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Sample Preparation for Secretome Analysis in A*-Type Strains of *Xanthomonas citri* subsp. *citri*, the Causal Agent of Asiatic Citrus Canker Disease

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ABSTRACT

Xanthomonas citri subsp *citri* (*Xcc*) is responsible for one of the most destructive diseases of citrus fruits, Asiatic citrus canker. Based on the bacterial host range, the pathogenic forms, A, A*, and A^w have been identified. A*-type strains are not well characterized and they have been reported to differ from other A-type strains on the basis of biochemical and genetic characteristics. In this study, we attempted to develop a medium to mimic *in planta* conditions for inducing protein secretion of the A*-type strain-NIGEB-088. We also attempted to achieve efficient extraction of *Xcc* A*-type extracellular proteins, the key mediators of plant-bacterium interaction. Results obtained by this study demonstrate that the m3 (mMM1) can induce *Xcc* A*-type strains to secret extracellular proteins in amounts detectable by two dimensional gel electrophoresis (2-DE). We also show that Pyrogallol red-molybdate methanol (PRMM) is more effective for precipitation of *Xcc* A*-type secreted proteins than other assessed procedures of protein precipitation, i. e. acetone, TCA, and sodium deoxycholate.

Keywords: *Xanthomonas citri* subsp. *citri*, Asiatic citrus canker disease, *in vitro* secretome analysis, secreted protein extraction

Introduction

Xanthomonas citri subsp *citri* (*Xcc*) causes Asiatic citrus canker in all citrus species and many Rutaceae (Verniere *et al.,* 1998; Das, 2003). The disease agent is known to be composed of three pathogenicity forms A, A*, and A^w (Moreira *et al.,* 2010; Soltaninejad *et al.,* 2010). Although genetically close, these forms of the disease agent have been shown to differ in host range (Morira *et al.,* 2010). A-type strains have shown the widest range of hosts and produce citrus canker specific lesions in all citrus species, while A* and A^w strains exhibit a host range restricted to the Mexican lime (*Citrus aurantifolia*) and alemow (*C. macrophylla*), respectively (Bui Thi Ngoc *et al.,* 2009).

Pathogenicity in Xanthomonas in general, and in *Xanthomonas citri* subsp. *citri* in particular, depends mostly on a protein injection nanomachinery, the so-called type III secretion system (T3SS) or injectisome (Cornelis, 2006; Alavi *et al.,* 2008; Dor Salomon *et al.,* 2011; Guo *et al.,* 2011; McDermott *et al.,* 2011). The type III secretion system is shown to be immediately activated when the disease agent contacts the host plant cell surface. The T3SS nanomachinery operates as an injection needle to secrete protein effectors essential for bacterial pathogenesis directly into the

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host plant cell (Astua-Monge *et al.,* 2005; Cornelis, 2006; Kay and Bonas, 2009).

In *Xcc* A-type strains, genomic regions responsible for expression of proteins involved in physical structure of T3SS, as well as the proteins secreted via the nanomachinery system, have already been identified (da Silva *et al.,* 2002). For A*-type strains little investigation into the T3SS apparatus and the genetic basis for their pathogenicity and host specificity has been conducted. We attempted to analyze the secretome of an *Xcc* A*-type strain (NIGEB-088) isolated from a citrus orchard in southern Iran using two-dimensional gel electrophoresis (2-DE). However, proteomics for *in-vitro* analysis of proteins has limitations, with the quantity and quality of extracted proteins a major concern (Kazemi-pour *et al.,* 2004; Chevallet *et al.,* 2007; Wang *et al.,* 2009). This constraint is a particular concern when working with bacterial extracellular proteins, usually secreted at low levels into the culture medium.

The aim of this study was to show the efficacy of a modified minimal medium (mMM1) to induce secretion of proteins by *Xcc* A*-type strains, some of which could potentially be involved in bacterial pathogenicity and to assess the effectiveness of four methods of extracting bacterial proteins secreted into an aqueous phase of mMM1. The efficacy of each experimental method was evaluated by 2-DE.

