REVISTA ARGENTINA DE
MICROBIOLOGÍA

ORIGINAL ARTICLE

A 14–20 kDa protein binds to the upstream region of the *phtM* operon involved in the synthesis of phaseolotoxin in *Pseudomonas syringae* pv. *phaseolicola* NPS3121

Ariel Álvarez-Morales[a], Alejandro Hernández-Morales[b], Jackeline L. Arvizu-Gómez[c,*]

[a] *Centro de Investigación y de Estudios Avanzados del IPN, Irapuato, Gto., Mexico*
[b] *Unidad Académica Multidisciplinaria Zona Huasteca, Universidad Autónoma de San Luis Potosí, San Luis Potosí, Mexico*
[c] *Secretaría de Investigación y Posgrado, Centro de Innovación y Transferencia de Tecnología (CENIT²), Universidad Autónoma de Nayarit, Tepic, Nayarit, Mexico*

Abstract *Pseudomonas syringae* pv. *phaseolicola* is a phytopathogenic bacterium in beans that produces a phytotoxin called phaseolotoxin, in whose synthesis a group of genes that belong to the ''Pht cluster'' are involved. This cluster comprises 23 genes arranged in 5 transcriptional units, two monocistronic (*argK*, *phtL*) and three polycistronic (*phtA*, *phtD*, *phtM*) operons, whose expression is increased at 18 °C, correlating with the production of phaseolotoxin by the bacterium. So far, the regulatory mechanisms involved in phaseolotoxin synthesis are poorly understood and only the requirement of low temperatures for its synthesis has been demonstrated. Therefore, in this study we searched for regulatory proteins that could be involved in the phaseolotoxin synthesis, focusing on the regulation of the *phtM* operon. Gel shift assays showed that the promoter region of the *phtM* operon contains binding sites for putative regulatory proteins, which are encoded outside the Pht cluster and are independent of the GacS–GacA two-component system. Deletion assays with the promoter region of the *phtM* operon show that the binding site for a putative transcription factor is located within a 58 bp region. The putative transcription factor of the *phtM* operon has an apparent molecular mass in the 14–20 kDa range. Furthermore, the results demonstrate that the transcription factor recognizes and binds the upstream *phtM* region as monomer o multimer of a single polypeptide. Our findings provide new insights into the regulatory mechanisms involved in phaseolotoxin production, and suggest that the Pht cluster was integrated into the global regulatory mechanism of *P. syringae* pv. *phaseolicola*.

* Corresponding author.
  E-mail address: lizzeta28@gmail.com (J.L. Arvizu-Gómez).

Una proteína de 14–20 kDa se une a la región río arriba del operón *phtM* involucrado en la síntesis de faseolotoxina en *Pseudomonas syringae* pv. *phaseolicola* NPS3121

Resumen   *Pseudomonas syringae* pv. *phaseolicola* es una bacteria patógena del frijol que produce una fitotoxina conocida como faseolotoxina. En la síntesis de dicha molécula se encuentran involucrados un grupo de genes que conforman el denominado *cluster* Pht. Este *cluster* comprende 23 genes, ordenados en 5 unidades transcripcionales —2 monocistrónicas (*argK, phtL*) y 3 poliscistrónicas (*phtA, phtD, phtM*)—, cuya expresión se encuentra incrementada a 18 °C, en correlación con la producción de faseolotoxina por la bacteria. Hasta ahora, los mecanismos regulatorios involucrados en la síntesis de faseolotoxina han sido poco estudiados. El objetivo de este trabajo fue buscar y caracterizar proteínas regulatorias involucradas en la síntesis de faseolotoxina, colocando el foco en la regulación del operón *phtM*. Los ensayos de cambio en la movilidad electroforética mostraron que la región promotora del operón *phtM* contiene sitios de unión para posibles proteínas regulatorias, codificadas fuera del *cluster* Pht e independientes del sistema de dos componentes GacS-GacA. El sitio de unión para estos factores de transcripción está localizado en una región de 58 pb. Los resultados demuestran, además, que el probable factor de transcripción del operón *phtM* tiene una masa molecular aparente de 14-20 kDa y que reconoce y se une a la región río arriba de *phtM* como monómero o multímero de un solo polipéptido. Este estudio aporta nuevos hallazgos dentro de los mecanismos regulatorios involucrados en la síntesis de faseolotoxina, los que indican que el *cluster* Pht se integró a los mecanismos regulatorios de *P. syringae* pv. *phaseolicola*.

Introduction

Phaseolotoxin   [N$^\delta$(N′-sulfodiaminophosphinyl)-ornithyl-alanyl-homoarginine], is an extracellular, nonhost-specific and chlorosis-inducing toxin, considered to be the major virulence factor of the phytopathogenic bacterium *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of the disease in beans (*Phaseolus vulgaris* L.) known as ''Halo Blight''[32]. This is considered one of the most common diseases of beans in temperate and cold regions, and is of economic importance in the world due to major field crop losses. The halo blight disease is characterized by the development of a chlorotic zone or halo around the necrotic infection site. This halo results from the action of phaseolotoxin released from the bacteria in the infection site and its diffusion into the surrounding leaf tissues[21–23]. Phaseolotoxin acts as reversible inhibitor of the ornithine carbamoyltransferase enzyme (OCTase; EC2.1.3.3) which catalyses the conversion of ornithine to citruline, a reaction common to both arginine biosynthesis and the urea cycle, thus resulting in host cell death. In addition, phaseolotoxin facilitates the systemic invasion of the plant, contributing significantly to the virulence of the pathogen[11,34]. In this manner, phaseolotoxin is a key element for halo blight disease development. However, non-phaseolotoxin-producing strains have also been detected in bean fields with epidemiological importance and causing the same lesions in the plants except the absence of chlorotic halos[13,28]. The production of phaseolotoxin by *P. syringae* pv. *phaseolicola* is mainly temperature-dependent, with high levels of this metabolite at 18 °C–20 °C, while no detectable amounts of the toxin are present at 28 °C–30 °C, corresponding to the optimal growth temperature for this bacterium[15,25]. Genes necessary for the synthesis of phaseolotoxin are encoded within a 30.24 kb pathogenicity island (PAI) termed the ''Pht cluster'', which contains 23 genes arranged into five transcriptional units, including two monocistronic (*argK* and *phtL*) and three polycistronic operons; one comprising 11 genes from *phtA* to *phtK* with an internal promoter driving expression of *phtD* to *phtK* and a third large polycistronic operon comprising 10 genes from *phtM* to *phtV*[1,12,30,31]. The function of only few of these genes has been elucidated; *argK* encoding a phaseolotoxin-insensitive OCTase, which provides resistance to the bacterium to its own toxin[24,27]; *desI* coding for a fatty acid desaturase[16], *amtA* encoding an amidinotransferase[17]; *phtU* coding an L-amino acid ligase[3] and *phtL*, whose product has a regulatory function on both the phaseolotoxin cluster (Pht) and genomic genes[1,14]. The presence of the Pht cluster has also been reported in the other known phaseolotoxin-producing pathovars *P. syringae* pv. actinidiae (kiwi pathogen) and in a single strain of *P. syringae* pv. syringae CFBP3388, although in the latter the cluster organization is poorly conserved[33,35]. So far, it is still unknown whether this cluster contains all the elements necessary for phaseolotoxin production. Recently, the PSPPH_4550 gene encoding for a putative non-ribosomal peptide synthetase, located outside the Pht cluster, has been identified and shown to be involved in the synthesis of phaseolotoxin in *P. syringae* pv. *phaseolicola* NPS3121. However, this gene appears to be related to bacterial fitness, in which the Pht cluster genes have been integrated after a presumed horizontal gene-transfer event[9].

