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Identification of an extracellular infection-induced glyceraldehyde-3-phosphate dehydrogenase of the phytopathogenic proteobacterium *Pseudomonas syringae* pv tomato DC3000

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ABSTRACT

According to molecular biology, genomic and proteomic data, the phytopathogenic gamma-proteobacterium *Pseudomonas syringae* pv. tomato DC3000 produces a number of proteins that may promote infection and draw nutrients from plants. Remarkably, *P. syringae* DC3000 strain possesses three paralogous *gap* genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes with different predicted molecular sizes and metabolic functions. As GAPDH was shown to be a virulence factor in other microbial pathogens, in the current study, we analyzed the expression levels of each paralogous *gap* gene by real-time PCR to understand the actual impact of their protein products on *P. syringae* virulence. We found that all of them were strongly induced during the infection process. Nevertheless, proteomic analysis of culture supernatants revealed that only Class I GAPDH1 encoded by the *gap*1 gene was identified as an extracellular protein in infective cells. These results strongly suggest that this GAPDH should play a role in the infective process, including its well-know enzymatic function in the glycolytic metabolic pathway.

KEYWORDS

GAPDH; Secretome; MALDI-TOF; Gene Expression; Infective State; Bacterial Speak; RNA

1. INTRODUCTION

The gammaproteobacterium *Pseudomonas syringae* pv. tomato DC3000 is an aerobic, rod-shaped, polar, motile and a Gram negative bacteria [1-3], which showed a fluorescent pigmentation in King B medium and oxidatively metabolizes glucose. This pathogenic is also characterized by Levan-positive colonies on nutrient agar medium supplemented with sucrose, exhibits positive hypersensitive reaction in the presence of tobacco leaves; it is oxidase, arginine-dehydrolase negative and does not produce pectolytic enzymes (were potato rot-negative) [4,5].

Disseminated primarily by water, *P. syringae* pv. tomato DC3000 has the potential to move through plug greenhouses very quickly; thus, infested seedlings could become an important inoculum source for field epiphytotics [6,7]. Consequently, this bacterium can survive up to 20 years in the crevices and cavities of the coat of tomato seeds causing bacterial speck, a disease characterized by small brown-black leaf spots sometimes surrounded by chlorotic margins, and dark superficial specks on green fruits specks on ripe fruit may become sunken, and are surrounded by a zone of delayed ripening [2,8,9]. Stunting and yield loss, particularly if young plants are infected, considerably reduced the market value of speckled fruit [9].

This pathogenicity of *P. syringae* DC3000 strain has been the subject of several studies, and it has emerged as an important model organism in molecular plant pathology because of its genetic tractability [10-12], and its genome has been fully sequenced [9]. Remarkably, the pathogenicity of DC3000 resembles that of most animal and other plant pathogens in the Gammaproteobacteria, which relys on a Type III secretion system (TTSS) to re-

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lease virulence effector proteins into host cells [13,14]. Other studies have shown that DC3000 strain encodes a wide range of proteins that should be involved in virulence [12]. One of these proteins is glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a highly conserved enzyme during evolution with a key role in the glycolytic and gluconeogenic pathways. GAPDH catalyzes the redox-linked reversible phosphorylation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, in the presence of $NAD(P)^+$ and inorganic phosphate [15]. Most glycolytic Class I GAPDHs so far studied are homotetrameric proteins with 34 - 38 kDa subunits, and the native oligomer has a molecular weight of 140 - 150 kDa [16,17]. In addition to its classic metabolic roles, GAPDH has been claimed to be involved in a number of diverse cellular processes unrelated to glycolysis ("moonlighting protein"), and many of these alternative roles are dependent on the ability of GAPDH to modify its subcellular localization. GAPDH was used as a model standard protein, or control, in gene regulation and catalytic mechanism related studies, because of its high degree of structural conservation across species [18]. Furthermore, with the advent of the molecular biotechnology, GAPDH recently emerged as a useful model for innovative methods to advance our understanding of the mechanisms through which cells organize and express their genetic information, despite its general consideration as a housekeeping enzyme [19]. Regarding its possible specific role in pathogenic organisms, the presence of a surface-associated GAPDH has been reported in all streptococcal strains tested so far [20,21], and in other diverse microorganisms like *Candida albicans*, *Schistosoma bovis*, *Mycoplasma genitalium*, and *Staphylococcus* spp. [22]. GAPDH was shown to be secreted by several pathogenic bacteria, like *Streptococcus pyogenes* and a number of enteropathogenic *Escherichia coli* strains [20,21,23]. It was claimed that secreted GAPDH has a role in signal transduction to their host cells, rendering them probably more susceptible to bacterial infection [21,24]. Three *gap* genes encoding distinct GAPDH proteins are present in the genome of *P. syringae* pv. tomato DC3000 [25]. Whether any of these bacterial GAPDHs are extracellular proteins involved in the plant infection process remains to be established. In any case, their possible secretion and association to the cell membrane might have biotechnological implications.

