Analysis of the molecular basis of *Xanthomonas axonopodis* pv. *citri* pathogenesis in *Citrus limon*

**Florencia Siciliano**  
Instituto de Biología Molecular y Celular de Rosario  
FByF, UNR, Suipacha 531, 2000  
Rosario, Argentina  
Tel: 0341 435 6369  
Fax: 0341 439-0465  
E-mail: florisciliano@yahoo.com.ar

**Pablo Torres**  
Fundación Instituto Leloir  
Av. Patricias Argentinas 435  
Buenos Aires, Argentina  
Tel/Fax: 011 238 7500  
E-mail: PTorres@leloir.org.ar

**Lorena Sendin**  
Estación Experimental Agroindustrial Obispo Colombres  
Casilla N° 9, Las Talitas, 4101  
Tucumán, Argentina  
Tel: 0381 427 6561  
Fax: 0381 427 6404  
E-mail: lorenasendin@arnet.com.ar

**Carolina Bermejo**  
Instituto de Biología Molecular y Celular de Rosario  
FByF, UNR, Suipacha 531, 2000  
Rosario, Argentina  
Tel: 0341 435 6369  
Fax: 0341 439 0465  
E-mail: carober29@yahoo.com.ar

**Paula Filippone**  
Estación Experimental Agroindustrial Obispo Colombres  
Casilla N° 9, Las Talitas, 4101  
Tucumán, Argentina  
Tel: 0381 427 6561  
Fax: 0381 427 6404  
E-mail: mpaula@unt.edu.ar

**Gabriel Vellice**  
Estación Experimental Agroindustrial Obispo Colombres  
Casilla N° 9, Las Talitas, 4101  
Tucumán, Argentina  
Tel: 0381 427 6561  
Fax: 0381 427 6404  
E-mail: biotecnologia@eeao.org.ar

**Jackie Ramallo**  
Estación Experimental Agroindustrial Obispo Colombres  
Casilla N° 9, Las Talitas, 4101  
Tucumán, Argentina  
Tel: 0381 427 6561  
Fax: 0381 427 6404  
E-mail: jramallo@eeao.org.ar

**Atilio Castagnaro**  
Estación Experimental Agroindustrial Obispo Colombres  
Casilla N° 9, Las Talitas, 4101  
Tucumán, Argentina  
Tel: 0381 427 6561  
Fax: 0381 427 6404  
E-mail: atilio@eeao.org.ar

This paper is available online at http://www.ejbiotechnology.info/content/vol9/issue3/full/20/
Analysis of the molecular basis of *Xanthomonas axonopodis* pv. *citri* pathogenesis in *Citrus limon*

Adrian Vojnov  
Fundación Instituto Leloir  
Av. Patricias Argentinas 435  
Buenos Aires, Argentina  
Tel/Fax: 011 238 7500  
E-mail: avojnov@leloir.org.ar

María Rosa Marano*  
Instituto de Biología Molecular y Celular de Rosario  
FByF, UNR, Suipacha 531, 2000  
Rosario, Argentina  
Tel: 0341 435 6369  
Fax: 0341 439 0465  
E-mail:mmarano@fbioyf.unr.edu.ar

Financial support: Agencia de Promoción Científica y Tecnológica (PICT-02 No: 08-10740).

Keywords: canker, DSF, extracellular polysaccharide, quorum sensing.