The regulatory mechanisms that control phaseolotoxin gene expression in *P. syringae* pv. *phaseolicola* are just beginning to be elucidated. The expression of Pht cluster genes occurs selectively during growth at low temperature (18 °C), while at 28 °C only basal levels of expression are observed for these genes. This is consistent with the permissive temperature for phaseolotoxin synthesis, with the exception of the expression of *phtL* which was detected at both temperatures[1]. The way *P. syringae* pv. *phaseolicola* regulates the expression of these genes and the synthesis of phaseolotoxin in relation to temperature is poorly understood. The molecular basis involved in the signal transduction pathway and/or regulatory networks that participate in this process have not been elucidated yet. The analyses of transcriptional fusions of intergenic regions of Pht cluster genes suggest that low temperature regulation occurs at the transcriptional level. Furthermore, analysis of the promoter regions identified in the Pht cluster showed that the divergent promoters for *argK* and *phtA* present a −35 and −10 region that is characteristic of the negatively controlled promoters, while the promoter regions for *phtD*, *phtL* and *phtM* did not show any similarity to consensus sequences for bacterial sigma factors. Although a common mechanism of transcriptional regulation for *phtD* and *phtM* has been suggested due to the presence of conserved regions in the promoters of these operons[1]. So far, the studies focused on the identification of regulatory proteins involved in the expression of Pht cluster genes are very scarce. A regulatory function for the PhtL protein has been suggested based on the lack of *phtM* operon expression in a *phtL*[−] background[1]. However this has not been confirmed yet. Similarly, a regulatory function for the *phtABC* genes has been proposed, participating in the transcriptional repression of the *argK* gene[2]. So far, only the IHF (Integration Host Factor) protein has been identified as involved, in a direct manner, in the regulation of Pht cluster genes, particularly in the *phtD* operon[4]. For these reasons, the goal of this study aims to delve into the regulatory pathways involved in the phaseolotoxin synthesis in *P. syringae* pv. *phaseolicola* by identifying and/or characterizing regulatory proteins that could participate in the regulation of the expression of Pht cluster genes by focusing on the *phtM* operon.

Materials and methods
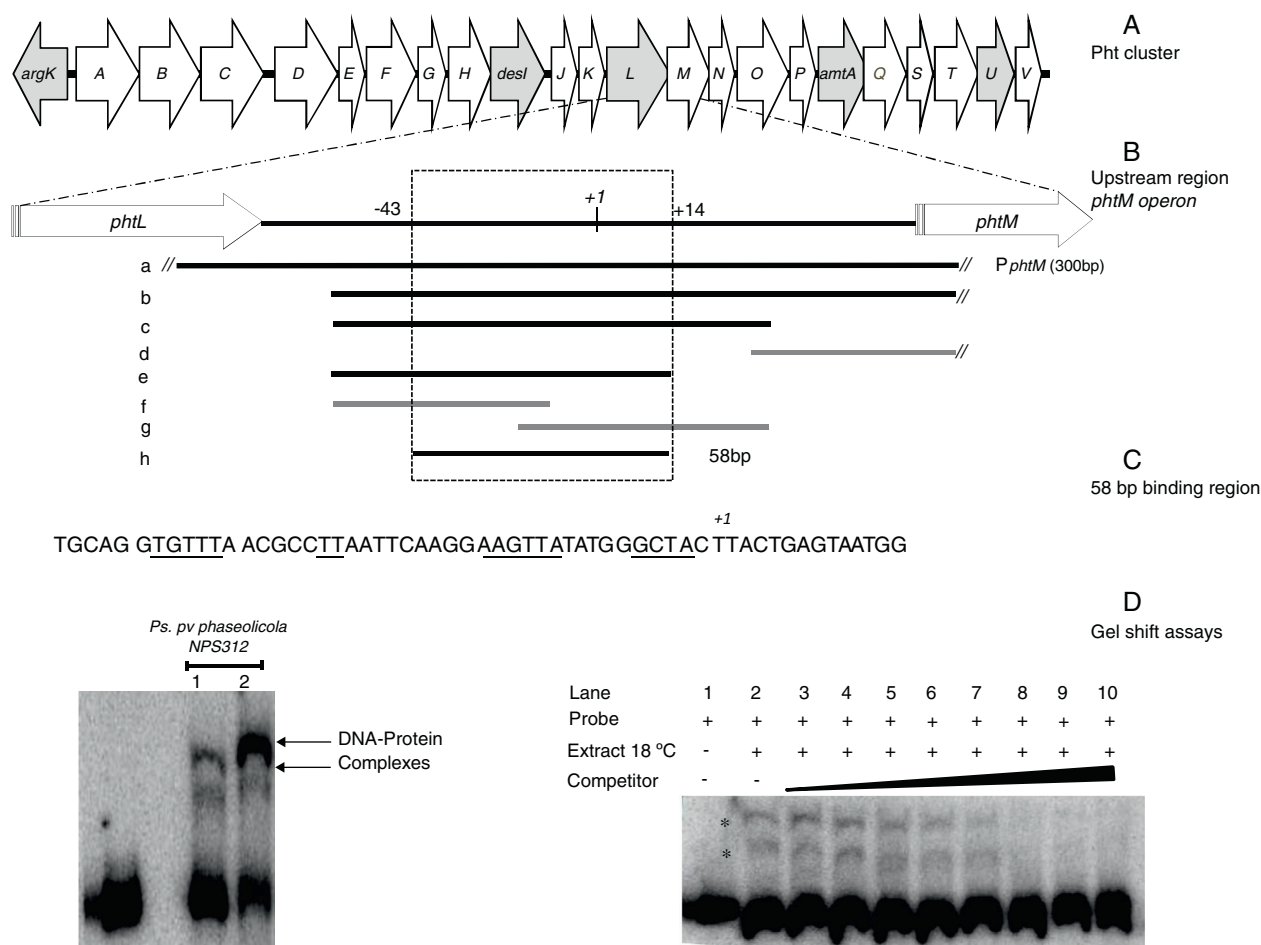
Bacterial strains, media and growth conditions

P. syringae strains: pv. *phaseolicola* NPS3121 wild type (wt), *gacA*[−] mutant, pv. *phaseolicola* CLY233, and pv. tomato DC3000 were grown on M9 minimal medium at 18 °C or 28 °C[1,7,9,28]. Pre-inocula (25 ml) of *P. syringae* strains were grown overnight at 28 °C in M9 medium with glucose (0.8%) as carbon source. The cells were inoculated into 50 ml M9 minimal medium at OD_{600nm} 0.1 and the cultures were incubated at 18 °C or 28 °C until they reached early stationary phase (OD_{600nm} 1.0). When required, antibiotics were added to cultures of *P. syringae* pv. *phaseolicola* NPS3121 wt (rifampin 50 μg/ml) and *gacA*[−] mutant strains (rifampin 50 μg/ml, kanamycin 70 μg/ml).

