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Cloning and heterologous overexpression of three *gap* genes encoding different glyceraldehyde-3-phosphate dehydrogenases from the plant pathogenic bacterium *Pseudomonas syringae* pv. tomato strain DC3000

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ABSTRACT

The gammaproteobacterium *Pseudomonas syringae* pv. tomato DC3000 is the causal agent of bacterial speck, a common disease of tomato. The mode of infection of this pathogen is not well understood, but according to molecular biological, genomic and proteomic data it produces a number of proteins that may promote infection and draw nutrients from the plant. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a major enzyme of carbon metabolism that was reported to be a surface antigen and virulence factor in other pathogenic microorganisms, but its possible role in the infection process of *P. syringae* has so far not been studied. Whole-genome sequence analyses revealed the occurrence in this phytopathogenic bacterium of three paralogous *gap* genes encoding distinct GAPDHs, namely two class I enzymes having different molecular mass subunits and one class III bifunctional D-erythrose-4-phosphate dehydrogenase/GAPDH enzyme. By using genome bioinformatics data, as well as alignments of both DNA and deduced protein sequences, the three *gap* genes of *P. syringae* were one-step cloned with a His-Tag in pET21a vector using a PCR-based strategy, and its expression optimized in *Escherichia coli* BL21 to achieve high yield of the heterologous proteins. In accordance with their distinct molecular phylogenies, these bacterial *gap* genes encode functional GAPDHs of diverse molecular masses and nicotinamide-coenzyme specificities, suggesting specific metabolic and/or cellular roles.

Introduction

Tomato is an outstanding crop grown in many countries around the world and under diverse climates, including relatively cold regions, due to the development of vegetable crops under cover. By the volume of its production, tomato remains the first vegetable in the world [1,2]. However, tomato fields are potential targets of pathogenic bacteria that cause diseases ranging from small spots on the foliage to the almost total defoliation of the plant, with severe consequences on photosynthesis and yield potential [3,4] One of these diseases is bacterial speck caused by the gram-negative bacterial strain *Pseudomonas syringae* pv. tomato DC3000, a member of the gamma subgroup of the Proteobacteria [5]. This bacterium has emerged as an important model organism in molecular plant pathology because of its genetic tractability, and its 6.5-Mb genome has been fully sequenced [6,7]. *P. syringae* pv. tomato DC3000 causes leaf lesions characterized by small brown–black specks, 1–3 mm in diameter, with a yellow chlorotic halo [8], and it can survive up to 20 years in the crevices and cavities of the tomato seed coat. These lesions constitute a severe blemish on fruit for fresh market and greatly reduce yield potential [9].

Up to now, the virulence molecular mechanisms of *P. syringae* are not well understood. This strain encodes a wide range of proteins that are involved in virulence, and 298 genes (ca. 5% of the total) have been identified in the virulence category [7]. One of these proteins is glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1], 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

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[1] Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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inorganic phosphate [10].GAPDH family members are classified into the ubiquitous class I enzymes that utilize NAD+(EC 1.2.1.12), NADP+(EC 1.2.1.13) or either (EC 1.2.1.59), class II of archaeal NAD(P)+-dependent GAPDHs, and class III bifunctional enzymes (erythrose-4-phosphate dehydrogenase/GAPDH) that are prevalent among gamma-proteobacteria [11]. Most glycolytic class I GAPDHs studied so far are homotetrameric proteins with 34–38 kDa subunits, and the native oligomer has a molecular mass of 140–150 kDa [12–14].

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”) [15–19]. Many of these alternative roles are dependent on the ability of GAPDH to modify its subcellular localization. In particular, it has been reported to bind DNA and RNA [15,16] and regulate transcription [17,18] in cell nuclei. Besides, GAPDH interacts with a number of small molecules [20] and disease-associated proteins [21,22], and operates as a fusogenic protein [23] and in nuclear membrane assembly [24]. A functional GAPDH has been reported as a cell wall component in budding yeasts [25]. Usually, GAPDH was used as a model protein or control in gene regulation and catalytic mechanism related studies, as well as a standard in Northern and Western blots, because of its high degree of structural conservation across species [18]. However, despite its general consideration as a housekeeping enzyme, GAPDH recently emerged as a useful model for innovative biotechnological methods [26].

Regarding its role in some pathogenic organisms, the presence of a surface-associated GAPDH has been reported on all streptococcal strains tested so far (21), as well as in microorganisms as diverse as *Candida albicans*, *Schistosoma bovis*, *Mycoplasma genitalium*, and *Staphylococcus* spp. [27]. Furthermore, GAPDH was shown to be secreted by several pathogenic bacteria, like *Streptococcus pyogenes* and enteropathogenic *Escherichia coli* strains [28–30]. It was claimed that secreted GAPDH has a role in signal transduction to their host cells, rendering them more susceptible to bacterial infection [28,31]. Since three GAPDH-encoding genes are present in the genome of *P. syringae* pv. tomato DC3000 [5,7], it remains to be established whether any of these GAPDHs are extracellular proteins involved in the plant infection process. Their possible secretion and association to the cell membrane might have biotechnological implications that are worth to be further investigated.