Materials AND METHODS

Bacterial strain and culture condition

Xanthomonas citri subsp. *citri*, strain NIGEB-088, was isolated from the Mexican lime in southern Iran (Soltaninejad *et al.,* 2010). Bacterial cells were overnight grown (28˚C/180 rpm) in yeast extract (3 g/l) and peptone (5 g/l) (YP) or modified minimal medium (m3) [20 mM NaCl, 10mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 1 mM $CaCl_2$, 0.16 mM KH_2PO_4, 0.32 mM K_2HPO_4 and 10 mM sucrose] (Mehta and Rosato, 2001). The culture medium was supplemented with 0.1% w/v yeast extract. When indicated, the host-plant leaf extract (PlEx) was added at a concentration of 0.1% v/v to accelerate protein secretion.

Plant extract preparation

Preparation of a plant extract preparation (PlEx) free of host-plant proteins was accomplished through trituration, autoclaving, or boiling. In trituration, one gram of fresh *C. aurantifolia* leaves was ground with a sterilized mortar and pestle, centrifuged at 5000 x g for 10 min, and filtered through a 0.2 µm filter disc.

For autoclaving, one gram of fresh *C. aurantifolia* leaves was added to 10 ml distilled water and autoclaved at 121˚C for 20 min to produce sterilized PlEx. To remove plant debris, the solution was centrifuged at 5000 x g for 10 min and the supernatant was collected.

For boiling, 10 g of fresh *C. aurantifolia* leaves were added to 100 ml distilled water and boiled for one hour. The mixture was centrifuged twice at 5000 x *g* for 20 min. The aqueous phase was collected and stored at -20˚C until use (Kazemi-pour *et al.,* 2004).

Bacterial growth and Sample preparation

To determine the nature of bacterial strains in culture media (YP or m3) and to facilitate sample preparation, bacterial growth was measured in a spectrophotometer at 600 nm, and a growth curve was established (data not shown). Bacterial samples were prepared by overnight growth of *Xcc* A*-type strain (NIGEB-088) in YP or m3. The cultures were then diluted 1:100 in fresh medium and grown at 28˚C with constant shaking to obtain an optical density (OD) of 1.1 at 600 nm. Bacterial cultures were harvested at the

stationery phase of growth and centrifuged at 13000 x *g* for 25 min. To remove bacterial residue, the aqueous phase was filtered through a 0.2 µm filter disc. The filtrate used for protein precipitation.

Protein precipitation

Bacterial extracellular proteins secreted into culture media were extracted using the following procedures: (i) acetone precipitation (Kazemi-pour *et al.*, 2004), (ii) a TCA-based method (Rajalingam *et al.*, 2009) with some minor modifications, (iii) sodium deoxycholate-TCA precipitation (Arnold and Ulbrich-Hofmann, 1999), or (iv) PRMM precipitation (Caldwell and Lattemann, 2004). The concentration of secreted proteins was determined according to Bradford (1976).

Protein electrophoresis by SDS-PAGE and 2-DE

(i) SDS-PAGE was performed following standard procedures for a small-scale (Mini protean II system; BioRad, Hercules, CA, USA). 12% w/v acrylamide gels were prepared as described by Laemmli (1970). Resolved proteins were detected by silver staining.

(ii) For 2-DE, the first dimension of electrophoresis was performed in individual 3 mm wide IPG gel strips (7 cm in length) in the linear pH range of 4-7 (Bio-Rad, Hercules, CA, USA). Protein samples were solubilized by in gel rehydration at 20 °C in 170 µl of lysis buffer containing 8 M urea, 4% w/v CHAPS, 20 mM DTT, 0.2% v/v carrier ampholytes, and 0.001% w/v bromophenol blue. Strips were rehydrated under passive conditions for 12-14 h at 20 °C. The first dimension was performed in an isoelectrofocusing (IEF) cell (Bio-Rad, Hercules, CA, USA). The IEF was performed at 50 V for 15 min, followed by a first linear 50-150 V gradient for 30 min and a second linear 150-250 V gradient for 15 min. Subsequently, a linear voltage ramping step was applied for 2 h at 4000 V. As a final focusing step, the maximum voltage of the ramp step was maintained at up to 20, 000 volt hours (Vh). Prior to the second dimension, the IPG strips were equilibrated in a buffer containing 6 M urea, 2.5% w/v SDS, 0.375 M Tris-HCl (pH 8.8), 30% v/v glycerol, and 130 mM DTT for 15 min and for 20min in the same buffer with 135 mM iodoacetamide replacing the DTT. For the second dimension of electrophoresis, the equilibrated strips were loaded onto 10% w/v acrylamide gels, and SDS-PAGE was performed at 25 V for 30 min followed by 40 min at 200V. 2-DE experiments were repeated in three independent replicates.