In this study we analyzed the expression level of each paralogous *gap* gene of *P. syringae* pv. tomato DC3000 both in the infective and resting states by one-step quantitative RT-PCR, looking for possible differences in mRNA expression between these two physiological status. Besides, the protein secretome of infective cells was analyzed by MALDI-TOF mass spectrometry in order to identify which of the three GAPDHs of this phytopathogenic bacterium exhibits an extracellular localization. In this way, the overexpressed Class I GAPDH1 was identified in the secretome of infective cells, suggesting a possible role of this protein in the virulence of *P. syringae* pv. tomato DC3000.

2. MATERIALS AND METHODS

2.1. Organisms and Growth Conditions

Pseudomonas syringae pv tomato DC3000 cells used in this study were grown in LB medium with glucose (3 g/l) at 28˚C in the presence of rifampicin 50 µg/ml, in the darkness with agitation. For infection studies in solid and liquid medium we adjusted the bacterial suspensions to 10^6 CFUs per milliliter with sterile distilled water.

2.2. Plant Material

Round Tomato (*Solanum lycopersicum* L.) leaflets from two-months old plants were detached with their petioles, from a biological culture of tomato plants grown under natural outdoors conditions in region of Sidi-Rehal (Casablanca, Morocco).

2.3. Plant sterilization Method

Leaflets were treated as follows: soaking in 10% (v/v) commercial bleach-sterilized distilled water for 2 min followed by soaking in 70% (v/v) ethanol-sterilized distilled water for 1 min; then rinsed tree times in sterilized distilled water and placed in sterile Petri dishes until de time of infection.

2.4. Bacterial Infection Method

Detached leaflets were inoculated by injection with a 10^6 of bacterial suspension (from either solid or liquid LB medium) and maintained at 28˚C under a 16 h photoperiod, with high relative humidity. All infection experiments were repeated at least three times. No-infected detached leaflets inoculated with sterile distilled water and maintained in LB medium were used in all experiments as controls.

2.5. Total RNA Isolation

Total RNAs was successfully extracted from bacterial suspensions with Aurum™ Total RNA Mini Kit (Bio-Rad). The final product yielded 260/280 nm ratios in the range 1.8 - 2.05 and its purity was confirmed by agarose gel electrophoresis. The nucleic acid concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific).

2.6. Real-Time PCR

Real-time quantitative PCR based on the use of the Pow-

er SYBR Green PCR Master Mix (Applied Biosystems) was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Reaction mixtures (12.5 µl) contained 6.5 µl of 2x SYBR Green RT PCR reaction mixture, 0.25 µl of iScript reverse transcriptase for one-step RT-PCR, 300 nM of each primer and 10 ng of isolated RNA solution.

2.7. Primers Used

Specific primers for each paralogous *gap* gene of *P. syringae* DC3000 strain were designed using Primer Premier 5.0 program (Biosoft International).