Biochemistry and molecular biology techniques

Genomic DNA of *P. syringae* pv. *phaseolicola* NPS3121 was isolated as previously described[8]. Routine molecular techniques were performed using standard protocols[29]. PCR products were amplified with High Fidelity DNA Polymerase and Platinum supermix (Invitrogen, California USA) and purified with the QIAquick® gel extraction kit (QIAGEN, Hilden, Germany). Primers were designed using the Vector NTI Software (Invitrogen) on the basis of the previously reported Pht cluster sequence (GenBank DQ141263[1]) (Fig. 1A). The oligonucleotides used in this study are listed under supplementary material 1. The biochemical procedures such as SDS-PAGE and electroblotting were performed according to standard protocols[29].

Electrophoretic mobility shift assays (EMSA)

The probes used in gel shift assays (Table 1) were obtained by PCR amplification using the oligonucleotide pairs designed in this study. The double-stranded probes were end-labeled with (γ^{32}P)-dATP using T4 polynucleotide kinase (Invitrogen). Gel shift assays were performed as previously described[4]. Briefly, protein extracts were prepared from *P. syringae* pv. *phaseolicola* NPS3121 wt strain grown in M9 minimal medium at 18 °C and 28 °C until reaching the early stationary phase (OD_{600nm} of 1.0). Cultures were centrifuged and the pellet was rinsed once with 1/20 volume of cold extraction buffer (25 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and 0.04 mM PMSF), the cell pellet was freeze-thawed once, diluted in 1/20 volume of extraction buffer, and sonicated (3 times for 15 s with intervals of 15 s) in an ice bath using a Virsonic 60 sonicator (Virtis Company Inc). The cellular debris was pelleted by centrifugation at $15\,000 \times g$ in a microcentrifuge, for 5 min at 4 °C and discarded. Total protein was measured using the Bradford method with a BSA standard curve as control[6]. The binding reactions contained approximately 10 ng of the probe, 30 μg of the appropriate protein extract, 0.5–1 μg poly(dI-dC) (Roche, Basel Switzerland), and 0.2 μg sonicated salmon sperm DNA, in a 20 μl total volume of binding buffer (25 mM Tris pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol) and were incubated for 30 min at room temperature. Protein-DNA complexes were separated from the unbound probe on 6.5% native polyacrylamide gels at 6 mA for 3–4 h, in 0.5× TBE buffer. Gels were vacuum-dried and exposed to a phosphor screen (Molecular Dynamics). The image was captured by scanning on a STORM 860 (Molecular Dynamics) and analyzed with Quantity One software (BIO-RAD, Berkeley, CA). To determine the specificity of the DNA-protein complexes observed, competition assays were carried out using increasing concentrations of specific and non-specific competitor DNA. A 300 bp-*Pvu*II fragment of the pUC19 plasmid was used as non-specific competitor. To delimit the binding site of the putative regulatory protein and/or to determine the localization of the DNA–protein complex, competition assays were performed with an excess of unlabeled wild-type probes, listed in Table 1. When crude extracts of *P. syringae* pv. *phaseolicola* NPS3121 *gacA*[−] mutant, *P. syringae* pv. tomato DC3000 and *P. syringae* pv. *phaseolicola* CLY233 strains were assayed, the conditions of the gel shift assays

Figure 1 Gel shift assays. (A) Graphic representation of the Pht region. Each arrow represents an individual gene, with the direction of the arrow indicating the direction of transcription. Gray arrows indicate genes whose function have been previously reported. (B) Detailed view of the *phtM* operon upstream region indicating the fragments used as unlabeled DNA competitors. The *phtL–phtM* intergenic region consists of 149 bp. The black bars represent the probes that were able to compete the DNA–protein complex, while the gray bars represent the probes unable to compete the complex. Fragment ''h'' corresponds to the 58 bp-region defined as binding site for protein. The truncated bars (//) are not represented to scale, whose length extends beyond what they are representing. (C) Nucleotide sequence of the 58 bp binding region, showing the DNA regions common to promoters of *phtD* and *phtM* in bold type and underlined. (D) (Left) Gel shift assays evaluating binding capacity of the P_{phtM} region using crude protein extracts of *P. syringae* pv. *phaseolicola* NPS3121 grown at 28 °C (1) or 18 °C (2) in M9 minimal medium; and (right) the gel shift competition assay performed using the ''h'' probe (58 bp) as competitor of the labeled P_{phtM} fragment. The concentration of unlabeled DNA competitors was as follows: lanes 1 and 2, no DNA competitor, lane 3, 25 ng; lane 4, 50 ng; lane 5, 60 ng; lane 6, 100 ng; lane 7, 150 ng; lane 8, 200 ng; lane 9, 250 ng; lane 10, 300 ng. The asterisks (*) indicate the DNA–protein complexes.

were similar to those described above. The gel shift assays evaluating the upstream region of the *phtD* operon were performed using the P_{phtD} probe previously reported under the conditions described above[4].

Southwestern assays

The Southwestern assays were performed as previously described with some modifications[19]. Briefly, protein extracts (70 μg) prepared from *P. syringae* strains, under similar conditions to those used on the gel shift assays, were fractionated on a 12% sodium dodecyl sulfate (SDS) denaturing gel. The gels were transferred to a 0.45 μm pore size nitrocellulose membrane by electroblotting under

the following conditions: 10 min at 30 V, 10 min at 40 V and 40 min at 50 V. Blots were incubated with shaking in buffer TNE-50 (10 mM Tris pH7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) for 5 min and subsequently incubated with shaking in blocking buffer SW (25 mM HEPES pH 8.0, 1 mM DTT, 10% glycerol, 50 mM NaCl, 1 mM EDTA, 2.5% low-fast skim milk) at 4 °C for overnight. The blot was processed by washing one time in 50 ml of TNE-50 buffer and incubated in hybridization buffer consisting in TNE-50 buffer, labeled target DNA (200 ng) with 0.5–1 μg of nonspecific competitor poly(dI-dC) DNA (Roche) at room temperature for 8 h. The blot was processed by washing two times in 50 ml of TNE-50 buffer and exposed to a phosphor screen (Molecular Dynamics). The image was captured by scanning on a STORM 860 (Molecular Dynamics) and analyzed with Quantity One software (BIO-RAD). Pre-stained

Table 1 Probes used in gel mobility shift assays

Probe	Oligonucleotide pairs	Positions of forward/ reverse primers[a]	Amplicon length (bp)	Competitor in gel-shift competition assays[b]
phtM				
a (P$_{phtM}$)	L100078–L100079	−131 to +168	300	+
b	L100145–L100079	−64 to +168	233	+
c	L100145–L100148	−64 to +39	104	+
d	L100063–L100079	+56 to +168	112	−
e	L100145–L100144	−64 to +14	78	+
f	L100145–L100146	−64 to −11	53	−
g	L100147–L100148	−17 to +39	57	−
h	L100143–L100144	−43 to +14	58	+
phtD				
P$_{phtD}$	L100074–L100075	−111 to +188	300	−

[a] Positions with respect to the transcription start site of the *phtM* and *phtD* operon.
[b] Fragments capable of competing the P$_{phtM}$–protein complex (+). Fragment unable to compete the P$_{phtM}$–protein complex (−).

protein markers were included in the assays for molecular mass estimation in Southwestern blots. To determine the specificity of the DNA–protein complexes observed, BSA protein was electrophoresed simultaneously. Additionally, competition assays were carried out using specific and non-specific competitor DNA. A 300 bp-*Pvu* II fragment of the pUC19 plasmid was used as non-specific competitor at 1 μg of concentration. For the competition assays, the protein extracts were electrophoresed in duplicate in the same gel. After completion of the blocking, the blot membrane was divided into two parts; one was used as control employing the conditions above mentioned, while the other was used for the competition assays, in which the unlabeled competitor was added to TNE-50 hybridization buffer. To determine the ability to compete the DNA–protein complexes, the images of both blots were fused using Quantity-one software (BIO-RAD).

