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Xanthomonas citri subsp. *citri* surface proteome by 2D-DIGE: Ferric enterobactin receptor and other outer membrane proteins potentially involved in citric host interaction

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ABSTRACT

Xanthomonas citri subsp. *citri* (XAC) is the causative agent of citrus canker, a disease of great economic impact around the world. Understanding the role of proteins on XAC cellular surface can provide new insights on pathogen-plant interaction. Surface proteome was performed in XAC grown *in vivo* (infectious) and *in vitro* (non-infectious) conditions, by labeling intact cells followed by cellular lysis and direct 2D-DIGE analysis. Seventy-nine differential spots were analyzed by mass spectrometry. Highest relative abundance for *in vivo* condition was observed for spots containing DnaK protein, 60 kDa chaperonin, conserved hypothetical proteins, malate dehydrogenase, phosphomannose isomerase, and ferric enterobactin receptors. Elongation factor Tu, OmpA-related proteins, Oar proteins and some Ton-B dependent receptors were found in spots decreased *in vivo*. Some proteins identified on XAC's surface in infectious condition and predicted to be cytoplasmic, such as DnaK and 60 KDa chaperonin, have also been previously found at cellular surface in other microorganisms. This is the first study on XAC surface proteome and results point to mediation of molecular chaperones in XAC-citrus interaction. The approach utilized here can be applied to other pathogen-host interaction systems and help to achieve new insights in bacterial pathogenicity toward promising targets of biotechnological interest.
Biological significance: This research provides new insights for current knowledge of the *Xanthomonas* sp. pathogenicity. For the first time the 2D-DIGE approach was applied on intact cells to find surface proteins involved in the pathogen-plant interaction. Results point to the involvement of new surface/outer membrane proteins in the interaction between XAC and its citrus host and can provide potential targets of biotechnological interest for citrus canker control.

1. Introduction

Xanthomonas citri subsp. *citri* (XAC) is the causal agent of citrus canker, a serious disease with worldwide distribution that has severe economic impact on the citrus industry [1]. The pathogen induces the formation of necrotic spots on leaves, branches and fruits and is pathogenic to grapefruit, some limes and sweet orange [1]. The complete genome sequence of XAC has been published by a Brazilian research consortium [2].

Several studies have been conducted in order to elucidate the XAC mechanisms involved in the plant-pathogen interaction, including the identification of hrp genes (*h*ypersensitive *r*esponse and *p*athogenicity related genes) [3] and avirulence genes [4]. Virulence mechanisms of this pathogen have been recently investigated screening a XAC mutant library [5]. Laia and co-authors [6] also reported new genes of XAC involved in pathogenesis and adaptation using a transposon-based mutant library.

Proteomic approaches were previously used to study the *in vitro* effect of host leaf extract on XAC [7] and to investigate *in vivo* interaction of XAC and *Citrus sinensis* [8]. Similarly, XAC total proteomic analysis upon *in vivo* condition revealed proteins from the Types III and IV Secretion Systems and proteins related to xanthan gum biosynthesis as involved in virulence [9].

Previous proteomic studies revealed that the vast majority of the identified proteins on a cell are the most abundant ones, whereas proteins present in much lower concentrations are usually not detected

Abbreviations: XAC, *Xanthomonas citri* subsp. *citri*; 2D-DIGE, Two-dimensional difference in gel electrophoresis; NA, Nutrient agar; NB, Nutrient broth; CFUs, Colony forming units.

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[10]. Membrane proteins are also difficult to detect using 2-D gel approach, particularly due to their low abundance, hydrophobic nature and high molecular weight [11]. Consequently, improved methods for enrichment of proteins present at low amounts are required, such as pre-fractionation procedures or more sensitive detection methods. Two-dimensional difference gel electrophoresis (2D-DIGE) has emerged as a more sensitive and more accurate analysis of protein abundance levels [12]. A 2D-DIGE comparative proteomic study was performed between biofilm and planktonic cells using total cellular extracts of *Xanthomonas axonopodis pv. citri* (strain Xcc99-1330). After *in vitro* cultivation under static condition in XVM2 [13], a medium which mimics plant environment and induces pathogenicity, major variations in the composition of outer membrane proteins including receptors and transporter proteins were observed to be associated to biofilm formation [14].

Proteomic analysis of cellular fractions allows identification of proteins that would not be detectable in a total cellular extract, especially if the proteins are not abundant and are exclusive to a particular cellular location. In this work, we used 2D-DIGE technology to detect changes of XAC surface proteins in response to host infection by comparison of cells grown *in vivo* (plant leaf, *i.e.* infectious condition) and *in vitro* (rich medium, *i.e.* non-infectious control condition). 2-DE profiles generated through intact cells labeling followed by bacterial lysis revealed differences in outer membrane proteins possibly related to host attachment and pathogenicity, many of them not identified before as involved with XAC pathogenicity or other phytopathogens. Methodology used here was shown to be suitable for preferential surface proteins labeling. Although several of the differential proteins detected here are known to be cytosolic, they have also been described in other organisms as having additional functionality at cell surface or extracellular milieu [15–18]. This additional function was named *moonlighting*. Many of the moonlighting proteins are highly conserved proteins, for example, metabolic proteins/enzymes or molecular chaperones, and their additional activities may occur only when the protein is at a different location from that it normally occupies [19]. Many bacteria secrete glycolytic enzymes which associate with the cell surface and then exhibit a moonlighting function [19].