- ***gap*1, PSPTO_1287 (gi|28868497)**
  Fw: GAAGAAGTCAACGCGCTGATG
  Rv: CCCAGGACCTTGGAGTGCT
- ***gap*2, PSPTO_2102 (gi|28869306)**
  Fw: GCACTCGGACCTGCACAAG
  Rv: CACCACTTCCTGCGAGTTCAC
- ***gap*3, PSPTO_0386 (gi|28867616)**
  Fw: TTTCTGGTCCAAGGCTGGTG
  Rv: AAGCCCCACTCGTTATCGAAC

2.8. Concentration of Extracellular Proteins

A 10-ml sample from overnight cultures of *P. syringae* pv tomato DC3000 strain grown in LB medium with glucose (3 g/l) in presence of tomato plant leaflets, was centrifuged for 15 min at 5000 g at 4˚C. The cell-free supernatant was mixed with ice-cold 100% (w/v) trichloroacetic acid up to a final concentration of 7% (w/v) trichloroacetic acid. The protein precipitation was accomplished by incubation of the mixture on ice for 1 h, and then washed with cold acetone. The precipitated protein was collected by centrifugation and eventually resuspended in 50 µl of SDS-PAGE sample buffer containing: 1% (p/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, 0.01% (w/v) bromophenol blue and Tris-HCl (60 mM, pH 6.8). A saturated Tris solution was added to neutralize the sample.

2.9. Protein Gel Electrophoresis (1D SDS-PAGE)

Prior to SDS-PAGE analysis, protein samples were incubated at 100˚C for 3 min. Then, the protein samples were loaded onto a 12% polyacrylamide gel and separated on a Mini Protean II cell (Bio-Rad, Hercules, CA), according to the method of Laemmli [26]. SDS-PAGE was performed at 120 V for 1 h, using a Tris-glycine buffer (25 mM Tris; 192 mM Glycine; 0.1% SDS; pH 8.3). The apparent molecular masses of the proteins were estimated using the Precision Plus Protein Standard (Bio-Rad) and the analysis software Quantity One (Bio-Rad). Western blots after SDS-PAGE and transfer to nitrocellulose membranes were carried out as previously described [27] using anti-GAPDH rabbit antibodies [25].

2.10. MALDI-TOF Mass Spectrometry Identification of Proteins

1) Sample preparation. Protein bands of interest were automatically excised from 1D SDS-PAGE gels after electrophoresis and Coomassie Brilliant Blue R-250 staining. The gel pieces were washed with distilled water (50 µl) during 5 min, then destained with 50 µl acetonitrile twice and vacuum dried during 15 min. The gel pieces were then chemically reduced with 10 mM dithiothreitol in 50 mM ammonium bicarbonate (*Ambic*) for 30 min at 56˚C, incubated with acetonitrile during 5 min, and then alkylated with 55 mM iodoacetamide in 25 mM *Ambic* at room temperature for 20 min in the dark. After that, gel pieces were washed with 25 mM *Ambic* twice, followed by acetonitrile, and dried under vacuum. Gel pieces were dried by shrinking in acetonitrile followed by vacuum drying. Then, all gel pieces were incubated with 12.5 ng/µl of sequencing-grade modified trypsin (Promega) in 50 mM *Ambic* for 30 min at 4˚C. After digestion, the supernatants were recovered. Peptides were then incubated overnight with 10 µl of 50 mM *Ambic* at 37˚C, and eventually extracted from the gel pieces twice with a 0.5% (v/v) trifluoroacetic acid at room temperature during 15 min.

2) MALDI-TOF mass spectrometry. analyses were performed on an Autoflex mass spectrometer (Bruker Daltonics) in the Servicio de Proteómica of IBVF (CSIC-US). All mass spectra were calibrated externally with a peptide mass standard kit (Bruker) and internally with trypsin autolysis peaks.

3) Database search. The peptide mass fingerprinting and peptide fragment ion data obtained from MALDI-TOF analyses were used to search for protein candidates in the NCBI database and genomes databases by using Mascot (Matrix Science) software programs.

