60	1476.12±49	1179.86±50
3	761.9±40	566.47±6	636.5±26
4	503.83±20	470.18±7	630.8±26
5	452.62±16	390.83±9	466.36±20
6	365.35±10	378.17±9	357.87±20
7	237.49±11	323.17±8	304.7±21
8	204.86±9	264.11±8	245.17±20
9	170.70±9	254.11±8	222.72±9
10	147.31±3	180.21±9	188.63±5
11	123.62±4	139.26±9	169.6±6
12	82.61±4	113.85±8	144.26±2
13	66.63±6	84.92±9	132.44±1
14	34.09±1	76.72±7	80.96±9
15		61.39±6	25.45
16			22.65
	6551.40	6501.70	6536.87

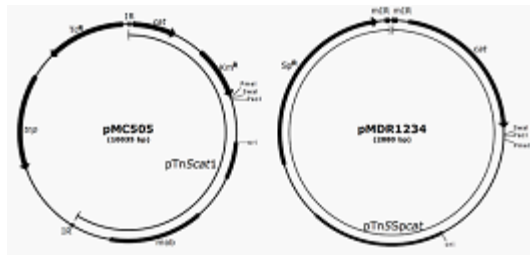
**Table 2.** Insertional mutants of *Pseudomonas syringae* pv. *maculicola* M2. DNA was digested with *PacI*, *PmeI* and *SwaI*. The fragments were tagged with transposable element. Mutants detected by Southern-blot were indicated with asterisk.

Mutants	<i>PacI</i> (kb)Fragment (sizes)	<i>PmeI</i> (kb)Fragment (sizes)	<i>SwaI</i> (kb)Fragment (sizes)
li	3A (736.70)	12E (43.82)	6W (246.3)
2i	4A (256.8, 201.3)	8E (238.44)	7W (263.78)
6i	6A (226.07, 100.06)	5E (352.8, 35.58)	3W (397.96, 226.77)
7i	10A (92.49, 39.26)	6E (332.54, 43.93)	3W (28.74)*
9	3A (247.52)	3E (326.7, 221.13)	9W (108.78)
16	2A (1049.56, 279.93)	7E (167.53, 136.26)	2W (860.02)
18	5A (226.23, 188.64)	11E (122.03)	8W (133.7, 113.06)
21	1A (1120.14, 535.32)	1E (1133.8)	1W (886.58)
M1	2A (881.67, 472.68)	13E (47.83, 28.93)	1W*
M2	12A (46.88, 37.25)	2E (1149.66, 76.32)	5W (296.83, 162.14)
M7	1A (23.86)*	2E (30.8)*	11W (116.49, 55.65)



## DISCUSSION

*Pseudomonas syringae* pathovars are widely distributed and considered plant pathogens with significant economic and environmental impacts, for this reason are an important model for the research on plant-pathogen interaction<sup>15</sup>. In this sense, *Pseudomonas syringae* pv. *maculicola* M2 infects plants of families Brassicacea and similar to others pathovars, required an arsenal of pathogenicity factors to infect the hosts, these important genes for pathogenicity and virulence