Abbreviations:  
DSF: diffusible signal factor  
EPS: extracellular polysaccharide  
Rpf: regulation of pathogenicity factors  
Xac: *Xanthomonas axonopodis* pathovar *citri*  
Xcc: *Xanthomonas campestris* pv. *campestris*

*Xanthomonas axonopodis* pathovar *citri* (Xac) causes bacterial citrus canker, a serious disease of most citrus species. *Xanthomonas campestris* pv. *campestris* (Xcc) is the causal agent of black rot disease in cruciferous plants. In Xcc, cell-cell signaling is mediated by diffusible signal factor (DSF). Synthesis of DSF depends on RpfB and RpfF. DSF perception and signal transduction have been suggested to involve a two-component system comprising RpfC and RpfG. It has been proposed that these proteins participate in a signal transduction system linking changes in the environment to the synthesis of DSF and the expression of virulence genes. Although the cluster of the rpf genes in Xac has synteny with the corresponding cluster in Xcc, two genes (rpfH and rpfI) are absent in Xac. To investigate DSF-mediated regulation during *Xac*-Citrus limon interaction, we constructed two strains of Xac, one with a mutation in the rpfF gene, leading to an inability to produce DSF, and one with a mutation in the rpfC gene leading to an overproduction of DSF. These mutants also show decreased levels of extracellular cyclic β-(1,2)-glucans and decreased production of endoglucanase and protease extracellular enzymes. The Xac DSF-deficient rpfF and the DSF-hyper producing rpfC mutants are both severely compromised in their ability to cause canker symptoms in lemon leaves compared to the wild-type. Here we provide evidence that rpf genes in Xac are involved in controlling virulence factors mediated by DSF.

*Xcc* is one of Xanthomonas species most extensively studied at the genetic level. The complete genome sequences of *Xac* and *Xcc*, revealed that the two bacteria have more than 80% of their genes in common and that their chromosomal gene order is largely conserved (da Silva et al. 2002). The ability of *Xcc* to elicit disease depends on several factors, including the synthesis of extracellular plant cell wall-degrading enzymes and the extracellular polysaccharide (EPS) xanthan (Tang et al. 1991). Production of these pathogenesis factors is regulated by a cluster of genes (A-I) called rpf (for regulation of pathogenicity factors) (Slater et al. 2000). Only two of these genes (rpfH and rpfI) are absent in Xac (da Silva et al. 2002). The predicted protein, RpfH, is structurally related to the sensory input domain of RpfC (Slater et al. 2000). RpfI positively regulates the synthesis of proteases, endoglucanases and EPS in *Xcc* (Dow et al. 2000). The locus corresponding to rpfI in Xac is occupied by a truncated copy of an insertion sequence (da Silva et al. 2002). The absence of rpfH and rpfI in Xac may be consistent with the distinct modes of entry of the two bacteria, with *Xcc* accessing the leaf mainly via hydathodes at the leaf margin and subsequently colonizing the xylem.

Citrus canker is an endemic disease of citrus in Argentina.

* Corresponding author
Analysis of the molecular basis of Xanthomonas axonopodis pv. citri pathogenesis in Citrus limon

vessels (Wallis et al. 1973) and Xac entering primarily through the stomates and colonizing the apoplasm of the fruit, leaf and stem tissue (Graham et al. 2004).

The major aim of this work is to identify genes involved in Xac pathogenicity, in particular, genes mediating cell-cell signaling. Our primary approach has been the isolation of additional mutants affected in the synthesis and perception of DSF. Such studies may provide a rational basis for the development of crop protection methods based on interference with intercellular signaling events.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The wild-type (8004) and rpfF (8523) and rpfC (8557) mutant strains of Xcc were described previously (Tang et al. 1991; Slater et al. 2000). The wild-type strain of Xac was obtained from cankerous leaves of infected lemon trees in the province of Tucumán, Argentina. Xcc and Xac strains were cultured in peptone-yeast extract-malt extract (PYM) nutrient medium (Vojnov et al. 2001), supplemented with D-glucose at a final concentration of 2% (w/v) at 28°C. For growth on plates, PYM was solidified with 1.5% (w/v) agar to make PYMA. L medium contained bacotryptone, 10 g/l; yeast extract, 5 g/l; sodium chloride, 5 g/l and D-glucose, 1 g/l. E. coli strains were grown at 37°C in Luria-Bertani (LB) broth and on LB agar (Dow et al. 2003). Bacterial growth was measured in an spectrophotometer at 600 nm. When required, the antibiotics, Rifampicin (Rif) and Kanamycin (Km) were added to the growth media at concentrations of 10 µg/ml and 50 µg/ml, respectively.