Results

Boiling is an appropriate method of PlEx preparation

Of the three methods of PlEx preparation, boiling resulted in a PlEx sample showing a minimum number of plant protein bands (Fig. 1). This procedure triggered the bacterial cells to secrete proteins into the culture medium and apparently had no additional effect on bacteria protein expression (Fig. 2). Trituration and autoclaving were ineffective in removing host-plant proteins from the PlEx mixture, which may have an inhibitory effect on expression of bacterial secreted proteins.

PRMM-based precipitation is the most efficient method of extracting proteins secreted by *Xcc* A*-type strain

To compare the amount of bacterial-secreted proteins extracted by each method, an equal quantity of initial bacterial cells (approximately 1.0×10^6 cfu/µl) was used (Suss *et al.,* 2006). After growing, bacterial cells were pelleted, and extracellular proteins, secreted into the culture

supernatant, were extracted. Quantitative comparison of the four extraction methods was carried out. Acetone, TCA, and SDC precipitation methods were capable of producing only minimally sufficient secreted proteins, 0.03, 0.04, and 0.05 µg respectively. Pyrogallol red-molybdate methanol resulted in the highest amount (0.1 µg) of protein secreted into the extracellular environment by *Xcc* A*-type strain NIGEB-088. To verify qualitatively, secreted proteins extracted by each of the four procedures were compared by SDS-PAGE. PRMM was shown to be the most effective approach for extracting bacteria-secreted proteins from culture supernatant in the presence or absence of PlEx (Fig. 2). It produced the maximum number of protein bands on acrylamide gel and the highest degree of resolution.

Proteins secreted by *Xcc* A*-type strain could be detected by 2-DE

Proteins precipitated by the four methods were verified by 2-DE. As with SDS-PAGE, no significant differences were detected among acetone, TCA, and SDC procedures in terms of numbers of protein spots on the gel (Fig. 3A, 3B, and 3D, respectively). In contrast, the PRMM-based extraction method resulted in a sufficiently scoreable number of protein spots extracted from PlEx-supplemented mMM1. The majority of bacterial proteins secreted by *Xcc* A*-type strain, NIGEB-088, into the culture supernatant were in a pI range of 4-5.5 with molecular mass ranging from 60 to 90 kDa (Fig. 3C).

Discussion

Asiatic citrus canker (ACC), caused by *Xanthomonas citri* subsp. *citri*, is a devastating citrus disease worldwide (Bruning and Gabriel, 2003). Narrow host range strains (A*-type), which cause disease exclusively to Mexican lime (*C. aurantifolia*), have been detected in India, South-East Asia, and Persian Gulf region countries, including Iran, leading to considerable economic losses (Verniere *et al.,* 1998, Soltaninejad *et al.,* 2010). In spite of their economic significance, little is known of the genetic basis of pathogenicity and host specificity of the A*-type strains. To expand knowledge of bacterial strategies to overcome the host-plant cells, we examined an *Xcc* A*-type strain to develop an efficient culture medium capable of mimicking *in-planta* conditions of bacterial pathogenicity-involved protein expression. We also aimed to identify an effective method of extracting secreted proteins involved in pathogenicity and host specificity of the bacterium.

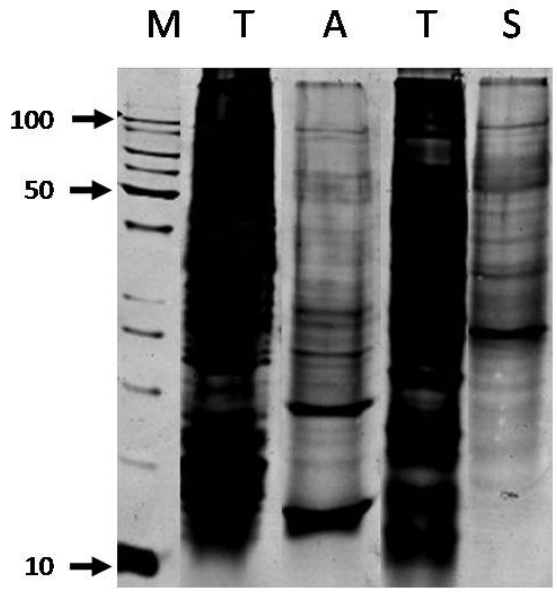

Figure 1. Acrylamide gel electrophoresis of PlEx proteins, extracted by trituration (T, lanes 2 and 4), autoclaving (A, lane 3), and boiling (S, lane 5) methods. Trituraton and autoclaving produced more plant proteins, posing problems for detection of bacterial secreted proteins by 2DE. The arrows indicate protein molecular weights of 100, 50, and 10 kDa.

