

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

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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.**

Invasion and colonization of the host mesophyll is accomplished through natural openings and plant tissue lesions (Graham et al. 2004). Most commercial citrus cultivars are moderately to highly susceptible to *Xac*. Thus, there is considerable need for a better understanding of the process of infection to pave the way for the development of biotechnology strategies that will support sustainable management of the disease.

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.

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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.

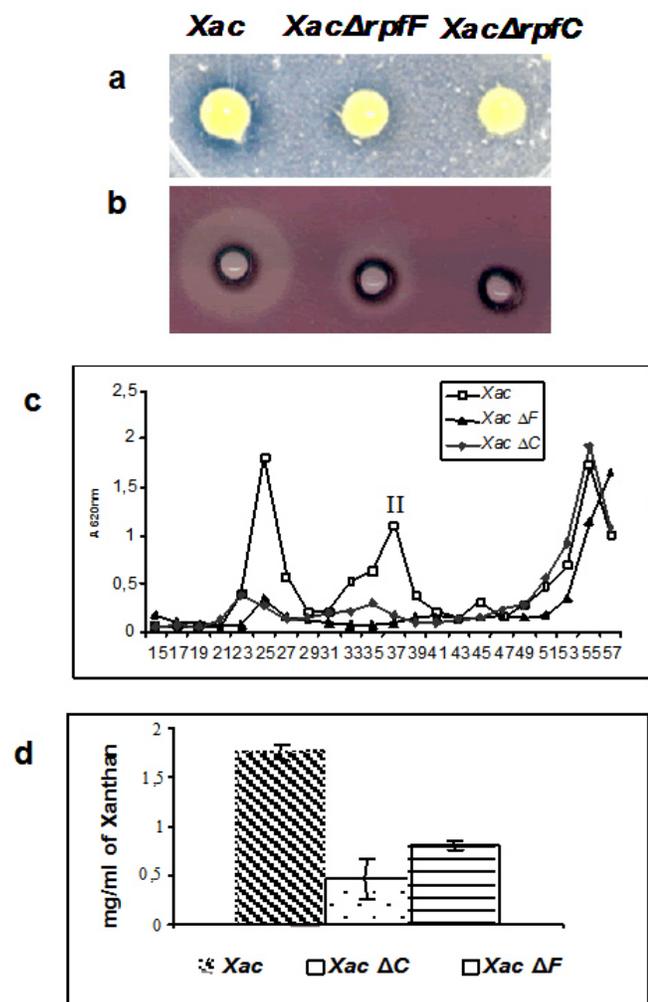


Figure 1. Phenotypic analysis of *Xac rpfF* and *rpfC* mutants.
 (a) Protease production.
 (b) Plate test for endoglucanase activity.
 (c) Elution profile of LPS, I and extracellular cyclic β-(1,2)-glucan, II.
 (d) Xanthan production.

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 bactotryptone, 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 a 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 10⁸ 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).

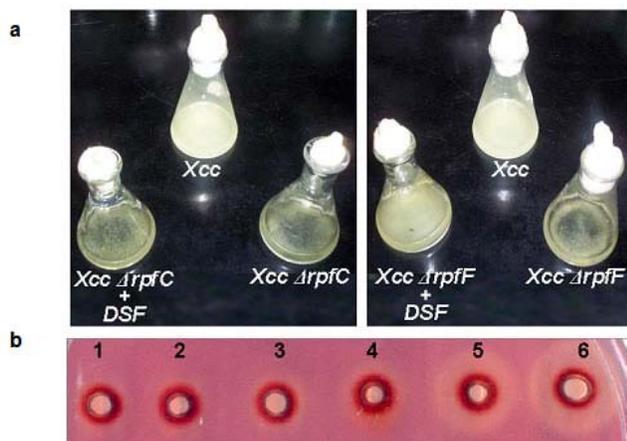


Figure 2. Role of DSF extracted from *Xcc* $\Delta rpfC$ mutants.

(a) Strains of *Xcc* with mutations in *rpfF* and *rpfC* genes grow in an aggregated fashion in L medium, whereas the wild-type strain grows in a dispersed fashion. Addition of DSF from the *Xcc* $\Delta rpfC$ mutant to *rpfF* strains, but not to *rpfC* strains of *Xcc*, causes dispersed growth.

(b) Bioassay of DSF extracted from culture supernatants of *Xcc* *rpfF* mutants (1), *Xcc* *rpfF* mutants (2), *Xcc* wild-type (3), *Xcc* *rpfC* mutant (4), *Xcc* wild-type (5) and *Xcc* *rpfC* strains (6).