Site-directed gene replacement of the Xanthomonas axonopodis pv. citri

Genomic DNA from the Xac strain was extracted according to Chen and Kuo (1993). PCR products, from the region encoding the wild-type Xac rpfF and rpfC genes, were amplified using primers designed from Xac sequences available in GenBank (da Silva et al. 2002) and cloned into the pGEM-Teasy vector (Promega). A cassette containing a Km resistance gene was inserted in the rpfF and rpfC cleavage sites, generating deletions in the rpfF and rpfC gene. These constructs were digested and cloned into the suicide vector pSac (Quandt and Hynes, 1993) and transformed into wild-type Xac by electroporation. Transformed bacteria were selected on PYM media supplemented with 5% sucrose and 50 µg/ml Km. Disruption of the rpfF and rpfC loci in the mutant strains was confirmed by PCR and Southern blot (data not shown).

Bioassay of DSF extracted from culture supernatants

DSF was extracted into ethyl acetate from culture supernatants of Xcc or Xac strains grown in PYM as described by Barber et al. (1997). The DSF bioassay is based on DSF’s ability to restore endoglucanase production to Xcc rpfF mutants as described in Barber et al. (1997).

Assays of extracellular enzymes and EPS

For measurement of protease and endoglucanase activity, Xac strains were grown in PYM medium. Enzyme activity in cell-free culture supernatants were measured by radial diffusion assays into substrate-containing agar plates as described by Slater et al. (2000). For measurements of EPS production, strains were grown in PYM medium supplemented with 2% (w/v) D-glucose for 24 hrs. EPS was precipitated from culture supernatants by ethanol, dried and weighed as described by Tang et al. (1991). Methods to isolate the cyclic glucan from culture supernatants, for size-exclusion chromatography on BioGel P4, have been described previously (Vojnov et al. 2001). Bacterial cultures were washed and resuspended in water to a concentration of 10^8 cfu ml⁻¹. Samples of these suspensions (50 µl) were spotted onto plates and allowed to dry before growth at 30°C. Plates were stained when necessary as described in Tang et al. (1991).
levels of cyclic
for protease and endoglucanase activities, as well as for
proteolytic activity was scored by detecting degradation of
The mutant strains
mutants with altered levels of DSF
Isolation of
RESULTS AND DISCUSSION
bacterial growth curves (Siciliano et al. 2005).
Citrus limon
Plant preparation and measurement of bacterial
growth in plant

Citeus limon variety Eureka was grown in a greenhouse at a
temperature of 18-25ºC. Xac and Xcc mutants were grown
in PYM media supplemented with the appropriate
antibiotics at 28ºC for 24 hrs. Cells were resuspended to a
concentration of 10⁶ cfu ml⁻¹. Xac strains were assayed for
pathogenicity by inoculation of bacteria into the lamina of
mature and young lemon leaves using three different
procedures, namely pressure infiltrating with a 2 ml syringe
without a needle, nicking the underside of the leaves with a
razor blade followed by spraying, and spraying without
nicking. The last method mimics the natural Xac infection
process, in which bacteria enter to the leaf through
openings in the leaf (via stomata), followed by colonization
of the apoplasms. Inoculated plants were maintained for 35
days in a growth cabinet, with temperatures ranging from
25-28ºC, high humidity, a photoperiod of 16 hrs light, and a
light intensity of 150 a 200 µE/sm². Disease progression
was monitored phenotypically and through analysis of
bacterial growth curves (Siciliano et al. 2005).