3. RESULTS AND DISCUSSION

Plant pathogenic bacteria possess a large number of genes that allow them to grow and cause disease on plants. Analyses of the molecular basis of pathogenesis in *P. syringae*. pv. tomato DC3000 reveal a complex and intimate interaction between bacteria and plant cells that depends on the coordinated expression of multiple pathogenicity and virulence factors. These include cytotoxins, polysaccharides, extracellular proteins, and the translocation of many of these proteins into plant cells by Type III (Hrp) secretion system [13,14,28].

Glyceraldehyde-3-phosphate dehydrogenase was described as one extracellular protein in a number of pathogenic microorganisms, both prokaryotes and eukaryotes [22]. Remarkably, genome sequence analysis re-

vealed the occurrence in the phytopathogenic proteobacterium *P. syringae*. pv. tomato DC3000 of three paralogous *gap* genes located in distant regions of the 6.5-Mb chromosome with different genomic organization: namely at ca. 1.41 (*gap*1), 2.28 (*gap*2) and 0.43 (*gap*3) Mb from the replication origin. Moreover, these paralogous genes are predicted to encode three GAPDH enzymatic proteins with distinct molecular and catalytic features, namely two Class I enzymes having different molecular mass subunits and one class III D-erythrose-4-phosphate dehydrogenase/GAPDH bifunctional enzyme [25].

In the present study total RNA was extracted as described in the Material and Methods section from both normal (bacteria in nutritive medium) and infective (bacteria in contact with tomato plant) *P. syringae*. pv. tomato DC3000 cells. RNA yields being in the range 70 - 100 µg per bacterial cell pellet. The determined A_{260}/A_{280} ratios ranged between 2.0 and 2.2 indicating high RNA purity and absence of proteins and polysaccharide contaminations. The integrity of extracted RNA preparations was further analyzed by migration in agarose gel electrophoresis (data not shown). Then the expression level of the three paralogous *gap* genes was subsequently determined by one-step real time-PCR. The obtained results clearly show that in the infective state there is a clear up-expression of transcripts of the three *gap* genes in comparison with the normal state of the bacterium (**Figure 1**). Moreover, we noticed a slight expression difference between the three *gaps* genes in this physiological state (data not shown).

These results are in agreement with previous reports claiming that the GAPDH is among the proteins involved in virulence of many pathogenic bacteria [29], and with

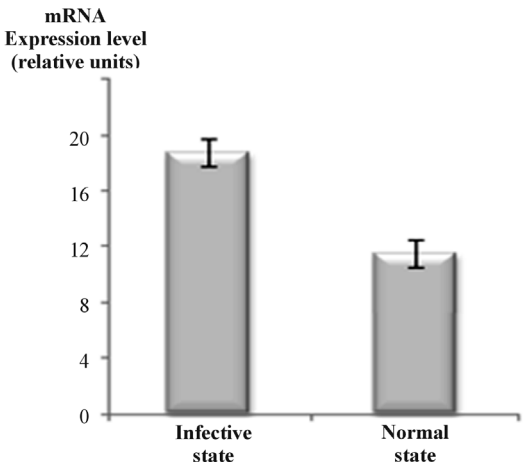

Figure 1. *P. syringae* pv. tomato DC3000 infective cells have higher expression levels of all the three *gap* genes when compared with normal cells, as estimated by real-time quantitative PCR analysis. Bars represent mean values ± SD of three independent experiments. Significant statistical differences in Student's test ($p \leq 0.05$).

others suggesting that a secreted GAPDH has a role in signal transduction to the host cells, rendering them more susceptible to bacterial infection [21,24].

However, three GAPDH-encoding genes are present in the genome of *P. syringae* pv. tomato DC3000 [25], it remains to be established whether any of these GAPDHs are actually extracellular proteins involved in the plant infection process. As described, the pathogenicity of DC3000 resembles those of most animal and plant microbial pathogens of the Gammaproteobacteria, which in the infective state secrete a number of proteins across their cell envelopes [30-32]. Plant-pathogen interactions involve complex processes including translocation of proteins across two cell membranes, the cytoplasmic and the outer membrane. For that, analyzing the *P. syringae* pv. tomato DC3000 secretome in the infective state is therefore of particular importance.