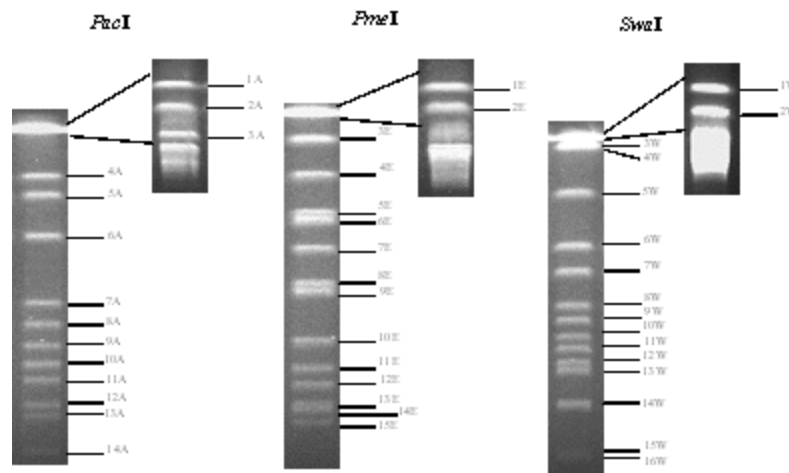


**Fig. 1.** Plasmids pMC505 and pMDR1234 used to obtain insertional mutants of *Psm*. pTn5cat1 and pTn5Spcat are the artificial transposable elements included in the plasmids. IR, Tn5 inverted repeats; mIR, “mosaic” inverted repeats; tnp, transposase gen; cat, promoterless chloramphenicol acetyltransferase; Tc, Km and Sp resistance genes for tetracycline, kanamycin and spectinomycin, respectively; mob, region for conjugal transference from RP4; and ori, ori<sub>ColE1</sub> replication origin from pBR322. The restriction sites for the rare cutting endonucleases are showed.

are often clustered in specific regions of the chromosome or plasmids<sup>16,17</sup>.

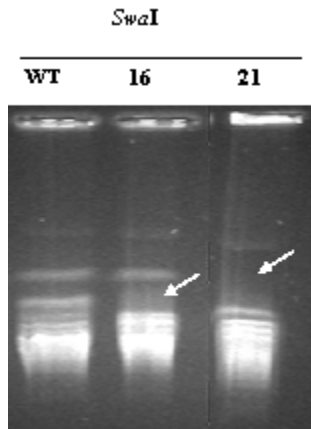
The PFGE analysis were initiated as a tool to mapping small genomes and to locate genes on the chromosomes<sup>18,19</sup>. Since to date, several physical maps have been constructed in a wide number of microorganisms with this technique, among these were found *Pseudomonas aeruginosa* C<sup>20</sup>, *Pseudomonas putida* KT2440<sup>21</sup>, *Pseudomonas syringae* pv. *phaseolicola*<sup>22</sup> and the apple proliferation phytoplasma strain AT<sup>23</sup>. All PFGE analysis for physical maps were facilitated by the rare cutting restriction endonucleases.

Restriction enzymes that can split the bacterial chromosome in a limited number of fragments (1 to 20) are required for the physical analysis of a genome<sup>24</sup>. The rare cutting restriction endonucleases *PacI*, *PmeI*, and *SwaI* were previously reported for construction physical maps in bacteria<sup>21,22,25,26</sup>. To construct the physical map of *Psm*, PFGE analysis of independent digestions with restriction enzymes *PacI*, *PmeI* and *SwaI* of wild type *Psm* chromosomal DNA were performed. The fragments produced using these enzymes were resolved: 14 fragments with *PacI* were obtained, 15 fragments with *PmeI*, and 16 fragments with *SwaI* (Fig.2). By PFGE analysis, the size of the bacterial chromosome of *Psm* was determinate to be ca. 6.53 Mb (Table 1). Among the size of chromosomes of the *P. syringae* pathovars reported were found: *P. syringae* pv. *phaseolicola* with 5.64 Mb in size<sup>22</sup>, and *P. syringae* pv. *tomato* DC3000 with 6.39 Mb in

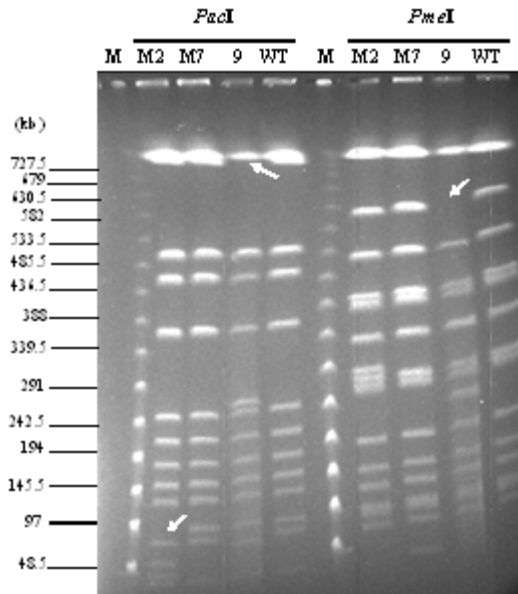


**Fig. 2.** Macrorestriction pattern by PFGE analysis from *P. syringae* pv. *maculicola* M2 chromosome digested with *PacI*, *PmeI* and *SwaI*, with 14, 15 and 16 fragments, respectively.