RESULTS AND DISCUSSION

Isolation of Xanthomonas axonopodis pv. citri
mutants with altered levels of DSF

The mutant strains ArpfF and ArpfC of Xac were screened
for protease and endoglucanase activities, as well as for
levels of cyclic β-(1,2)-glucan and xanthan. The
proteolytic activity was scored by detecting degradation of
milk proteins, seen as a zone of clearing around the
colonies. As shown in Figure 1a, the halo around the colony
of the rpffF and rpfc genes is reduced compared with the
wild-type Xac strain. The activities of endoglucanase, an
enzyme involved in plant cell wall degradation, was
measured by a plate diffusion assay, where the zones of
clearing indicate degradation of carboxymethylcellulose
(CMC). Similar to protease activity, endoglucanase activity
was found to be reduced in both rpffF and rpfc mutants
(Figure 1b).

As shown in Figure 1c and Figure 1d, cyclic β-(1,2)-
glucan and xanthan in the Xac ArpfF and ArpfC mutants
are reduced compared to wild-type Xac. Based on these
results and given that these pathogenicity factors are
induced by DSF, we can infer that DSF levels are altered in
the Xac ArpfF and ArpfC mutants.

Xac DSF reverses the phenotype of Xcc rpffF
mutants

Strains of Xcc carrying mutations in the rpffF and rpfc
genes grow as matrix-enclosed aggregates in L medium,
whereas the wild-type Xcc strain grew in a dispersed
fashion. (Dow et al. 2003). We investigated the behavior of
the Xcc ArpfF (8523) and ArpfC (8557) mutants in the
presence of DSF extracts taken from the Xac ArpfF mutant.
As shown in Figure 2a, wild-type Xcc grows in a dispersed
fashion, but Xcc rpffF and rpfc mutants do not. Addition of
DSF extracted from the Xac ArpfC mutant reverses the
phenotype of mutant 8523, but not mutant 8557. Based on
these findings, we conclude that the Xac ArpfC mutant
produces DSF, which induces bacteria to grow in a
dispersed manner, and that DSF from Xcc and Xac likely
share structural similarities.

To confirm that Xac is able to produce DSF, we analyzed
DSF extracted from wild-type and Xac mutants using the
DSF bioassay developed by Barber et al. (1997), which
relies on the restoration of endoglucanase activity to an
rpffF mutant strain of Xcc. Ethyl acetate extracts of culture
supermatants from mutant and wild type strains were
assayed for DSF activity. As expected the extracts of the
Xac ArpfC mutants show bigger halos compared with the
wild-type, while the rpffF strain extracts show no halos
indicative of CMC degradation (Figure 2b). Extracts from
Xac DSF-hyper producing rpfc and DSF-non-producing
rpffF mutant strains were used as controls (Figure 2b).

Reduction of virulence on lemon leaves is
preferentially associated with mutation of the rpfc
gene in Xac

To test whether the mutations in the rpffF and rpfc genes of
Xac affect general fitness in plant, bacteria suspensions
were inoculated at a concentration of 10⁶ cfu ml⁻¹, followed
by measurements of bacterial growth and observation of
canker symptoms. Figure 3 shows symptoms on lemon
leaves 35 days post-infection (d.p.i.) using the methods
Analysis of the molecular basis of Xanthomonas axonopodis pv. citri pathogenesis in Citrus limon

described above (nick and spray, A; spray alone, B; and pressure infiltration, C) with XacΔrpfF and ΔrpfC mutants and wild-type Xac. Although both mutants show reduced pathogenicity compared with wild-type, the DSF-hyper producing mutant (ΔrpfC) is more severely compromised in its ability to cause canker disease in citrus. These results are most clearly evident with the spray inoculation method (B). Plants inoculated with MgCl₂ show no disease symptoms. Further study of DSF role in the plant immune response and other regulatory mechanisms should advance our understanding of the adaptation of bacteria to parasitic life within plants and may allow us to develop tools to control Xanthomonas infection that function across plant species.

ACKNOWLEDGMENTS

We thank Dr. Marcelo A. Dankert for his continuous support.

Atilio Castagnaro, María Rosa Marano and Adrián Vojnov are members of the Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas.

REFERENCES


Siciliano, F. et al.


