Mutation in the \textit{xpsD} gene of \textit{Xanthomonas axonopodis} pv. \textit{citri} affects cellulose degradation and virulence

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Abstract

The Gram-negative bacterium \textit{Xanthomonas axonopodis} pv. \textit{citri}, the causal agent of citrus canker, is a major threat to the citrus industry worldwide. Although this is a leaf spot pathogen, it bears genes highly related to degradation of plant cell walls, which are typically found in plant pathogens that cause symptoms of tissue maceration. Little is known on \textit{Xac} capacity to cause disease and hydrolyze cellulose. We investigated the contribution of various open reading frames on degradation of a cellulose compound by means of a global mutational assay to selectively screen for a defect in carboxymethyl cellulase (CMCase) secretion in \textit{X. axonopodis} pv. \textit{citri}. Screening on CMC agar revealed one mutant clone defective in extracellular glycanase activity, out of nearly 3,000 clones. The insertion was located in the \textit{xpsD} gene, a component of the type II secretion system (T2SS) showing an influence in the ability of \textit{Xac} to colonize tissues and hydrolyze cellulose. In summary, these data show for the first time, that \textit{X. axonopodis} pv. \textit{citri} is capable of hydrolyzing cellulose in a T2SS-dependent process. Furthermore, it was demonstrated that the ability to degrade cellulose contributes to the infection process as a whole.

Key words: citrus canker, transposome, type II secretion system.

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Introduction

Citrus canker is a highly destructive disease of citrus crops, which occurs worldwide and affects most of the commercial varieties of citrus. The disease is caused by the bacterium \textit{Xanthomonas axonopodis} pv. \textit{citri}. In areas where citrus canker has been established, its control is very difficult. Control is extremely difficult in areas where the disease is already firmly established, and is based on eradication programs involving the heavy use of copper compounds. Bacterial plant pathogens, such as xanthomonads deliver various virulence proteins that play a role during infection. Knowledge on the complete repertoires of specific types of effectors would be beneficial towards understanding the mechanisms by which bacteria interact with plants, thereby causing diseases (Collmer \textit{et al.}, 2002, Gutman \textit{et al.}, 2002, Roden \textit{et al.}, 2004, Chang \textit{et al.}, 2005, Nomura and He, 2005).

The complete genome sequencing of \textit{X. axonopodis} pv. \textit{citri} revealed several open reading frames that were annotated as related to pathogenicity and virulence (da Silva \textit{et al.}, 2002). Unexpectedly, given that the symptoms caused by \textit{X. axonopodis} pv. \textit{citri}, a leaf-spot pathogen, are not typically associated to massive degradation of the plant-cell wall, such sequences include a number of genes that share similarity with those that code for plant cell wall degradation in other organisms. Plant cell walls are comprised mainly of cellulose and hemicellulose as major components, and also lignin, pectin, among others. Apparently there is no correlation between citrus canker symptoms and citrus tissue degradation caused by cell-wall cleavage. Interestingly, \textit{X. axonopodis} pv. \textit{citri} was found to express cell-wall degrading enzymes when grown in a medium that supposedly mimics the environment of plant intercellular spaces (Astua-Monge \textit{et al.}, 2005).

In \textit{Erwinia} species, plant-cell wall cleavage is a key step for the pathogenesis. In fact, virulence depends basically on the production and secretion of host-cell wall degrading enzymes (Matsumoto \textit{et al.}, 2003, Corbett \textit{et al.}, 2005). To date, most of the proteins that have been characterized in bacterial plant pathogens, and also involved in the degradation of several components of plant-cell walls, are
secreted by the type II secretion system (T2SS) (Jha et al., 2005). A functional T2SS is known to be required for virulence and the secretion of cell-wall degrading enzymes, in at least two xanthomonads that are vascular pathogens in their plant hosts, *Xanthomonas oryzae* pv. *oryzae* and *Xanthomonas campestris* pv. *campestris* (Chen et al., 2005; Lee et al., 2005; Jha et al., 2007).

Although *X. axonopodis* pv. *citri* harbors the T2SS, little is known regarding the effects of this system and the proteins that are delivered during its action. This study reports on the role of *xpsD*, a T2SS component, in the ability of *X. axonopodis* pv. *citri* to cause the disease and degrade cellulose.