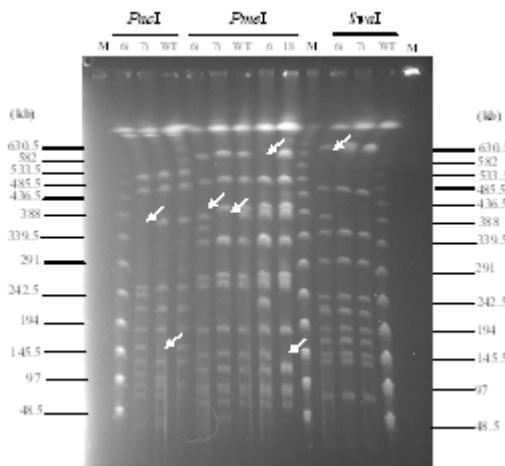
size<sup>27</sup>, *P. syringae* pv. *syringae* B301D-R with 6.03 Mb in size and an average G+C of 59.2%<sup>15</sup>, and recently, *P. syringae* pv. *maculicola* CFBP 1657 with 6.05 Mb in size<sup>28</sup>.



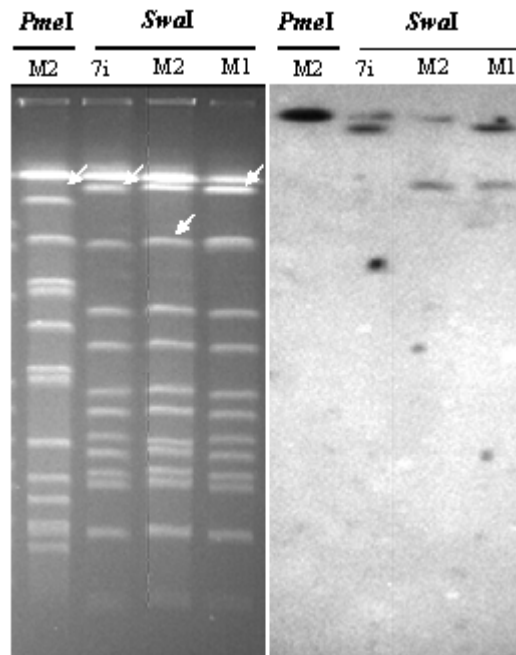
**Fig. 3.** Large-sized fragments by PFGE from *Psm* DNA chromosome digested with *SwaI*. (WT) *Psm* wild type strain, (16, 21) *Psm* pTn5*cat* mutants. Arrows indicated the fragment in which was inserted the transposable elements.



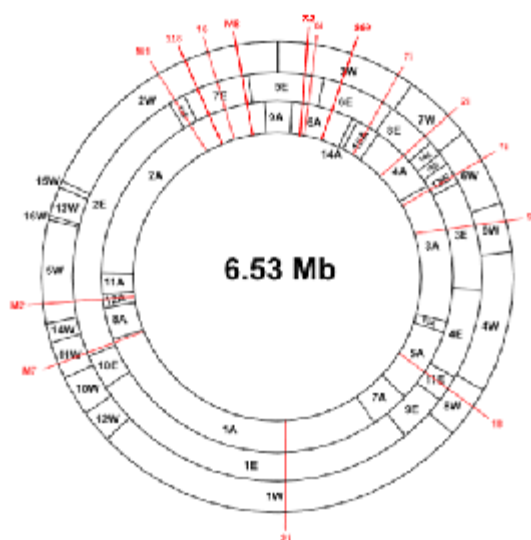
**Fig. 4.** PFGE analysis of *Psm* insertion mutants digested with *PacI* and *PmeI*. (M2, M7) *Psm* pTn5*cat*1 mutants, (9) *Psm* pTn5*cat* mutant and (WT) *Psm* wild type strain. (M) Size markers  $\lambda$  DNA ladder. Arrows indicated the fragment in which was inserted the transposable elements.