SDS-PAGE fractionation

Molecular mass (MM) determination of unknown proteins by SDS-PAGE was performed as described previously[26,36]. Briefly, crude extracts (80 μg) prepared from *P. syringae* pv. *phaseolicola* NPS3121 wt strain grown in M9 minimal medium at 18 °C, were electrophoresed on a 12% SDS-polyacrylamide gel under similar conditions to those mentioned above. The lane containing protein extracts was sliced uniformly into molecular mass intervals. Gel slices were crushed into 1.5 volumes of renaturation buffer (3% Triton X-100, 20 mM Hepes, 100 mM NaCl, 5 mg/ml BSA, 3 mM ZnCl$_2$, 3 mM MgCl$_2$, 2 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine–HCl) and incubated overnight at 4 °C. The polyacrylamide debris was discarded by centrifugation, and the supernatant was then used to evaluate the DNA binding activity by gel shift assays using the reaction conditions previously mentioned. The P$_{phtM}$ probe was used in the gel shift assays. Prestained protein markers were included in the assays for molecular mass estimation. The proteins within the molecular mass intervals of 14–20 kDa and 30–45 kDa were analyzed by this technique.
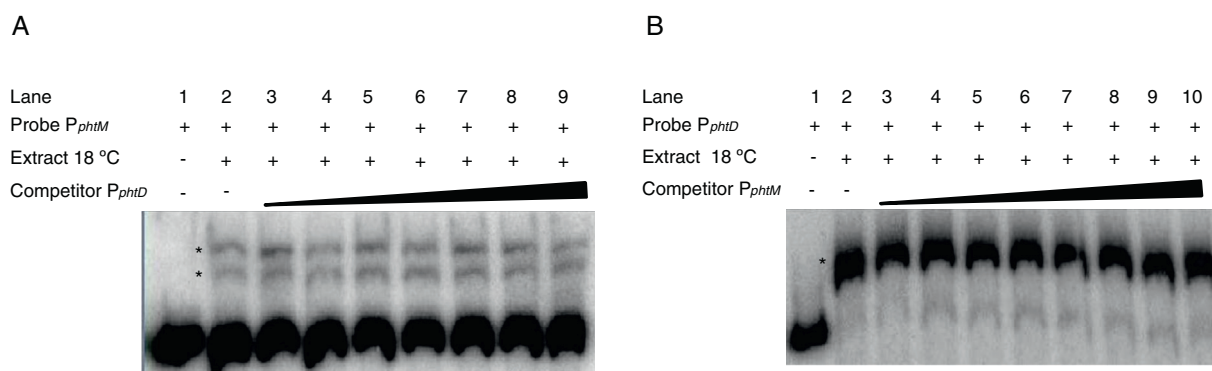
Results

The promoter region of the *phtM* operon contains binding sites for putative regulatory proteins

In order to identify potential transcriptional regulators of the *phtM* operon, we began this study by conducting an EMSA, to evaluate the binding activity of the *phtM* promoter region to proteins present in the extracts from *P. syringae* pv. *phaseolicola* NPS3121 grown at 18 °C (the optimal temperature for toxin production) or 28 °C. A 300 bp radiolabeled DNA fragment (P$_{phtM}$), spanning positions −131 to +167 relative to the transcription start site of the *phM* operon[1] was used as probe (Fig. 1B). Mobility shift assays showed the presence of two specific DNA–protein complexes with extracts of cells grown at 18 °C and 28 °C (Fig. 1D) suggesting that the *phtM* promoter region contains a binding site for a putative regulatory protein whose presence, on the basis of the gel shift assays, is independent of temperature. Furthermore, the formation of two DNA–protein complexes also suggests the possible binding of two regulatory proteins and/or multimer protein on the *phtM* promoter region.

The *phtM* and *phtD* operons do not share a common mechanism of regulation

Previous works had suggested a common mechanism of transcriptional regulation for *phtM* and *phtD* operons due to the presence of conserved sequences in the promoter regions of both genes, which could be recognized by a similar or unique transcriptional regulator[1]. In order to evaluate whether the *phtM* and *phtD* promoters regions (P$_{phtM}$ and P$_{phtD}$ respectively) are targets of the putative regulatory protein, revealed through the gel shift assays, gel-shift competition assays were performed using the promoter region of the *phtD* operon (P$_{phtD}$)[4], as competitor into gel shift assays that evaluate the binding activity of the *phtM* promoter region (P$_{phtM}$) and *vice versa*. The P$_{phtM}$ and P$_{phtD}$ regions contain the conserved sequences previously mentioned[1]. The results of these assays showed that the addition of the

A

Lane	1	2	3	4	5	6	7	8	9
Probe P$_{phtM}$	+	+	+	+	+	+	+	+	+
Extract 18 °C	-	+	+	+	+	+	+	+	+
Competitor P$_{phtD}$	-	-							

B

Lane	1	2	3	4	5	6	7	8	9	10
Probe P$_{phtD}$	+	+	+	+	+	+	+	+	+	+
Extract 18 °C	-	+	+	+	+	+	+	+	+	+
Competitor P$_{phtM}$	-	-								

Figure 2 Gel shift competition assays. (A) Gel shift competition assays using the P$_{phtD}$ region as competitor of the P$_{phtM}$ fragment. The concentrations of unlabeled DNA competitor used were: lanes 1 and 2, no DNA competitor; lane 3, 50 ng (0.26 pmol); lane 4, 60 ng (0.30 pmol); lane 5, 100 ng (0.51 pmol); lane 6, 150 ng (0.77 pmol); lane 7, 200 ng (1 pmol); lane 8, 250 ng (1.3 pmol) and lane 9, 300 ng (1.5 pmol). (B) Gel shift competition assays evaluating the competing capacity of the P*phtM* fragment on the P$_{phtD}$–IHF interaction. The competitor was used in increasing concentrations: 50 ng (0.26 pmol); 60 ng (0.30 pmol); 100 ng (0.51 pmol); 150 ng (0.77 pmol); 200 ng (1 pmol); 250 ng (1.3 pmol) and 300 ng (1.5 pmol). The asterisks (*) indicate the DNA–protein complexes.