### Materials and Methods

#### Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in the present study are listed in Table 1. *Xanthomonas axonopodis* pv. *citri* 306 strain, already described (da Silva et al., 2002), was routinely cultured on nutrient broth (NB) medium [containing per liter, 15 g of peptone, 3 g of yeast extract, 6 g of NaCl and 1 g D(+)-glucose] at 28 °C, while *Escherichia coli* cultures were grown on Luria-Bertani medium at 37 °C. Kanamycin (15 μg/mL) was used for selection in *E. coli* and *X. axonopodis* pv. *citri*. The plasmid for site-directed mutagenesis was prepared from the *E. coli* host strain by the alkaline lysis method.

#### DNA manipulation

Insertion points were confirmed by DNA sequencing with the forward and reverse primers of EZ::TN (Epicentre Technologies, Madison, WI) (Table 2). For genomic DNA isolation, strains were cultured overnight in 50 mL of NB. Cultures were then adjusted to optical density of 0.6 at 600 nm in sterile NB, and DNA was isolated using the CTAB (hexadecyltrimethylammonium bromide) method (Ausubel et al., 1998). DNA was stored in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at -20 °C. For Southern blotting and hybridization, 2 μg of genomic DNA from *X. axonopodis* pv. *citri* were digested with EcoRI and EcoRV, separated by electrophoresis in an 0.8% agarose gel, and transferred onto Hybond-N nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England) in 5 M NaOH, with subsequent cross-linking by exposure to UV irradiation. Hybridization was carried out at 65 °C. Southern blotting analysis was performed with digoxigenin (DIG)-labeled PCR products corresponding to a 399-bp fragment of *aph* gene as probe, by using the PCR DIG Probe Synthesis Kit and DIG DNA Labeling and Detection Kit (Roche Diagnostics, Indianapolis, IN). The analysis was used to confirm single EZ:TN insertions and homologous recombination.

#### Construction of mutant strains

For random insertion mutagenesis, the EZ::TN < KAN-2 > transposome complex, a mixture of the transposon EZ::TN < KAN-2 > and EZ::TN transposase (Epicentre Technologies, Madison, WI), was introduced by electroporation directly into *X. axonopodis* pv. *citri* 306 electrocompetent cells. These cells were prepared as described by Amaral et al. (2005). A Gene Pulser II Electroporator (Bio-Rad, Hercules, CA) was used to transform 50 μL of

| Strain or plasmid | Characteristics | Source or reference |
|-------------------|----------------|---------------------|
| **Escherichia coli** | | |
| DH5α | F′ φ80d lacZΔM15 Δ(lacZ-argF)U169 endA1 deoR recA1 hsdR17(rK- mK+) phoA supE44 lacI thi-1 gyrA96 relA1 | Gibco |
| **Xanthomonas axonopodis pv. citri** | | |
| 306 | Wild type, Ap′ | R.P. Leite |
| 30E9 | Kan′, XpsD- | This study |
| ΔxpsD | Kan′, site-directed XpsD- | This study |
| **Plasmid** | | |
| pCR2.1-TOPO | vector pUC18 derivative, kanr, bla (Apr), lacZ | Invitrogen |
| TOPO-xpsD | PCR-amplified xpsD (725-1307) in pCR2.1-TOPO; Kan′ | This study |
of the Student two-sample groups (wild-type and mutant strains) was tested by means of the hypothesis of equivalent CMC degradation in the two strains. 

To confirm the phenotypes, the site-directed ΔxpsD mutant was produced by using the primers XpsD_EcoRI_F and XpsD_EcoRI_R to PCR amplify an internal fragment of the xpsD region from X. axonopodis pv. citri 306 genomic DNA. The 583-kb PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), thereby generating TOPO-xpsD, and then electroporated into E. coli, whereupon the plasmid DNA was extracted and electroporated into X. axonopodis pv. citri.

Plate assay

Carboxymethyl cellulose (CMC) (0.5%) was incorporated into agar plates with culture medium (0.1% NaNO₃, 0.1% K₂HPO₄, 0.1% KCl, 0.05% MgSO₄, 0.05% yeast extract, 0.1% glucose and 1.7% agar). Colonies were grown at 28 °C for 48 h and then washed off with water. The plates were flooded with 1% Congo Red for 30 min and then dried and spread onto NB plates, with proper antibiotics. Colonies were scored by any visual modification on lesion. Bacterial growth in citrus leaves was measured by harvesting leaf discs for each X. axonopodis pv. citri mutant strain. The leaves were ground with a mortar and pestle in 1 mL of 0.01 M phosphate buffer, pH 7.2. The solution was serially diluted and spread onto NB plates, with proper antibiotics. The mean number of colonies in plates of the proper dilutions was calculated.