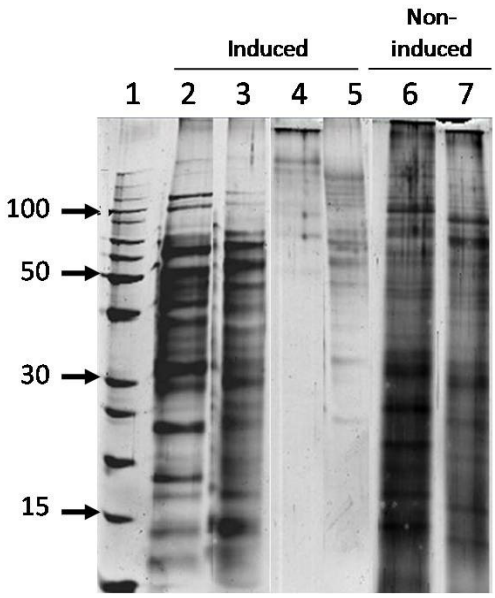

Figure 2. Silver stained SDS-PAGE displaying t he quality of secreted proteins extracted by four methods under induced, with PIEx (lanes 2-5), and non-induced, without PIEx (lanes 6-7), conditions. Lane 1: protein marker; lanes 2 and 6: PRMM precipitated proteins; lanes 3 and 7: TCA precipitated proteins; lane 4: acetone precipitated proteins; and lane 5: sodium deoxy-cholate precipitated proteins. The arrows indicate protein molecular weights of 100, 50, 30 and 15 kDa.