P$_{phtD}$ competitor in increasing concentrations does not interfere with the formation of protein–P$_{phtM}$ complexes, indicating that this P$_{phtD}$ site is not target of the putative regulatory protein of the *phtM* operon (Fig. 2A). Likewise, similar results were obtained when the P$_{phtM}$ region was used as competitor into the gel shift assays that evaluate the binding activity of the *phtD* promoter region (P$_{phtD}$), indicating that P$_{phtM}$ is not a target of the *phtD* promoter binding protein, which has been previously identified as the IHF protein[4] (Fig. 2B). These results suggest that these proteins, as revealed through the gel shift assays, are specific for the regulation of each operon.
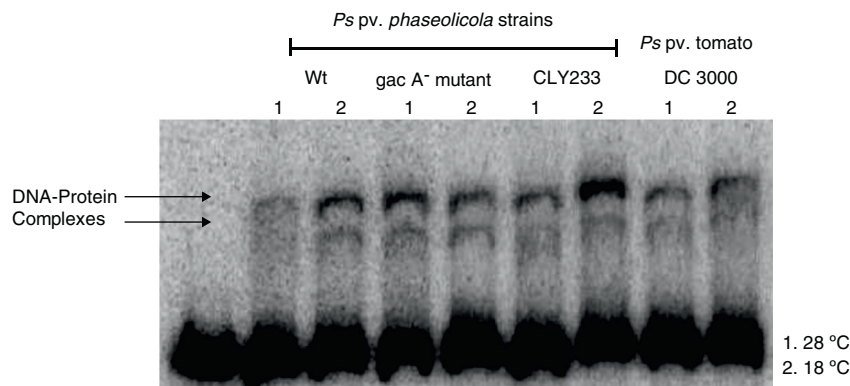
The presence of the putative regulatory proteins of the *phtM* operon is independent of the Pht region and of the GacS–GacA two-component system

Since previous studies suggest that genes within the Pht cluster, particularly the *phtL* gene, could be involved in the regulation of the *phtM* operon[1], we decided to evaluate whether the putative transcription factors of the *phtM* operon as revealed through the mobility shift analysis were encoded outside or within the Pht cluster. Gel-shift assays using crude extracts from *P. syringae* pv. *phaseolicola* strain CLY233, a non-toxigenic strain lacking the Pht cluster and *P. syringae* pv. *tomato* DC3000 (non-phaseolotoxin producer) grown at 18 °C and 28 °C in M9 minimal medium were performed. The incubation of the P$_{phtM}$ fragment with the protein extracts previously mentioned showed the presence of two identical DNA–protein complexes to those obtained with protein extracts from *P. syringae* pv. *phaseolicola* NPS3121 (Fig. 3). Gel-shift competition assays with specific and non-specific probes indicated that the observed DNA–protein binding was specific for the P$_{phtM}$ region (data not shown). These results indicate that the putative transcription factor binding upstream *phtM* is encoded by a gene located outside the Pht region and this is not specific for the phaseolotoxin synthesis due to the presence of this protein in other nonphaseolotoxin-producing *P. syringae* pathovars.

Furthermore, because previous reports have suggested the influence of the GacS–GacA two component system on the expression of the Pht cluster genes by its effect on regulatory proteins[9], we decided to evaluate whether the putative transcription factors binding upstream *phtM* are part of the hierarchical regulatory network of the GacS/GacA system. Gel-shift assays were performed using protein extracts from *P. syringae* pv. *phaseolicola gacA*⁻ mutant grown at 18 °C and 28 °C in M9 minimal medium. Mobility shift assays showed the formation of two specific DNA–protein complexes in a similar position to that obtained with the wild type strain (Fig. 3). These assays suggest that the presence of the putative transcription factors binding upstream *phtM* is independent of the regulatory pathway of the GacS–GacA two-component system.

The binding site for the putative transcription factors of the *phtM* operon is located within a 58 bp region

In order to characterize the *cis*-acting elements involved in the binding of the putative regulatory proteins of the *phtM* operon, we performed gel shift competition assays to delimit the binding site of these proteins. Different fragments of the P$_{phtM}$ (Fig. 1B) region were used as unlabeled competitors in increasing concentrations. The results of these assays showed that the retarded band was effectively competed by fragments b, c, e and h (Table 1), thus indicating that the binding site of the regulatory protein is located in a 58 bp region that spans positions −43 to +14, relative to the *phtM* operon transcription start site (Fig. 1C). Although fragments such as f and g, which span shorter regions within the h fragment (Fig. 1B, Table 1), were used in the competition assays, these were unable to compete the DNA–protein binding (supplementary material II). To evaluate the presence of conserved sequences to the 58 bp region within the genome of the bacterium as well as in related *P. syringae* strains (*P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* B728), BLAST analyses were performed using the microbial nucleotide database

Figure 3 Gel shift assays with crude extracts of different pathovars of *P. syringae*. Radiolabeled P$_{phtM}$ fragment was incubated with protein extracts of *P. syringae* strains:pv. *phaseolicola* NPS3121 wt and *gacA$^-$* mutant; pv. *phaseolicola* CLY233 and pv. tomato DC3000, grown at 28 °C (1) and 18 °C (2) in M9 minimal medium.

optimizing for megablast, discontiguous megablast and BLASTn algorithms using default parameters. The results of these analyses showed that the ''h'' sequence does not exhibit similarity with any other region of the chromosome. These analyses showed that the 58 bp region matched itself, with 100% identity in the regions of the available sequences of *P. syringae* pv. *phaseolicola*, such as the complete genome of the1448A strain (GenBank accession number CP000058.1); phaseolotoxin synthesis gene cluster of NPS3121 strain (DQ141263.1) and argK-tox gene cluster of pathovar *phaseolicola* (AB237164.1). Furthermore, the results showed that no similarity of the 58 bp region was found with sequences of related *P. syringae* strains, with the exception of phaseolotoxin producer bacterium *P. syringae* pv. actinidae ICMP 9853 (CP018202.1) (data not shown).
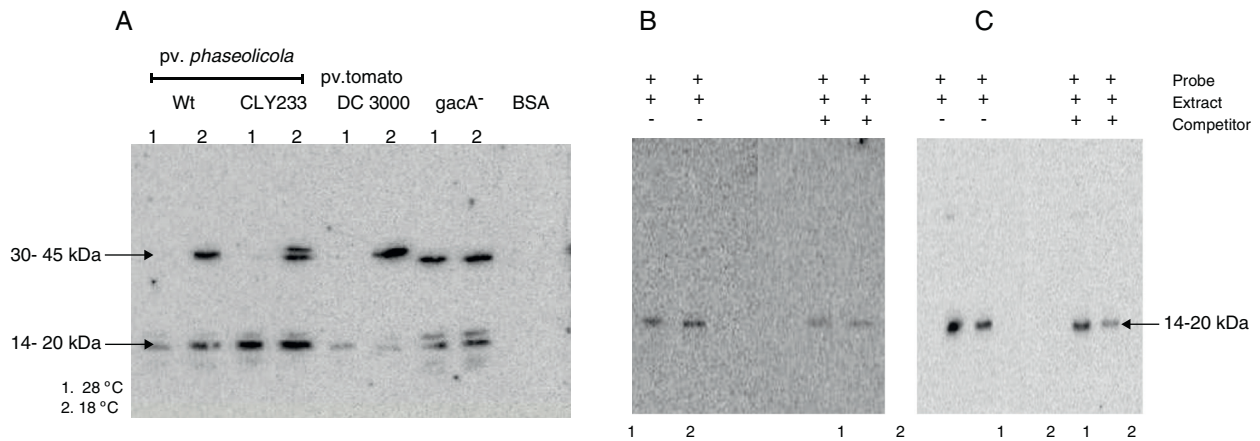
The putative transcription factor of the *phtM* operon has an apparent molecular mass in the 14–20 kDa range