This is the first report describing changes in XAC surface proteome, especially upon *in vivo* growth. The proteomic approach described here can help to identify membrane and/or surface proteins that participate on other pathogen-host interaction processes.

2. Material and methods

2.1. Bacterial strain and culture conditions

XAC genome strain (strain 306) was used in the present study. Cells were grown on nutrient agar (NA) at 28 °C and in NB liquid medium (5 g L^{-1} peptone, 3 g L^{-1} meat extract) on a rotary shaker at 200 rpm and 28 °C. For proteomic analyses, XAC was grown *in vivo* on detached *Citrus aurantifolia* leaves (infectious condition) and *in vitro* in NB (non-infectious condition, control), as described below (Section 2.2).

2.2. Plant inoculation and bacterial recovery

Young leaves of *Citrus aurantifolia* measuring approximately 7 cm along the main midrib and without visible lesions were used for bacterial infection. The inoculum was prepared by growing the bacterium for 72 h in NA. Cell mass was suspended in distilled water and adjusted to $OD_{595\ nm} = 0.3$ (approximately 10^8 CFU·mL^{-1}). This suspension was then used to infiltrate the intercellular spaces of 18 young leaves using a plastic syringe, after puncturing the tissue with a needle. Infiltration was performed in quadruplicate until the plant tissue was soaked with the bacterial suspension, totalizing 72 young leaves. The inoculated detached leaves were introduced in 50 mL Falcon flasks containing ~5 mL distilled water and were maintained at ~25 °C [20] for virulence induction. Cells recovery by exudation [8] was monitored by CFU counting until 7 days.

Cells for protein extraction were obtained by exudation as follows. Seven days after the inoculation, when typical disease symptoms were visible, 6 mm diameter leaf disks were cut out, decontaminated with 1% sodium hypochlorite for 30 s and 70% alcohol for 1.5 min, followed by washing with distilled water. Each disk was shredded with a blade and then immersed in 1 mL of distilled water for one hour at 4 °C to allow the bacteria to exudate to the water. The suspension obtained was centrifuged at 10.000 *g* for 10 min, and cells were washed with cold PBS buffer (0.136 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0017 M KH_2PO_4, pH 8.5) and used for cell surface protein labeling with CyDyes (GE Healthcare) followed by cellular lysis (Section 2.3).

XAC growth curve in 20 mL NB medium culture was performed at 28 °C and 200 rpm in order to determine the end of the logarithmic phase. Based on the results, cells for proteomic analysis were grown in NB medium for 16 h (until OD_{595nm} ~ 1.9), collected by centrifugation (10,000 *g*, 20 min, 4 °C), washed with PBS buffer at 4 °C, and used for cell surface protein labeling with CyDyes (GE Healthcare) followed by cellular lysis (Section 2.3).

2.3. Protein labeling and extraction

Labeling procedure was performed on intact bacterial cells, prior to cell disruption, allowing surface protein detection as described by Hagner-McWhirter [21], with modifications, according to manufacture' s instructions. Four replicates of XAC samples from *in vivo* (infectious condition) or *in vitro* (non-infectious condition) were randomly labeled with CyDyes DIGE Fluors minimal dyes (GE Healthcare), also according to manufacturer's instructions, as designed in Table S1. Briefly, intact cells (~1×10^7) were labeled with 400 pmol of CyDyes (Cy3 or Cy5) in a total volume of 200 μL. An internal standard was prepared by pooling a mixture with equal amounts of each of the eight samples and Cy2-labeled. The labeling mixtures were incubated 30 min in dark on ice bath, and reaction was interrupted by adding 1 μL of 10 mM lysine. Cells were then washed with PBS buffer to remove dyes excess prior to cell disruption.

Labeled cells were suspended in 150 μL lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT) and kept on ice for 1 h with occasional vortexing. The soluble protein fraction was recovered by lysate centrifugation (12,000 *g*, 20 min, 4 °C) and stored at −20 °C. Protein concentration was determined by residual unbound copper method using 2D-Quant Kit (GE Healthcare). BSA was used as standard and absorbance was measured at 480 nm as an inverse function of the protein concentration.

Efficiency of specific labeling of surface proteins was evaluated by SDS-PAGE (12.5% acrylamide gel). Cells grown in NB medium were labeled with Cy3, washed with PBS to remove dye excess, and disrupted with 150 μL lysis buffer followed by centrifugation to eliminate remaining intact cells. Sample lysate was centrifuged at 40,000 rpm for 2 h at 4 °C (Himac Ultracentrifuge CP90WX, Hitach) for separation of membranes and cytosolic fractions. The pellet (membrane enriched fraction) was suspended in 100 μL lysis buffer without DTT and 20 μg of each sample were separated by SDS-PAGE [22] ran at 110 V. Fluorescent protein bands were acquired using Ettan DIGE Imager scanner (GE Healthcare) and total proteins were revealed by silver staining.

2.4. 2D-DIGE

For 2-D analysis, the proteins were focused at pH 3–10 (linear) in 13 cm IPG strips (GE Healthcare). Each strip was rehydrated overnight with a mixture of 50 μg of Cy3-labeled sample, 50 μg of Cy5-labeled sample, and 50 μg of Cy2-labeled internal pooled standard (labeling according to Table S1), which were diluted to 250 μL in DeStreak Rehydration Solution and 0.5% IPG buffer (GE Healthcare). IEF was performed with the Ettan IPGphor III system (GE Healthcare) at 20 °C using the

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