Results

Construction of a knockout mutant library and isolation of cellulose degradation deficient X. axonopodis pv. citri mutants

To confirm the presence of functional genes for CMC degradation in X. axonopodis pv. citri, a collection of nearly 3,000 knock-out mutants was produced by random insertion of a cassette for resistance to kanamycin.

To evaluate the capacity of X. axonopodis pv. citri in hydrolyzing cellulose-containing material, we performed a global screening to search strains for lack-of-function (Figure 1 A and B). Individual transformants from the random mutant libraries were replicated onto squared CMC plates for phenotypic screening. Carboxymethyl cellulose (CMC) is a high-molecular weight polymer that mimics cellulose without being transported into cells (Kim et al., 2000). Cell colonies that hydrolyze CMC can be clearly identified by the surrounding halo after treatment with Congo red. For more reliable screening of mutants with modified CMC activity, selection criteria were changed from the intensity of the clear zones apparent below transformant colonies on CMC plates, to the size of clear-zone formation around the colonies, thereby obtaining a few candidate mutant strains.
From the knock-out mutants produced by random insertion, the transformant named 30E9 exhibited a smaller, superficial clear zone on the CMC plate, suggesting that it is merely partially functional (Figure 1C and D). The ability of the mutant 30E9 to promote CMC degradation from agar plates was very weak and limited, only occurring below the colony. This inconsiderable CMC degradation remained stable for several days after inoculation. Areas with CMC degradation were significantly lower on spots with the 30E9 mutant strain than on those with the wild-type \( t \) test with paired two samples for mean, \( p < 0.001; n = 24 \) spots analyzed per each strain) (Table 3). To check whether the CMC degradation in the mutant strain could be induced by the plant tissue, infected foliar discs were placed on a CMC plate (Figure 1D). Despite the fact that the wild-type strain showed CMCase activity below the edge of the discs, the mutant strain had little effect.

To identify the site of transposon integration, we determined the DNA sequence adjacent to the \textit{kanR} cassette for this strain. The 30E9 mutant had inactivation of the gene \textit{xpsD} (GenBank accession n. AAM38377.1), with the transposon located at the position 4,178,942 in the genome (GCACGATG \textless{} KAN-2 \textgreater{} TCCAGAA). Since the transposon EZ::TN \textless{} KAN-2 \textgreater{} shows no origin of replication it cannot be rescued by digesting genomic DNA of the mutant strain, therefore the TAIL-PCR technique was used to map the site of cassette insertion. Southern blot analysis verified that a single insertion was responsible for the phenotype (Figure 2A).

**Interruption of \textit{X. axonopodis pv. citri} \textit{xpsD} gene results in the modification of bacterial secretion, disease symptoms and multiplication on citrus leaves**

The screening of the transposon library led to the identification of a clone with an impaired ability to hydrolyze cellulose. Also, the lack-of-function for the gene investigated in this study had effect on bacterial secretion (Figure 2B), disease symptoms (Figure 3 A-E), and growth \textit{in planta} (Figure 4). Nevertheless, no apparent differences in symptom severity were observed on comparing mutant and wild-type strains when the inoculation method (spray) reproduced natural-infection phenomena (Figure 3F and G).

### Table 3 - Hydrolysis of CMC by wild-type and mutant strains.

| \textit{X. axonopodis pv. citri} strain               | CMCase activity index\(^{*,a}\) | Standard deviation |
|-----------------------------------------------------|--------------------------------|--------------------|
| Wild type                                           | 5.07a\(^{b}\) \pm 0.1487       | 0.7285             |
| XpsD-defective mutant (30E9)                        | 0.86b\(^{b}\) \pm 0.0804       | 0.3941             |

\(^{*}\)\((H^2 - C^2)/ C^2\) \((H\) - diameter of the halo, \(C\) - diameter of the colony).  
\(^{a}\)Average \pm standard error of the means \((n = 24)\).  
\(^{b}\)Statistically significant differences \((p < 0.001)\), as determined by the Student \(t\) test, are indicated by letters “a” and “b” for comparison to the strains.