Once the binding site for the *phtM* regulatory protein had been delimited to a 58 bp region, we evaluated the presence of putative *cis*-acting elements within the *phtM* promoter region using a transcription factor search program (BPROM, http://www.softberry.com). However the *in silico* analysis did not yield any positive results regarding transcription factors able to recognize this region. On the other hand, the search of inverted or direct repeats within the 58 bp region by REPuter software[18] using standard parameters showed the presence of three inverted repeats and two palindromic sequences with variable position and length within this region (data not shown). Therefore, in order to characterize the putative transcription factors binding upstream of the *phtM* operon, we performed Southwestern assays to estimate the molecular mass of these proteins. Protein extracts from *P. syringae* pv. *phaseolicola* NPS3121 strains wt and *gacA$^-$* mutant, *P. syringae* pv. *phaseolicola* CLY233 and from *P. syringae* pv. tomato DC3000 grown at 18 °C and 28 °C, which contain the putative transcription factors, were electrophoresed on 12% SDS-polyacrylamide gels and incubated with the P$_{phtM}$ fragment. BSA protein was electrophoresed simultaneously and used as control to

validate results. The assays identified a clear DNA binding activity with an apparent molecular mass in the 14–20 kDa range in all evaluated cell extracts. Likewise, a DNA binding activity was identified with an apparent molecular mass in the 30–45 kDa range solely with the protein extracts from *P. syringae* pathovars grown at 18 °C with the exception of the *gacA$^-$*mutant, in which the DNA binding activity was observed at both temperatures (Fig. 4A). Competition assays with specific probes indicated that the DNA binding activity observed in the 30–45 kDa range appears to be unspecific because only those in the 14–20 kDa range decrease in the presence of increasing concentrations of competitor (data not shown).

The 14–20 kDa regulatory protein binds to the *phtM* promoter as monomer or multimer of a single polypeptide

To validate the above results, two types of additional experiments were performed: 1) Southwestern assays using the shorter fragments (''c'' and ''h'') as probes of the *phtM* promoter, which contain the binding site for the putative regulatory proteins and, 2) Molecular Mass (MM) Fractionation assays. The Southwestern assays were performed using protein extracts from *P. syringae* pv. *phaseolicola* NPS3121 wt strain grown at 18 °C and 28 °C, which were electrophoresed on 12% SDS-polyacrylamide gels and incubated with the ''c'' fragment (104 bp) of the *phtM* promoter region. The results of these assays showed only the presence of a polypeptide with DNA binding activity with molecular mass in the 14–20 kDa range (Fig. 4B). Likewise, the Southwestern assays performed using the ''h'' fragment (58 bp) as probe, corresponding to the minimal binding region of *phtM* regulatory proteins, identified a similar DNA binding activity in the 14–20 kDa range (Fig. 4C). The competition assays showed that the DNA–protein binding observed is specific to the region (Fig. 4B, C). These results indicate that a putative regulatory protein of the *phtM* operon has an apparent molecular mass in the 14–20 kDa range. However, given the constraints of the Southwestern assays using proteins under denaturing conditions, it is possible that the *phtM* protein

Figure 4 Southwestern assays and competition assays. (A) Southwestern assays with crude extracts of different pathovars of *P. syringae* grown at 28 °C (1) or 18 °C (2) using the P$_{phtM}$ fragment as probe. The arrows indicate the DNA–protein complexes. (B) Southwestern assay and competition assay using the ''c'' fragment of the upstream region of the *phtM* operon as probe (left) and as competitor (right). Fragment ''c'' contains the binding site for the putative regulatory protein. (C) Southwestern assay and competition assay using the ''h'' fragment of the *phtM* operon upstream region as probe (left) and as competitor (right). Fragment ''h'' corresponding to the 58 bp-region defined as binding site for the protein. 1 = extract of *P. syringae* pv. *phaseolicola* NPS3121 wt grown at 28 °C, and 2 = extract of *P. syringae* pv. *phaseolicola* NPS3121 wt grown at 18 °C.

may bind this region either as a monomer or a multimer of a single polypeptide.

Finally, strong evidence concerning the molecular mass of the P$_{phtM}$ binding protein was obtained through SDS-PAGE-molecular mass (MM) fractionation. Protein extracts from *P. syringae* pv. *phaseolicola* NPS3121 grown at 18 °C were electrophoresed on 12% SDS-polyacrylamide gels and fractions of molecular mass in 14–20 kDa range and 30–45 kDa range were excised and eluted from the gel. Proteins of each fraction were renatured and evaluated by gel shift assays for P$_{phtM}$ binding activity. The results showed that the incubation of the P$_{phtM}$ fragment (a) with proteins of molecular mass in the 14–20 kDa interval were capable to produce retarding signals in identical position to those obtained with crude extracts from the wt strain, unlike proteins in the 30–45 kDa range, which were unable to produce a DNA–protein complex (Fig. 5). Furthermore, the results of P$_{phtM}$ binding activity of the 14–20 kDa fraction showed the formation of three other DNA–protein complexes that are not observed in the crude extract binding profile. These complexes might represent multimers of the *phtM* binding proteins. The results of these assays demonstrate that the *phtM* binding protein has a 14–20 kDa molecular mass and it does not require additional proteins for its binding to the *phtM* promoter where it binds as monomer or multimer of a single polypeptide.
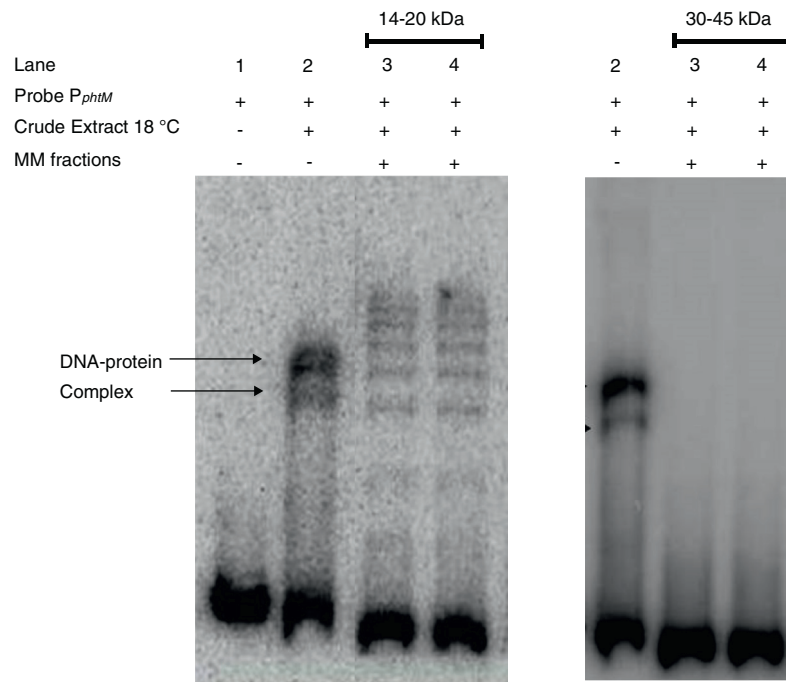
Discussion

Phaseolotoxin is an important virulence factor of *P. syringae* pv. *phaseolicola* and a key element for halo blight disease development in beans. The coding ability for the synthesis of this compound lies mainly in genes that can be found in the Pht cluster, and whose expression is mainly regulated by low temperature[1]. The knowledge about the regulatory pathways involved in the expression of the Pht cluster genes, and

phaseolotoxin synthesis is very scarce, and studies focusing in this issue are necessary, to expand our understanding about the molecular basis governing these processes. In this work we initiated the search and characterization of transcription factors involved in the expression of genes of the phaseolotoxin synthesis (Pht cluster); particularly of the *phtM* operon in *P. syringae* pv. *phaseolicola* NPS3121.