In the present work, we successfully cloned and optimized overexpression in *E. coli* of three paralogous *gap* genes encoding different GAPDHs identified in the genome of *P. syringae* pv. tomato DC3000. Some molecular and catalytic parameters of the recombinant GAPDHs of this phytopathogenic bacterium have been determined revealing the diverse structural and functional features of these enzymes.

Materials and methods

Bioinformatics research: search, alignment and sequences analysis

Searches for sequences of *gap* genes in EMBL/DDBJ/GeneBank databases, and multiple alignments of GAPDH sequences, were performed using the bioinformatics programs Blast and Clustal X v.2.0, respectively. Domain structures of the predicted proteins were analyzed with the ProDom (http://prodom.prabi.fr/prodom/current/html/home.php), SUPERFAMILY (http://supfam.cs.bris.ac.uk/SUPERFAMILY/) and Pfam (http://pfam.sanger.ac.uk/) bioinformatics tools.

Organisms and growth conditions

P. syringae was cultured in LB medium with glucose (3 g/l) at 28 °C in the presence of rifampicin (50 μg/ml) with agitation (200 rpm) in the dark. Unless otherwise indicated, *E. coli* Top10 and BL21 strains were grown in LB medium at 37 °C with agitation (200 rpm), and ampicillin was employed for selection of transformants. Where indicated, an additional carbon source -glycerol, succinate or glucose- was added at a final concentration of 2% (w/v) to the LB medium. When necessary, ampicillin and isopropyl-β-D-thiogalactopyranoside (IPTG) were added at concentrations of 50–100 μg/ml and 1 mg/ml, respectively.

DNA manipulation and cloning strategy

-DNA isolation and purification. A modified phenol–chloroform method was used for the extraction of chromosomal DNA from *P. syringae* pv. tomato DC3000 cells. Purified DNA was eventually washed with 70% (v/v) ethanol, air dried and resuspended in 100–200 μl of TE buffer. DNA concentration was estimated after 100-fold dilution in 1 ml of TE and measuring ultraviolet absorbance at 260 nm.-*Primers Design.* Specific primers for each putative *gap* gene of *P. syringae* were designed using Primer Premier 5.0 (Biosoft International). At each primer end, a new restriction site was added as shown in Table 1 to facilitate directional cloning.-*Cloning strategy*

Step 1. *Amplification by PCR.* To check primers, a PCR amplification of the complete open reading frames (ORF) of the three *gap* genes was carried out with their specific complementary oligonucleotides, using the genomic DNA from *P. syringae* pv tomato as a template. The reaction mixture contained 100 mM Tris–HCl buffer (pH 8.3), 1.5 mM $MgCl_2$, 0.8 mM of each deoxynucleoside triphosphate, 0.08 μM of each primer, 50 ng of DNA, and 1 U of *GoTaq* DNA Polymerase (Promega). Amplification was performed in a DNA thermal cycler (Perkin–Elmer Cetus, Norwalk, CT) programmed for 2 min at 95 °C and 35 cycles of 40 s at 94 °C, 40 s at 60 °C, and 1 min at 72 °C. In the end, a final cycle of 7 min at 72 °C was added. In this way, new restriction sites were created to facilitate directional one-step cloning of the amplified DNA fragments. Amplification products were resolved by electrophoresis in a 0.8% agarose gel according to Sambrook [32] and detected by staining with ethidium bromide. After agarose gel electrophoresis the PCR-amplified DNA fragments were purified with Wizard SV Gel and PCR Clean-Up System kits (Promega).

Step 2. *Digestion of inserts and plasmid, and ligation.* The plasmid pET-21a (Novagen, Cambridge, UK) and the three PCR-amplified DNA fragments (1–1.5 kb) were sequencially cleaved, first with *Nde*I endonuclease overnight and purified; then, the second cleavage was carried out also overnight by *Bam*H1 for plasmid pET-21a and the *gap1* and *gap3* inserts, or by *Hind*III for plasmid pET-21a and the *gap2* insert. The reaction mixtures were carried out according to the manufacturer’s recommendations of each restriction enzyme. All DNA ligations were carried out with T4 DNA ligase (New England BioLabs, MA), as recommended by the manufacturer. Ligation reactions between the vector and the inserts were analyzed by PCR using T7 vector-specific primers and a combination of vector-specific and insert-specific primers.

Step 3. *Transformation into non-expressing host E. coli Top10.* After DNA ligation, recombinant plasmids were transformed into chemically-competent *E. coli* Top 10 “One shot” (Invitrogen), which was grown on low salt LB agar plates containing 50 μg/ml ampicillin. Plates were then incubated overnight at 37 °C and recombinant colonies were selected.

Step 4. *Colony PCR and sequencing.* Before growing colonies for plasmid isolation, subcloning was verified by analyzing the presence of the appropriate insert and its orientation by single-colony PCR using T7 vector-specific primers and a combination of vector-specific and insert-specific primers. Then, plasmids were isolated by E.Z.N.A R Plasmid Mini Kit I, and reading frame verified by sequencing.