**Fig. 5.** PFGE analysis of *Psm* insertion mutants digested with *PacI*, *PmeI* and *SwaI*. (6i, 7i) *Psm* pTn5*cat*1 mutants, (6, 18) *Psm* pTn5*cat* mutants and (WT) *Psm* wild type strain. (M) Size markers  $\lambda$  DNA ladder. Arrows indicated the fragment in which was inserted the transposable elements.



**Fig. 6.** PFGE and Southern-blot analysis from *Psm* mutants digested with *PmeI* and *SwaI*. (M1, M2, 7i) *Psm* pTn5*cat*1 mutants. Plasmid pTn5*cat*1 was used as probe. Arrows indicated the fragments in which was tagged with the transposable elements.



**Fig. 7.** Physical map of the chromosome of the *Pseudomonas syringae* pv. *maculicola* M2. The insertional positions are indicated with red number in different regions of genome.

A physical map of *Psm* (Fig. 7) was made using insertional mutants of *Psm* obtained using transposable elements. Artificial transposable elements pTn5*scat1* and pTn5*scat* endowed with restriction sites for *PacI*, *PmeI* and *SwaI* (unpublished) were used to obtain a collection of insertional mutants, each with an extra restriction site for these endonucleases. Many insertional mutants were evaluated by PFGE and 11 were selected to determine the fragment in which the transposable element was inserted. The analysis was strengthened with Southern-blot, where the transposable elements were found. The fragments with no insertions were arranged using data from the partial restriction experiments. The size and location of fragments 15W and 16W made them very difficult to assess their position, in Figure 7 these fragments can be as drew or in an inverted position.

However, additional analysis for insertional mutants show genes homologous to *Pseudomonas syringae* pv. *tomato* DC3000 and were related in pathogenesis in *Psm* (unpublished data). These genes were localized with its position into the chromosome and were reported *hrpZ*, *hrpR*, *hrpA* and *gacS*. The phytopathogenic *Pseudomonas syringae* strains harbour a TTSS involved in the delivery of effector proteins into

plant cells. Among these *hrp* genes that encoding for TTSS we found *hrpA*, the structural gene encoding Hrp pilin and is an essential pathogenicity determinant for *Pseudomonas syringae* pv. *tomato* DC3000<sup>29,30</sup>; also HrpA pilin subunit is involved for the Hrp pilus elongation and that the effector protein HrpZ is delivered only by the pilus tip<sup>31</sup>. HrpR and HrpS proteins are involved in activation of the *hrp* regulon and considered as bacterial enhancer-binding proteins (bEBPs) that operate as a highly co-dependent hetero-hexameric complex<sup>32</sup>. GacS is a sensor kinase and the response regulator GaCA both members of a two-component system involved in the pathogenicity in fluorescent pseudomonads<sup>33</sup>, GacS/GacA system is required for virulence and GacA functions as a central regulator in *Pseudomonas syringae* pv. *tomato* DC3000<sup>34</sup>. However, the complete characterization of these insertional mutants involved in the pathogenicity of *Psm* remain in study.

The physical map constructed in this study will help to understand genes order in the chromosome of *Psm* and its evolution when compared with other related *P. syringae* pathovars genomes. Although the correct position of the smallest *SwaI* fragments was not detected by partial digestions, further analysis with insertional mutants of *Psm* could determinate the order in the physical map. Finally, this study reports the first physical map of *Psm* constructed with synthetic transposable elements for the analysis of insertional mutants.