The analysis of the upstream region of the *phtM* operon (P$_{phtM}$) by electrophoretic mobility shift assays, clearly show the presence of two specific retarding signals, indicating that binding sites for regulatory proteins are found within this region. These results also indicated the possible binding of two regulatory proteins and/or multimer protein on the *phtM* promoter region, whose presence is independent of the temperature. Although the idea of a common mechanism of regulation for the *phtD* and *phtM* operons has been postulated, on the basis of the presence of six conserved sequences in the promoters of both genes[1]. The results of the gel competition assays using the *phtM* promoter region as competitor in the gel shift assays that evaluate the *phtD* promoter, clearly demonstrate that the upstream *phtM* region (P$_{phtM}$) is not a target of the IHF protein, which has already been identified as a protein binding to the *phtD* promoter[4]. These results indicate that the identity of the putative regulatory proteins binding *phtM*, as revealed in the gel shift assays, does not correspond to an IHF protein. Furthermore, the results of the gel competition assays using the *phtD* promoter region as competitor in gel shift assays that evaluate the *phtM* promoter strongly suggest that specific regulatory pathways are involved in the expression of each transcriptional unit of the Pht cluster. However, we still cannot rule out the possibility that a common mechanism of regulation exists between the *phtD* and *phtM* operons, since the conserved sequences in these regions might be targets for a different protein, whose presence could not be observed under the conditions used in this study.

Figure 5 The *phtM* promoter binding protein corresponds to a 14–20 kDa polypeptide. Protein extract of *P. syringae* pv. *phaseolicola* NPS3121 (wt) grown at 18 °C was analyzed by SDS-PAGE, and Molecular Mass (MM) fractions in the 14–20 kDa and 30–45 kDa range were isolated in gel slices. Proteins were eluted, renatured and evaluated by EMSA. The P*phtM*–protein complex formation was detected in the 14–20 kDa fraction. MM fractions were evaluated in duplicate.

The assays with *P. syringae* pv. *phaseolicola* strain CLY233 (which lacks the Pht cluster) and *P. syringae* pv. tomato DC3000 (non phaseolotoxin-producer) show the formation of two DNA–protein complexes in identical position to that obtained with our working strain, indicating that the putative regulatory proteins of *phtM* are not encoded within the Pht cluster, thus ruling out the PhtL protein as responsible for the formation of the DNA–protein complexes observed. The regulation of the *phtM* operon by the PhtL protein has been suggested on the basis of a lack of expression of genes within the *phtM* operon in a *phtL*⁻ mutant background[1]. However, a previous work has postulated that the regulatory role of the PhtL protein on the Pht cluster genes may be indirect through its effect on the Fur global regulator[14]. Additionally, the results of these assays indicate that *phtM* binding proteins are non-exclusive of the *phaseolicola* pathovar due to their presence in other non phaseolotoxin-producing pathovars of *P. syringae*, further suggesting that these could be global regulators. These results confirm the previous conclusions made by our working group, which indicate that the Pht cluster of genes has been integrated into the global regulatory mechanisms of the bacterium after the horizontal transfer event[4]. Some authors suggest that this event allows the host cells to control the expression of transferred genes thus avoiding unregulated expression that could have harmful consequences besides having a high energy cost[10,20]. The minimal region necessary for the binding of the putative regulatory proteins of *phtM* was delimited to a 58 bp (−43 to +14) region, which still contains four of the six conserved sequences between the *phtD* and *phtM* promoters. Bioinformatics analysis showed that this region is not found in another part of the chromosome of the bacterium

and further that it is not conserved in related *P. syringae* strains. Likewise, the bioinformatic analyses did not identify any known target sequences for transcription factors within this region, therefore preventing any possible inference regarding the regulatory pathways involved in the process. Nevertheless, previous research has found that the position of the binding site on the DNA relative to the transcription start site is indicative of the regulatory function of the protein. Thus, the repressors have downstream binding sites in relation to the RNA polymerase binding site at −35, while the large majority of activators have upstream binding sites[5]. This idea could suggest a negative role for the proteins binding upstream of the *phtM* region. However, because some atypical activators have demonstrated binding in downstream sites to the RNA polymerase binding site at −35, so far a positive regulatory function for the binding *phtM* protein[5] could not be ruled out. More experimental evidence is necessary to determine its regulatory role. Experiments focused on the characterization of the binding protein to the *phtM* promoter may allow us to speculate about the action mode of the putative regulatory protein and about those characteristics or properties providing support to our ideas. In this sense, the Southwestern blot assays and SDS-PAGE-molecular mass (MM) fractionation using the a (P$_{phtM}$), c and h probes of the *phtM* promoter show a clear DNA-binding activity in proteins present in the 14–20 kDa range, indicating that the putative transcription factor of the *phtM* operon has a molecular mass within this range. Furthermore, on the basis of the constrained conditions of these assays, the results demonstrate that the putative transcription factor recognizes and binds to the upstream *phtM* region as monomer o multimer of a single polypeptide. Experimen-

tal work is still necessary to determine the identity of the regulatory protein of the *phtM* operon. In conclusion, the results of this study have allowed us to gain insight about the regulatory pathways involved in the phaseolotoxin synthesis and to further lay the groundwork for future research. From the data obtained in this study it is possible to device strategies aimed to the purification and identification of this putative regulatory protein of the *phtM* operon eventually leading to the identification of the gene/operon that encodes this protein.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

All authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ram.2017.07.003

References

1. Aguilera S, López-López K, Nieto Y, Garcidueñas-Piña R, Hernández-Guzmán G, Hernández-Flores J, Murillo J, Álvarez-Morales A. Functional characterization of the gene cluster from *Pseudomonas syringae* pv. *phaseolicola* NPS3121 involved in synthesis of phaseolotoxin. J Bacteriol. 2007;189:2834–43.
2. Aguilera S, De la Torre-Zavala S, Hernández-Flores J, Murillo J, Bravo J, Álvarez-Morales A. Expression of the gene for resistance to phaseolotoxin (*argK*) depends on the activity of genes *phtABC* in *Pseudomonas syringae* pv. *phaseolicola*. PLOS ONE. 2012;7:e46815. http://www.plosone.org/
3. Arai T, Kino K. A novel L-amino acid ligase is encoded by a gene in the phaseolotoxin biosynthetic gene cluster from *Pseudomonas syringae* pv *phaseolicola* 1448A. Biosci Biotechnol Biochem. 2008;72:3048–50.
4. Arvizu-Gómez J, Hernández-Morales A, Pastor-Palacios G, Brieba L, Álvarez-Morales A. Integration Host Factor (IHF) binds to the promoter región of the *phtD* operon involved in phaseolotoxin synthesis in *P. syringae* pv. *phaseolicola* NPS3121. BMC Microbiol. 2011;11:90. http://www.biomedcentral.com/bmcmicrobiol/
5. Babu M, Teichmann S. Functional determinants of transcription factors in *Escherichia coli*: protein families and binding sites. Trends Genet. 2003;19:754–79.
6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem. 1976;72:248–54.
7. Buell C, Joardar V, Lindeberg M, Selengut J, Paulsen I, Gwinn M, Dodson R, Deboy R, Durkin A, Kolonay J, Madupu R, Daugherty S,