**Figure 1** - Selection of \textit{X. axonopodis pv. citri} mutant colonies defective in CMC degradation. In panel A, Petri dish with 96 colonies grown on CMC medium, 2 days after inoculation; B, Halo of CMC degradation in Petri dish after colonies were washed off and Congo red staining was applied. C. Agar medium topped with CMC was seeded with lawn of mutant strain 30E9 (left) and wild-type (right) \((40 \mu L \text{ per spot})\), grown for 24 h, washed off and then stained with Congo red. In panel D, the same procedure as that in panel C, however wells were made in the medium before seeding the cells. In panel E, foliar discs of citrus plants were infiltrated, placed onto the medium and then removed after 24 h, whereupon plates were stained. All strains were grown at 28 °C in NB liquid medium and harvested at O.D.\(_{600}\) = 0.5 for inoculation. Unstained regions correspond to areas where CMC has been degraded.
As shown by protein profiles (Figure 2B), CMCases certainly are not the only enzymes whose secretion is affected by mutation in \( xps \) \( \text{D} \). In fact, the \( xps \) \( \text{D} \) gene characterized in \( X. \text{campestris} \) pv. \( \text{campestris} \) was involved in the secretion of a number of extra-cellular enzymes (Hu \( \text{et al.} \), 1992).

To confirm that during infection on leaves, the transposon was not excised from the bacterium due to selection, the strains were re-isolated from the infected tissues and plated using an appropriate antibiotic. Unlike the wild-type strain, the mutant strain was capable of growing on plates with kanamycin, and the 399-bp fragment of \( \text{aph} \) (kanamycin-resistance gene) was amplified by PCR.

A clear difference in symptomatology was found according to the method of inoculation used in the study. Although no differences were detected in leaves inoculated by spray (Figure 3A and B), we found clear altered symptoms in the inoculated zone of the mutant strain compared to the wild-type strain in infiltrated leaves (Figure 3C to G). The differences were especially notable at the first two days af-

**Figure 2** - A. Southern blot by using a fragment of \( \text{nptII} \) (399 bp) as a probe (1) to confirm single insertion. Total genomic DNA of the strain \( 30E9(\text{xpsD}) \) from \( \text{Xac} \ 306 \) digested with restriction enzymes (2: \( \text{EcoRI} \), 3: \( \text{EcoRV} \), and 4: \( \text{NcoI} \)) and wild strain (5). B. SDS-PAGE of the secreted proteins (supernatant) from \( Xanthomonas \ axonopodis \ pv. \ citri \) in the XVM2 medium. Lane 1, molecular mass marker; lane 2, wild-type strain; lane 3, \( \text{xpsD} \) defective strain.

**Figure 3** - Foliar symptoms of inoculation with \( X. \ axonopodis \ pv. \ citri \) in citrus. In panels A (mutant strain) and B (wild-type strain), symptoms of spray inoculation with \( \text{Xac} \) (\( \text{O.D.} \ 600 = 0.5 \)) on the abaxial surface of sweet orange leaves. The photographs were taken 30 days post-inoculation. In panels C, D, E, F and G, the strains were inoculated (\( \text{O.D.} \ 600 = 0.5 \)) into the side of the mid-vein (mutant strain, left; wild-type strain, right), and respective photographs taken 2, 4, 6, 8 and 10 days post-infiltration, in each case.

**Figure 4** - Growth curve of \( Xanthomonas \ axonopodis \ pv. \ citri \) (\( \text{Xac} \)) strains on leaves of susceptible sweet orange cultivar ‘Baia’ 0, 1, 3, 6 and 10 days after inoculation by syringe infiltration with needle. \( \text{Xac WT} \): wild-type strain; \( \text{Xac} 30E9(\text{xpsD}) \): \( \text{xpsD} \) mutant strain. Data represent the means of three assays.
ter inoculation (Figure 3C), by a delay of appearance of symptoms in the mutant strain, and at ten days (Figure 3G), when the wild-type strain showed necrosis in the inoculated zone, in opposition to the mutant strain.

To ascertain the influence of the xpsD genes in citrus canker development, the growth of X. axonopodis pv. citri was compared and differences between the two strains observed (Figure 4). A smaller population of the mutant strain was observed within 3 days after inoculation and remained until the stationary phase.

Discussion

In this study, we demonstrate that the T2SS exerts an influence on the ability of X. axonopodis pv. citri to colonize host tissues and is able to mediate the hydrolysis of cellulose. X. axonopodis pv. citri enters citrus tissues through stomata and wounds, and has been traditionally described as causing lesions where the center becomes raised and spongy, or corky (Graham et al., 1992; Graham et al., 2004), aspects not typically related to symptoms of plant-cell degradation. However, the results presented above showed that X. axonopodis pv. citri cells have the ability to hydrolyze cellulose.