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#### REFERENCES

1. Katagiri, F., Thilmony, R., and Yang-He, S. The *Arabidopsis Thaliana*-*Pseudomonas syringae* Interaction. In: *The Arabidopsis Book. The American Society of Plant Biologist*, 2002; pp



- 1–39.
2. Bereswill, S., Bugert, P., Völksch, B., Ullrich, M., Bender, C.L., Geider, K. Identification and relatedness of coronatine-producing *Pseudomonas syringae* pathovars by PCR analysis and sequence determination of the amplification products. *Appl. Environ. Microbiol.* 1994; **60**(8): 2924-30.
  3. Ritter, C., Dangl, J. The *avrRpm1* gene of *Pseudomonas syringae* pv. *maculicola* is required for virulence on *Arabidopsis*. *Mol. Plant Microbe Interact.* 1995; **8**(3): 444-453.
  4. Völksch, B., Weingart, H. Toxin production by pathovars of *Pseudomonas syringae* and their antagonistic activities against epiphytic microorganisms. *J. Basic Microbiol.* 1998; **38**(2): 135-45.
  5. Ichinose, Y., Taguchi, F., Mukaihara, T. Pathogenicity and virulence factors of *Pseudomonas syringae*. *J. Gen. Plant Pathol.* 2013; **79**(5): 285-296.
  6. Krzymowska, M., Konopka-Postupolska, D., Sobczak, M., Macioszek, V., Ellis, B.E., Henning, J. Infection of tobacco with different *Pseudomonas syringae* pathovars leads to distinct morphotypes of programmed cell death. *The Plant J.* 2007; **50**(2): 253-264.
  7. Willis, D.K., Rich, J.J., Hrabak, E.M. *hrp* genes of phytopathogenic bacteria. *Mol. Plant Microbe Interact.* 1991; **4**(2): 132-138.
  8. Petnicki-Ocwieja, T., Schneider, D.J., Tam, V.C., Chancey, S.T., Shan, L., Jamir, Y., Schechter, L.M., Janes, M.D., Buell, C.R., Tang, X., Collmer, A., Alfano, J.A. Genowide identification of proteins secreted by the Hrp system of *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. USA.* 2002; **99**(11): 7652-7657.
  9. Mudgett, M.B. New insights to the function of phytopathogenic bacterial Type III effectors in plants. *Annu. Rev. Plant Biol.* 2005; **56**: 509-531.
  10. Marsch-Moreno, R., Hernandez-Guzman, G., Alvarez-Morales, A. pTn5*cat*: A Tn5-derived genetic element to facilitate insertion mutagenesis, promoter probing, physical mapping, cloning, and marker exchange in phytopathogenic and other Gram-negative bacteria. *Plasmid.* 1998; **39**(3): 205-214.
  11. King, E.O., Ward, M.K., Raney, D.E. Two simple media for the demonstration of pyocyanin and fluorecin. *J. Lab. Clin. Med.* 1954; **44**(2): 301-307.
  12. Zhou, M., Bhasin, A., Reznikoff, W.S. Molecular genetic analysis of transposase-end DNA sequence recognition: cooperativity of three adjacent base-pairs in specific interaction with a mutant Tn5 transposase. *J. Mol. Biol.* 1998; **276**(5): 913-925.
  13. Sambrook, J., Russell, D.W. Cold Spring Harbor Laboratory Press (ed): *Molecular Cloning: A Laboratory Manual*, 3rd edn. New York, 2001; pp 6.25.
  14. Smith, C.L., Klco, S.R., Cantor, C.R. Pulsed field gel electrophoresis and the technology of large DNA molecules in genome analysis. In: *Genome analysis, a Practical Approach*. (Davies K, ed). Oxford IRL Press, 1988; pp. 41–72.
  15. Dudnik, A., Robert, D. Genome and transcriptome sequences of *Pseudomonas syringae* pv. *syringae* B301D-R. *Gen. Ann.* 2014; **2**(2): 1-2.
  16. Rahme, L.G., Mindrinos, M.N., Panopoulos, N.J. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bact.* 1992; **174**(11): 3499-3507.
  17. Rohmer, L., Kjemtrup, S., Marchesini, P., Dangl, J.L. Nucleotide sequence, functional characterization and evolution of pFKN, a virulence plasmid in *Pseudomonas syringae* pathovar *maculicola*. *Mol. Microbiol.* 2003; **47**(6): 1545-1562.
  18. Römling, U., Grothues, D., Bautsch, W., Tummier, B. A physical genome map of *Pseudomonas aeruginosa* PAO. *The EMBO J.* 1989; **8**(13): 4081-4089.
  19. Smith, C.L., Condemine, G. New approaches for physical mapping of small genomes. *J. Bact.* 1990; **172**(3): 1167-1172.
  20. Schmidt, K.D., Tümmler, B., Römling, U. Comparative genome mapping of *Pseudomonas aeruginosa* PAO with *P. aeruginosa* C, which belongs to a major clone in cystic fibrosis patients and aquatic habitats. *J. Bact.* 1996; **178**(1): 85-93.
  21. Ramos-Díaz, M.A., Ramos, J.L. Combined physical and genetic map of the *Pseudomonas putida* KT2440 chromosome. *J. Bact.* 1998; **180**(23): 6352-6363.
  22. De-Ita, M.E., Marsch-Moreno, R., Guzmán, P., Alvarez-Morales, A. Physical map of the chromosome of the phytopathogenic bacterium *Pseudomonas syringae* pv. *phaseolicola*. *Microbiology*, 1998; **144**: 493-501.
  23. Lauer, U., Seemüller, E. Physical map of the chromosome of the apple proliferation phytoplasma. *J. Bact.* 2000; **182**(5): 1415-1418.
  24. Fonstein, M., Haselkorn, R. Physical mapping of bacterial genomes. *J. Bact.* 1995; **177**(12): 3361-3369.
  25. Rainey, P.B., Bailey, M.J. Physical and genetic

