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A proteomic study of *Xanthomonas oryzae* pv. *oryzae* in rice xylem sap

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ABSTRACT

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the second most important rice pathogen, causing a disease called bacterial leaf blight. *Xoo* colonizes and infects the vascular tissue resulting in tissue necrosis and wilting causing significant yield losses worldwide. In this study *Xoo* infected vascular fluid (xylem sap) was recovered and analyzed for secreted *Xoo* proteins. Three independent experiments resulted in the identification of 324 different proteins, 64 proteins were found in all three samples which included many of the known virulence-associated factors. In addition, 10 genes encoding for the identified proteins were inactivated and one mutant displayed statistically a significant loss in virulence when compared to the wild type *Xoo*, suggesting that a new virulence-associated factor has been revealed. The usefulness of this approach in understanding the lifestyle and unraveling the virulence-associated factors of phytopathogenic vascular bacteria is discussed.

1. Introduction

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causal agent of bacterial leaf blight (BLB), a vascular disease of rice causing significant losses every year in Asia [1,2]. In fact *Xoo* seriously affects rice production in all tropical countries and is a major threat to world food production and nutrition. *Xoo* infects rice through wounding sites or by penetrating the leaves through hydathodes; once inside the plant, the bacteria colonizes and clogs xylem vessels ultimately leading to tissue necrosis and wilting [3]. In recent years, genome sequences of a number of *Xoo* strains allowed more detailed studies of the molecular systems involved in pathogenesis [4]. *Xoo* uses a large and diverse set of virulence-associated factors many of which are secreted including exopolysaccharides, effectors secreted via the Type II and Type III secretion systems, motility factors, adhesion molecules, iron transport and quorum sensing systems [5,6]. *Xoo* virulence has, to our knowledge, not been studied using proteomics and in particular, *in vivo* proteomics. Significantly, proteomics has resulted in important discoveries using other plant pathogens. For example, a study was conducted on the plant pathogenic fungus *Fusarium graminearum* by comparative proteomics of extracellular proteins expressed *in vitro* and *in planta*. Interestingly, it was established that many of the proteins expressed *in planta* are not expressed under various *in vitro* conditions, indicating specific *in planta* expression [7]. The extracellular proteome has also been studied in some phytopathogenic bacteria such as *Xanthomonas campestris* pv *campestris* (*Xcc*) [8] and *Xylella fastidiosa* [9]. Comparative proteomic analysis in pathogenic *Xanthomonas* bacteria was studied under varying growth conditions, such as the addition of host plant extracts in *Xanthomonas axonopodis* pv *citri* [10], in *X. axonopodis* pv *passiflora* [11], and *Xcc* [12]. It was demonstrated that many proteins are differentially expressed when the plant

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extract is added to the growing medium, being up- or down-regulated. Similarly, in phytopathogenic *Burkholderia glumae*, proteomics was used to determine the difference in protein profiles between the wild type and quorum sensing deficient mutants [13].

Several proteomic studies of rice proteins expressed in response to *Xoo* infection have been performed [14–16]. However, the expression of *Xoo* proteins *in planta* has thus far not been addressed. Importantly, *Xoo* undergoes inter-kingdom signaling via a protein called OryR, which responds to a plant low molecular weight compound(s) and regulates virulence [17,18]; this is direct evidence that in *Xoo*, gene expression is influenced by *in planta* growth. In this study, an *in vivo* proteomics approach was used in order to identify *Xoo* proteins that are expressed *in planta*. As secreted proteins are particularly important for *Xoo*–rice interactions and virulence [19], we focused mainly on identifying putative secreted proteins. Three biological replicate experiments resulted in the identification of 324 *Xoo* proteins in rice xylem sap. The genes encoding for 10 of the identified proteins were inactivated and one novel factor involved in virulence was identified.

2. Material and methods

2.1. Bacterial strains and growth conditions

The strain used in this study was *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) XKK.12, isolated in the Kerala State (India) and previously reported as being highly virulent to rice [17]. *Xanthomonas* was grown at 28 °C on peptone-yeast extract liquid medium (PY; 0.8% peptone, 0.2% yeast extract, 0.2% K_2HPO_4, 0.05% KH_2PO_4, 0.025% $MgSO_4$ $7H_2O$, 0.5% glucose w/v), peptone-sucrose agar plates (peptone 1%, sucrose 1%, agar 1.5% w/v) [17] or XOM2 media [20]. *Escherichia coli* DH5α [21] was grown at 37 °C in Luria-Bertani medium. When necessary, antibiotics for *Xoo* and *E. coli* growth were added to media at the following concentration: ampicillin, 100 μg/ml; gentamicin, 10 μg/ml.