Brinkac L, Beanan M, Haft D, Nelson W, Davidsen T, Zafar N, Zhou L, Liu J, Yuan Q, Khouri H, Fedorova N, Tran B, Russell D, Berry K, Utterback T, Van Aken S, Feldblyum T, D'Ascenzo M, Deng W, Ramos A, Alfano J, Cartinhour S, Chatterjee A, Delaney T, Lazarowitz S, Martin G, Schneider D, Tang X, Bender C, White O, Fraser C, Collmer A. The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. Proc Natl Acad Sci U S A. 2003;100:10181–6.
8. Chen W, Kuo T. A simple and rapid method for the preparation of gram negative bacterial genomic DNA. Nucleic Acids Res. 1993;21:2260.
9. De la Torre-Zavala S, Aguilera S, Ibarra-Laclette E, Hernández-Flores J, Hernández-Morales A, Murillo J, Álvarez-Morales A. Gene expression of Pht cluster genes and a putative non-ribosomal peptide synthetase required for phaseolotoxin production is regulated by GacS/GacA in *Pseudomonas syringae* pv. *phaseolicola*. Res Microbiol. 2011;20:1–11.
10. Dorman CJ. Regulatory Integration of horizontally-transferred genes in bacteria. Front Biosci. 2009;14:4103–12 [on-line].
11. Ferguson AR, Johnston J. Phaseolotoxin: chlorosis, ornithine accumulation and inhibition of ornithine carbamoyltransferase in different plants. Physiol Plant Pathol. 1980;16:269–75.
12. Genka H, Baba T, Tsuda M, Kanaya S, Mori H, Yoshida T, Noguchi M, Tsuchiya K, Sawada H. Comparative analysis of *argK-tox* clusters and their flanking regions in phaseolotoxin-producing *Pseudomonas syringae* pathovars. J Mol Evol. 2006;63:401–14.
13. González AI, Pérez de la Vega M, Ruiz ML, Polanco C. Analysis of the *argK-tox* gene cluster in nontoxigenic strains of *Pseudomonas syringae* pv. *phaseolicola*. Appl Environ Microbiol. 2003;69:4979–82.
14. González-Villanueva L, Arvizu-Gómez J, Hernández-Morales A, Aguilera-Aguirre S, Álvarez-Morales A. The PhtL protein of *Pseudomonas syringae* pv. *phaseolicola* NPS3121 affects the expression of both phaseolotoxin cluster (Pht) and Non-Pht encoded genes. Microbiol Res. 2014;169:221–31.
15. Goss RW. The relation of temperature to common and halo blight of beans. Phytopathology. 1970;30:258–64.
16. Hatziloukas E, Panopoulos N, Delis S, Prosen D, Schaad N. An open reading frame in the approximately 28-kb *tox-argk* gene cluster encodes a polypeptide with homology to fatty acid desaturases. Gene. 1995;166:83–7.
17. Hernández-Guzmán G, Álvarez-Morales A. Isolation and characterization of the gene coding for the amidinotransferase involved in the biosynthesis of phaseolotoxin in *Pseudomonas syringae* pv. *phaseolicola*. Mol Plant Microbe Interact. 2001;14:545–54.
18. Kurtz S, Choudhuri J, Ohlebusch E, Schleiermacher C, Stoye J, Giegerich R. REPuter the manifold applications of repeat analysis on genomic scale. Nucleic Acids Res. 2001;29:4633–42.
19. Latchman D. Transcription Factors A practical approach. 2nd ed. New York: Oxford University Press; 1999.
20. Lercher M, Pál C. Integration of horizontally transferred genes into regulatory interaction networks takes many million years. Mol Biol Evol. 2008;25:559–67.
21. Mitchell RE. Bean halo-blight toxin. Nature. 1976;260:75–6.
22. Mitchell RE. Isolation and structure of a chlorosis inducing toxin of *Pseudomonas phaseolicola*. Phytochemistry. 1976;15:1941–7.
23. Mitchell RE, Bieleski R. Involvement of phaseolotoxin in Halo blight of beans. Plant Physiol. 1977;60:723–9.
24. Mosqueda G, Van de Broeck G, Saucedo O, Bailey A, Álvarez-Morales A, Herrera-Estrella L. Isolation and characterization of the gene from *Pseudomonas syringae* pv. *phaseolicola* encoding the phaseolotoxin-insensitive ornithine carbamoyltransferase. Mol Gen Genet. 1990;222:461–6.
25. Nüske J, Fritsche W. Phaseolotoxin production by *Pseudomonas syringae* pv. *phaseolicola*: the influence of temperature. J Basic Microbiol. 1989;29:441–7.

26. Ossipow V, Laemmli U, Schibler U. A simple method to renature DNA-binding proteins by SDS-polyacrylamide electrophoresis. Nucleic Acid Res. 1993;21:6040–1.

27. Peet RC, Panopoulos N. Ornithine carbamoyltranferase and phaseolotoxin immunity in *Pseudomonas syringae* pv. *phaseolicola*. EMBO J. 1987;6:3585–91.

28. Rico A, López R, Asensio C, Aizpún MT, Asensio-S-Manzanera MC, Murillo J. Nontoxigenic strains of *Pseudomonas syringae* pv. *phaseolicola* are a main cause of halo blight of beans in Spain and escape current detection methods. Phytopathology. 2003;93:1553–9.

29. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning; a laboratory manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.

30. Sawada H, Kanaya S, Tsuda M, Suzuki F, Azegami K, Saitou NA. Phylogenomic study of the OCTase genes in *Pseudomonas syringae* pathovars: the horizontal transfer of the *argK*-tox cluster and the evolutionary history of OCTase genes on their genomes. J Mol Evol. 2002;54:437–57.

31. Sawada H, Suzuki F, Matsuda I, Saitou N. Phylogenetic analysis of *Pseudomonas syringae* pathovars suggests the horizontal gene transfer of *argK* and the evolutionary stability of *hrp* gene cluster. J Mol Evol. 1999;49:627–44.

32. Singh Uma S, singh Rudra P, Kohmoto Keisuke. Pathogenesis and host specificity in plant diseases. Histopathological, biochemical, genetic and molecular bases. 1st ed. Pergamon Press; 1995. p. 80.

33. Tamura K, Imamura M, Yoneyama K, Kohno Y, Takikawa Y, Yamaguchi I, Takahashi H. Role of phaseolotoxin production by *Pseudomonas syringae* pv. *actinidae* in the formation of halo lesions of kiwifruit canker disease. Physiol Mol Plant Pathol. 2002;60:207–14.

34. Templeton MD, Sullivan P, Shepherd M. The inhibition of ornithine transcarbamoylase from *Escherichia coli* W by phaseolotoxin. Biochem J. 1984;224:379–88.

35. Tourte C, Manceau C. A strain of *Pseudomonas syringae* which does not belong to pathovar *phaseolicola* produces phaseolotoxin. Eur J Plant Pathol. 1995;101:483–90.

36. Woo A, Dods J, Susanto E, Ulgiati D, Abraham L. A proteomics approach for the identification of DNA binding activities observed in the electrophoretic mobility shift assays. Mol Cell Proteomics. 2002;47:472–8.