- map of the *Pseudomonas fluorescens* SBW25 chromosome. *Mol. Microbiol.* 1996; **19**(3): 521-533.
26. Rawnsley, T., Tisa, L.S. Development of a physical map for three *Frankia* strains and a partial genetic map for *Frankia* Eu11c. *Physiol. Plant.* 2007; **130**(3): 427-439.
  27. Buell, C.R., et al. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. USA.* 2003; **100**(18): 10181-10186.
  28. Bartoli, C., Carrere, S., Lamichhane, J.R., Varvaro, L., Morris, C.E. Whole-genome sequencing of 10 *Pseudomonas syringae* strains representing different host range spectra. *Gen. Ann.* 2015; **3**(3): 1-3.
  29. Taira, S., Tuimala, J., Roine, E., Nurmiaho-Lassila, E.-L., Savilahti, H., Romantschuk, M. Mutational analysis of the *Pseudomonas syringae* pv. *tomato* *hrpA* gene encoding Hrp pilus subunit. *Mol. Microbiol.* 1999; **34**(4): 736-744.
  30. Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.-L., Kalkkinen, N., Romantschuk, M., He, S.Y. Hrp pilus: An *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl. Acad. Sci. USA.* 1997; **94**(7): 3459-3464.
  31. Li, Ch-M., Brown, I., Mansfield, J., Stevens, C., Boureau, T., Romantschuk, M., Taira, S. The Hrp pilus of *Pseudomonas syringae* elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ. *The EMBO J.* 2002; **21**(8): 1909-1915.
  32. Jovanovic, M., James, E.H., Burrows, P.C., Rego, F.G., Buck, M., Schumacher, J. Regulation of the co-evolved HrpR and HrpS AAA+ proteins required for *Pseudomonas syringae* pathogenicity. *Nat. Commun.* 2011; **2**(177): 1-9.
  33. Heeb, S., Haas, D. Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Mol. Plant Microbe Interact.* 2001; **14**: 1351-1363.
  34. Chatterjee, A., Cui, Y., Yang, H., Collmer, A., Alfano, J.R., Chatterjee, A.K. 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.* 2003; **16**(12):1106-1117.