Plant preparation and measurement of bacterial growth in plant

Citrus limon variety Eureka was grown in a greenhouse at a temperature of 18-25°C. *Xcc* and *Xcc* mutants were grown in PYM media supplemented with the appropriate antibiotics at 28°C for 24 hrs. Cells were resuspended to a concentration of 10^6 cfu ml⁻¹. *Xcc* 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 *Xcc* infection process, in which bacteria enter to the leaf through openings in the leaf (via stomata), followed by colonization of the apoplast. Inoculated plants were maintained for 35 days in a growth cabinet, with temperatures ranging from 25-28°C, high humidity, a photoperiod of 16 hrs light, and a light intensity of 150 a 200 $\mu\text{E}/\text{sm}^2$. 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 $\Delta rpfF$ and $\Delta rpfC$ of *Xcc* 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 *rpfF* and *rpfC* mutants is reduced compared with the wild-type *Xcc* 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 *rpfF* and *rpfC* mutants (Figure 1b).

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

Xcc DSF reverses the phenotype of *Xcc* *rpfF* mutants

Strains of *Xcc* carrying mutations in the *rpfF* and *rpfC* genes grew 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* $\Delta rpfF$ (8523) and $\Delta rpfC$ (8557) mutants in the presence of DSF extracts taken from the *Xcc* $\Delta rpfC$ mutant. As shown in Figure 2a, wild-type *Xcc* grows in a dispersed fashion, but *Xcc* *rpfF* and *rpfC* mutants do not. Addition of DSF extracted from the *Xcc* $\Delta rpfC$ mutant reverses the phenotype of mutant 8523, but not mutant 8557. Based on these findings, we conclude that the *Xcc* $\Delta rpfC$ mutant produces DSF, which induces bacteria to grow in a dispersed manner, and that DSF from *Xcc* and *Xcc* likely share structural similarities.

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

Reduction of virulence on lemon leaves is preferentially associated with mutation of the *rpfC* gene in *Xcc*

To test whether the mutations in the *rpfF* and *rpfC* genes of *Xcc* affect general fitness *in plant*, bacteria suspensions were inoculated at a concentration of 10^6 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

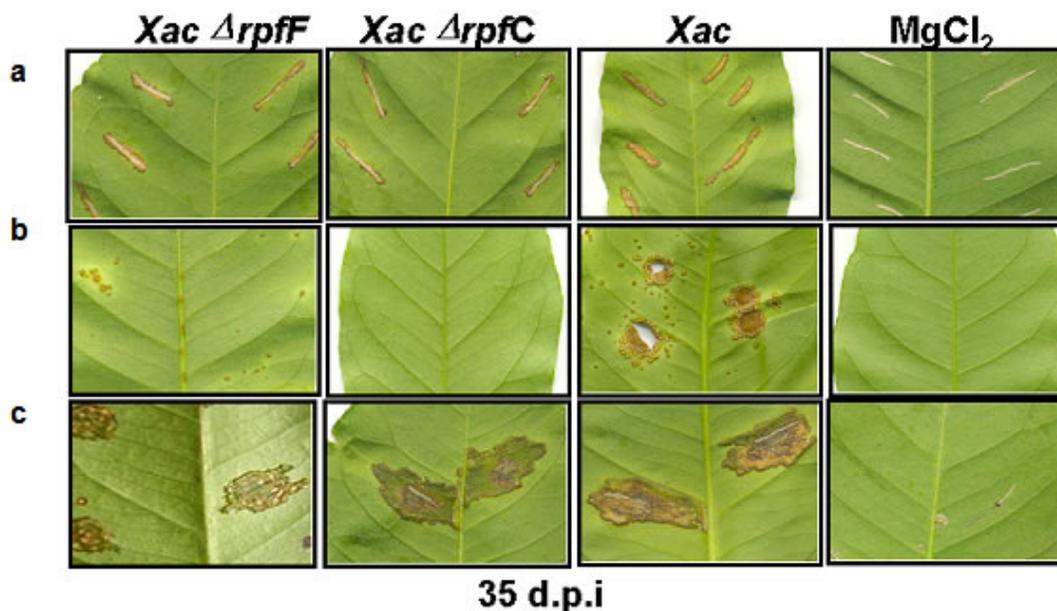


Figure 3. Macroscopic symptoms (canker) in *Citrus limon* variety Eureka. Each strain was inoculated into three different leaves. Infected plants were observed for disease symptoms (formation of cankers) over the course of 35 days under optimal temperature and humidity.

- (a) Inoculation by nick and spray
 (b) spray
 (c) pressure infiltration

described above (nick and spray, A; spray alone, B; and pressure infiltration, C) with *Xac ΔrpfF* and *ΔrpfC* mutants and wild-type *Xac*. Although both mutants show reduced pathogenicity compared with wild-type, the DSF-hyper producing mutant (*rpfC*) 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 $MgCl_2$ 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.

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