In order to ascertain the possible occurrence of an extracellular GAPDH during the infection process and identify which of the three GAPDHs is secreted, the extracellular proteins (secretome) released by *P. syringae* DC3000 cells in the infective state were analyzed by a proteomic approach in concentrates of culture supernatants. After protein resolution of concentrates by SDS-PAGE, automatic excision of selected protein bands from Coomasie blue-stained gels was performed, followed by MALDI-TOF peptide mass fingerprinting as described in Materials and Methods.

A number of protein bands with apparent molecular masses in the range 20 - 200 kDa were resolved in SDS-PAGE gels (**Figure 2**). In this figure we noticed the presence of a major band with the expected molecular mass for the GAPDH subunit (36.3 kDa). To verify this finding, this band with others in the range 20 - 50 kDa (see asterisks **Figure 2**) were selected for MALDI-TOF analysis.

The result of this analyses confirm that the suspected band (36.3 kDa) contain the *gap*1 gene product GAPDH1 as a main component (see **Figure 2** and **Table 1**). We have previously cloned the *gap*1 gene and expressed its protein product in *E. coli* BL21, being a member of the GAPDH family classified into the ubiquitous Class I enzymes that utilize NAD + (EC 1.2.1.12) [25]. This finding has allowed us to know which of the three GAPDHs from *Pseudomonas syringae* pv tomato DC3000 is extracellular.

Bioinformatic searches in NCBI database allow us to identify a number of extracellular proteins in the secretome of this phytopathogenic bacterium. As shown in **Table 1**, the Class I GAPDH1 was identified among other secreted proteins of *P. syringae* DC3000 cells. A similar scenario has been reported for pathogenic *E. coli* strains, namely the GAPDH A is secreted to the periplasmic compartment and the extracellular medium [20-

Table 1. Some extracellular proteins produced by *Pseudomonas syringae* pv tomato DC3000 cells in the infective state[a].

GI Accession Number	Protein Name (Predicted molecular mass)	Functional assignments
gi|28869499	Outer membrane porin OprF (36.4 kDa)	Outer membrane channel and peptidoglycan-associated (lipo) protein.
gi|28872543	Secreted protein Hcp (19.1 kDa)	Hcp1 family. Bacterial secretion system: Type VI secretion system effecttor. Functions in pilli and flagella biogenesis, nutrient acquisition, virulen-ce, and efflux of drugs and other toxins.
gi|28868497	**Glyceraldehyde 3-phosphate dehydrogenase 1 (GAPDH1), Type I (36.5 kDa)**	**Extracellular protein (this work).**
gi|422648999	4-hydroxypyruvate dioxygenase (37.0 kDa)	Oxidizes 4-hydroxyphenylpyruvate, a tyrosine and phenylalanine catabolite, to homogentisate, which can undergo a further non-enzymatic oxidation and polymerization into brown pigments that protect some bacterial species from light. In some bacteria, this enzyme has been studied as a hemolysin.
gi|28871145	Type III effector protein AvrPto1 (18.2 kDa)	Interaction with host via protein secreted by Type III secretion system. Negative regulation by pathogen of defense-related host MAP kinase-mediated signal transduction pathway. Negative regulation of microbe-associated molecular pattern-induced host innate immune response.
gi|28868581	Type III helper protein HrpW1 (42.8 kDa)	Protein with domains similar to harpins and pectate lyases, which can elicit the plant hypersensitive response and bind to pectate (polysaccharide binding). Interaction with host via protein secreted by Type III secretion system.

[a]Proteins identified by MALDI-TOF peptide mass fingerprinting of SDS-PAGE resolved culture media supernatants (cell-free), as described under Materials and Methods.