The lack of a halo of CMC degradation around colonies of X. axonopodis pv. citri was expected to result from the inactivation of either a gene for a major cellulose-degrading enzyme (Walker et al., 1994), a gene-regulator (Vincent-Sealy et al., 1999), or a component of the secretion system itself (Ray et al., 2000). The fact that the scant CMC degradation on agar plates only occurred below the colony, and furthermore that no surrounding degradation halo was detected through staining, all implies that the mechanism is cell bound (Zorregueta et al., 2000).

Studies indicated that when the xpsD gene was interrupted, extracellular enzymes polygalacturonate lyase, alpha-amylose and endoglucanase accumulated in the periplasm of Xanthomonas campestris pv. campestris (Hu et al., 1992). Likewise, our study has demonstrated by protein profile that X. axonopodis pv. citri which lacks a functional xpsD gene was clearly affected in its secretion.

XpsD is the outer membrane single protein of the xps cluster-encoded T2SS in Gram-negative bacteria, which requires a multicomponent assembly apparatus for the secretion of extracellular enzymes. Recent articles on several plant pathogens report a key role of the xps cluster during pathogenesis (Jha et al., 2005, 2007). This finding has shed light on the possibility of X. axonopodis pv. citri being capable of somehow modifying cellulose components in its environment, and is consistent with the effects of the T2SS found in other bacteria (Ray et al., 2000; Zhou and Ingram, 2000; Zorregueta et al., 2000; Jha et al., 2005, 2007). In fact, X. axonopodis pv. citri, like X. campestris pv. vesicatoria and X. campestris pv. campestris, harbors two gene clusters coding for different T2SSs (the xcs and xps type II secretion systems), whereas X. oryzae pv. oryzae encodes only the xps cluster. So far, there are no reports on the role played by the xcs-encoded T2SS in xanthomonads.

We had no means of complementing X. axonopodis pv. citri to evaluate whether wild-type xpsD genes are capable of inducing virulence and cellulose degradation, since amplification of the full gene (2,291 bp in length) from the genome or cosmid has so far been unsuccessful. Thus, complementation tests were not accomplished.

Although it remains unknown whether the degradation of cellulose can be unequivocally imputed to a major gene or is the result of the joint-action of a number of genes, it seems clear that such activity is mediated by the T2SS. Likewise, the same machinery is in some way responsible for the secretion of proteins that play important roles in virulence.

In Erwinia chrysanthemi, two endoglucanases (CelZ and CelY) are produced, however CelZ represents approximately 95% of the total carboxymethyl cellulase activity (Zhou and Ingram, 2000). The particular X. axonopodis pv. citri enzymes involved in cellulose hydrolysis have not, as yet, been identified. Even though many more insertion strains must be tested for this screen to reach saturation, these results suggest that the proteins related to CMC degradation seem to be exclusively secreted by the xps-encoded T2SS present in X. axonopodis pv. citri. Furthermore, even though incapable strains represent only a small fraction of the total number of sequences annotated as putatively involved in the process, this mutant strain could possibly make a significant difference for studies involving plant cellulose and the action of plant pathogens.

The multiplication of X. axonopodis pv. citri was significantly suppressed when inoculated into sweet orange leaves. The observation of reduced disease severity following inoculation with the 30E9 was different from that observed when xpsD-defective X. campestris pv. campestris was inoculated into cabbage (Hu et al., 1992). In fact, apart from these two pathogens infecting distinct plant hosts, a major difference between these xanthomonads is their mode of action. Whereas X. axonopodis pv. citri is a leaf-spot pathogen, X. campestris pv. campestris is a vascular bacterium. Moreover, in the spray process emulating natural infection, there were no visible modifications in lesions. Collectively, these data may indicate specific roles played by the T2SS according to the pathosystem.

The work described herein was designed to test the ability of X. axonopodis pv. citri in using the T2SS during infection and cellulose hydrolysis, in view to the adequate application of potential disease-control strategies. In summary, we have identified that X. axonopodis pv. citri is capable in hydrolyzing cellulose and that its T2SS has effects on the disease symptoms. These data demonstrate that the T2SS present in X. axonopodis pv. citri plays various and substantial biological and ecological roles that remain to be elucidated.
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