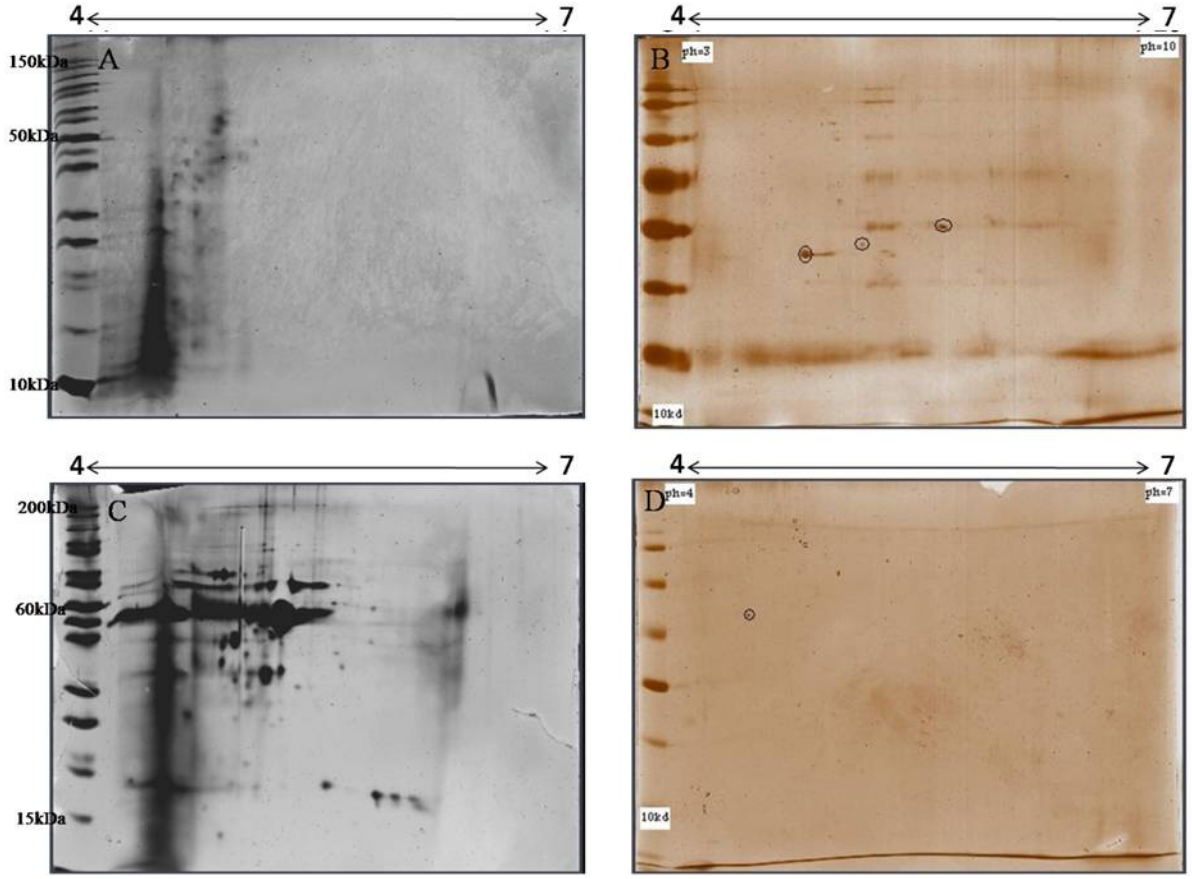

Figure 3. 2-DE profile of proteins secreted by *Xcc* A*-type strain, NIGEB-088, into the PIEx-supplemented mMM1. Secreted proteins were extracted by A: TCA; B: sodium deoxycholate (SDC); C: PRMM; and D: acetone. 2-DE was performed at a pH range from 4 to 7. PRMM seems to be the most appropriate method of extraction for bacterial proteins secreted by *Xcc* strain NIGEB-088.

Our results show that strain NIGEB-088 possesses a T3SS-like secretion system to deliver bacterial proteins directly into the extracellular environment. Generally, the results obtained by this research demonstrated the effectiveness of PRMM-based method to isolate the secretome of *Xcc* A*-type strain, NIGEB-088. When grown in mMM1 in the presence of the host-plant extract (PlEx) to induce protein secretion (Wengelnik and Bonas, 1996), the NIGEB-088 strain exuded proteins via its T3SS-like system into the culture supernatant. As illustrated in Figure 2. in PlEx-supplemented mMM1 (lanes 2 and 3), the given strain secreted more proteins than in non-supplemented mMM1 (lanes 6 and 7). This finding showed that the host-plant extract positively affects secretion of proteins when pathogenic bacterial cells are grown outside the host-plant cellular environment (Watt *et al.,* 2009). Apparently some plant molecules play a role in triggering the bacterium to activate its system of protein secretion. Although the nature of these molecules remains unknown, their biological properties must be conserved, so that bacterial cells remain active to secrete pathogenic factors into the culture medium.

The host plant active elements, essential for *in vitro* activity of the bacterium and protein secretion into the extracellular environment, are better preserved when using boiling as a method to prepare the plant extract (Figure 1). This procedure makes it possible to obtain bacterial-secreted proteins in amounts sufficient to be detected by two-dimensional gel electrophoresis (Fig. 3). Protein spot identification (data not shown) showed no homology with plant proteins, suggesting that the PlEx had no influence on protein composition of the bacterial culture soup.

Despite the experimental progress in 2-DE techniques, analysis of secreted proteins remains difficult due to their low concentrations when grown in culture media (Tjalsma *et al.,* 2004; Chitlaru *et al.,* 2006; Galan and Wolf-Watz, 2006; Watt *et al.,* 2009). We attempted to optimize an experimental platform to develop a method for accumulating secreted protein. While TCA and Acetone are usually used for bacterial secretome analysis by 2-DE (Cen *et al.,* 2010), in the present study, the PRMM-based precipitation approach produced the best results for extracting proteins secreted into the culture supernatant by bacterial cells of *Xcc* A*-type strain, NIGEB-088 (Figure 3C). Other tested procedures gave a poor yield of secreted proteins. In addition, the resolution and the quantity of secreted proteins obtained by these methods were apparently not adequate for 2-DE analysis. To deal with this constraint, proteins should be as pure as possible, which may explain the relatively poor outcome of the methods other than PRMM (Caldwell and Lattemann, 2004).

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