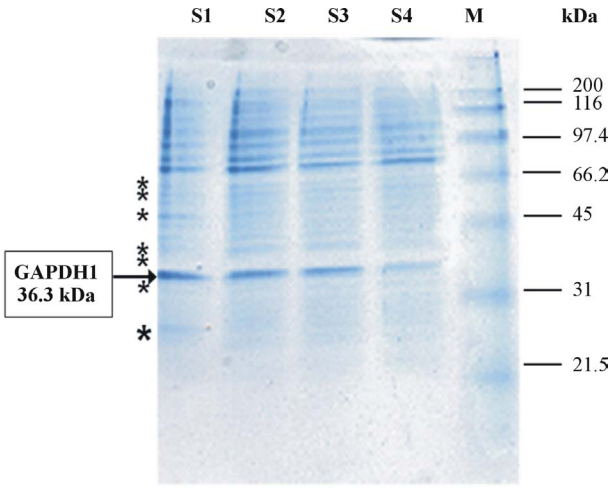

Figure 2. Coomassie Brilliant Blue-stained SDS-PAGE electrophoretogram showing the protein pattern of the secretome of *P. syringae* pv tomato DC3000 cells in the infective state. Protein concentrates (ca. 50 µg protein per lane) from culture supernatants of four independent experiments (S1-S4) were analyzed (S1-4 samples of Ps syringae celles in the infective state). The molecular masses of protein markers (M) are shown on the right side. The arrow on the left side indicates a major protein band with the expected molecular mass of the GAPDH subunit (36.3 kDa), of which the *gap*1 gene product GAPDH1 was identified as a main component by MALDI-TOF analysis. Asterisks indicate the approximate positions of other selected protein bands identified by MALDI-TOF analyses (see **Table 1**).

22]. Moreover, a post-translational modification of the secreted GAPDH has been described in the parasitic protist *Entamoeba histolytica*, while an extracellular form of GAPDH [E.C. 1.2.1.12] was reported as the major secreted protein produced by the endocarditis isolate *S. gordonii* FSS2 [33].

As is shown in **Table 1**, the proteomic analysis of *P. syringae* pv tomato DC3000 culture concentrates allowed us to identify other extracellular proteins already described to be involved in virulence of other pathogenic bacteria, the secretion of which having been previously demonstrated. As the secreted protein Hcp, which play an important role in bacterial fitness, allowing microbial pathogens to survive under conditions where it has to compete with other microorganisms for resources [34]. And the helper protein Hrp, a member of a protein family of secreted proteins encoded by the hypersensitive response and pathogenicity genes (*hrp*) that control the ability of major groups of plant pathogenic bacteria to elicit the hypersensitive response (HR) in resistant plants and to cause disease in susceptible plants [14,35,36]. A number of Hrp proteins share significant similarities with components of Type III secretion apparatus and flagellar assembly apparatus in animal pathogenic bacteria [13,14,30,36-39]. In fact, Type III effector protein AvrPto1, a secreted bacterial protein involved in negative regulation of defense-related host signal transduction pathways, was also identified in the *P. syringae* pv tomato DC3000 secretome (see **Table 1**).

Overall, our proteomic data should be considered a non-exhaustive representative sample of the *P. syringae* pv tomato DC3000 secretome. The concurrent release of Class I GAPDH1 with a number of virulence-involved secreted proteins strongly suggests that this dehydrogenase should be a *bona fide* extracellular protein that may have an active role in *P. syringae* pv tomato DC3000 pathogenicity.

4. CONCLUSION

In this study, we determined the expression levels of the three *gap* genes from the phytopathogenic bacterium *P. syringae* pv tomato DC3000 under infective and normal physiological conditions by one-step Real-Time PCR. Then, the secretome of infective cells was analyzed by MALDI-TOF peptide mass fingerprinting. It was found that there is a clear up-expression of the transcripts of the three *gap* genes in the infective state, in comparison with the normal state. However, only the Class I GAPDH1 isoform was found as an extracellular protein in infective cells, concurrently with a number of secreted proteins that are involved in the virulence of *P. syringae* pv. tomato DC3000.

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ABBREVIATION LIST

GAPDH: glyceraldehyde-3- phosphate dehydrogenase;
TTSS: type III secretion system;
NAD: Nicotinamide adenine dinucleotide;
LB: Luria Broth;
TCA: trichloroacetic acid;
Hrp: hypersensitive response and pathogenicity;
Hcp: Hemolysin Coregulated Protein.