2.2. Recombinant DNA techniques

Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with Klenow fragment of DNA polymerase, and transformation of *E. coli* DH5α were performed as described [22]. Plasmids were purified using the JetStar plasmid purification mini or midi kit (Genomed, GmbH, Germany); genomic DNA was isolated by sarcosyl-pronase lysis as described previously [23]. DNA was extracted from agarose gel by EuroGold gel extraction kit (EuroClone, Milano, Italy).

2.3. Inoculation of rice plants with Xoo XKK.12 and xylem sap collection

Xoo was grown in PY liquid medium at 28 °C for two days before being used for inoculation of rice plants. The bacterial concentration was adjusted to 1×10^9 CFU/ml with sterile demineralized water. Inoculation was carried out on the Italian rice cultivar Baldo, which showed high sensitivity to *Xoo* XKK.12 infection. Rice was grown and inoculation performed as previously described [24]. Three to four leaves per plant were inoculated using the clipping method [25] and water was used as negative control. Inoculated plants were kept for 24 h in humid chambers (>92% relative humidity) at 30±4 °C, and were then transferred to greenhouse conditions (26–28 °C, 65% relative humidity with a photoperiod of 16 h of light and 8 h of darkness) for disease development for an additional 13 days. Experiments were performed to test the virulence of *Xoo* mutants in comparison with *Xoo* wild type; one tray with 20 rice plants was used for each mutant, ten plants were inoculated with *Xoo* wild type and ten plants inoculated with one of the *Xoo* mutants. Inoculation experiments were performed in triplicate. Symptoms were evaluated by measuring the lesion length of the inoculated leaves. Significantly different lesion lengths values were established at $P<0.05$ in a Student's two-tailed t test for independent means.

Infected leaves were used for sampling of xylem sap as follows: infected plants were placed in a humidity chamber, the dried blighted part of the infected leaves were cut 2 cm from the edge of infection. Drops oozing out from the xylem were constantly collected using a 200 μl pipette during the subsequent 4 h (Fig. 1), placed in sterile Eppendorf tubes and centrifuged at 16,000× *g* at 4 °C for 30 min to remove bacterial cells. The experiment was repeated three times giving origin to three distinct xylem sap samples (approximately 500 μl for each sample) used for independent mass spectroscopy analysis.

2.4. Mass spectroscopy analysis of xylem sap samples

Preliminary experiments indicated that sugars and other non-proteinaceous contaminants found in xylem sap could efficiently be removed using SDS-PAGE gels. The collected xylem sap from inoculated and un-inoculated (negative control) rice leaves was diluted to 1× Laemmli sample buffer and boiled for 5 min and then run onto 4–10% Nupage gels (Invitrogen). The samples were allowed to run 1 cm into the gel and then the gels were stained with colloidal comassie blue (Pierce). The stained area of the gel was cut into 5 bands and processed for in-gel digestion with trypsin using standard procedures. Samples 2 and 3 were treated with nicotinyl-n-hydroxysuccinimide, as modification with cyclic amines has been shown to improve peptide fragmentation in MALDI-TOF/TOF instruments [26]. The resulting peptides were cleaned up using STAGE-tips [27]. LC-MALDI of the digests was performed using an Applied Biosystems 4800 mass spectrometer coupled with an Ultimate 3000 HPLC via a PROBOT target spotter (Dionex). The peptides were separated using an in-house packed column (20 cm×75 μm) containing Jupiter proteo resin (Phenomenex). The gradient ranged from 5-50% Acetonitrile in 0.1% TFA in 90 min. The gradient was developed at 300 nl/min and subsequently mixed with a 3 mg/ml solution of CHCA flowing at 1 μl/min and spots were acquired every 10 seconds using a PROBOT plate spotter. The top 10 precursor ions from each spot were subjected to MS/MS analysis with 1500 laser shots and a laser intensity of 2600.

MGF files were created using TS2Mascot.exe (MatrixScience) with the mass range parameter set to a minimum of 60 Da and a maximum mass equal to the mass of the precursor. Only monoisotopic peaks with a signal to noise ratio>10 were included in the final MGF.