Xanthan Induces Plant Susceptibility by Suppressing Callose Deposition[1][OA]

Maximina H. Yun, Pablo S. Torres, Mohamed El Oirdi, Luciano A. Rigano, Rocio Gonzalez-Lamothe, María Rosa Marano, Atilio P. Castagnaro, Marcelo A. Dankert, Kamal Bouarab[2], and Adrián A. Vojnov[2]*

Fundación Instituto Leloir, Instituto de Investigaciones Bioquímicas de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas and Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires C1405BWE, Argentina (M.H.Y., P.S.T., L.A.R., M.A.D., A.A.V.); Centre en Amélioration Végétale, Centre de Recherche sur les Mécanismes du Fonctionnement Cellulaire, Département de Biologie, Facultés des Sciences, Université de Sherbrooke, Sherbrooke, Quebec, Canada J1K2R1 (M.E.O., K.B.); Department of Biochemistry, Université de Montréal, Montreal, Quebec, Canada H3C 3J7 (R.G.L.); Instituto de Biologia Molecular de Rosario-Departmento Microbiología, Facultad de Ciencias, Bioquímicas y Farmacéuticas, Universidad National de Rosario, Rosario S2002LRK, Argentina (M.R.M.); and Estación Experimental Agroindustrial Obispo Colombres, Las Talitas, 4101-Tucuman, Argentina (A.P.C.)

Xanthan is the major exopolysaccharide secreted by *Xanthomonas* spp. Despite its diverse roles in bacterial pathogenesis of plants, little is known about the real implication of this molecule in Xanthomonas pathogenesis. In this study we show that in contrast to *Xanthomonas campestris* pv *campestris* strain 8004 (wild type), the xanthan minus mutant (strain 8397) and the mutant strain 8396, which is producing truncated xanthan, fail to cause disease in both *Nicotiana benthamiana* and Arabidopsis (*Arabidopsis thaliana*) plants. In contrast to wild type, 8397 and 8396 strains induce callose deposition in *N. benthamiana* and Arabidopsis plants. Interestingly, treatment with xanthan but not truncated xanthan, suppresses the accumulation of callose and enhances the susceptibility of both *N. benthamiana* and Arabidopsis plants to 8397 and 8396 mutant strains. Finally, in concordance, we also show that treatment with an inhibitor of callose deposition previous to infection induces susceptibility to 8397 and 8396 strains. Thus, xanthan suppression effect on callose deposition seems to be important for Xanthomonas infectivity.

The phytopathogenic bacterium *Xanthomonas campestris* pv *campestris* (*Xcc*) is the causal agent of black rot disease of cruciferous plants. *Xcc* produces a range of extracellular enzymes (including proteases, pectinases, and endoglucanase) and an extracellular polysaccharide (xanthan), both being essential for pathogenesis (Dow and Daniels, 2000). The expression cascade of these virulence factors is likely to occur in the determined place, at the appropriate stage of infection, and at a proper level to achieve successful infection by the pathogen (Dow and Daniels, 2000). In *Xcc*, the production of extracellular enzymes and xanthan is subjected to coordinate positive regulation by the *regulation of pathogenicity factors* gene cluster (Tang et al., 1991; Slater et al., 2000). Xanthan biosynthesis is regulated (at least in part) at a transcriptional level (Vojnov et al., 2001). The production of xanthan seems to be required at early stages of *Xcc* infection in leaf mesophyll tissue (Newman et al., 1994), but copious production of this polysaccharide is observed at later stages of pathogenesis in tissue undergoing necrosis (Vojnov et al., 2001). Similarly, the operon that controls the synthesis of exopolysaccharide in *Ralstonia solanacearum* is mainly activated at later stages of infection of tomato (*Solanum lycopersicum*; Kang et al., 1999).

The chemical structure of xanthan has been studied by several laboratories. It consists of a cellulosic backbone with (1→4)-β-D-Glc linkages and a trisaccharide side chain, β-D-Man-(1→4)-β-D-GlcUA-(1→2)-α-D-Man, (1→3) linked to every two Glcs (Fig. 1A). About half of the terminal Mans are substituted with ketal-pyruvate residues and the other half with acetyl residues, and all of the internal ones with acetyl residues (Jansson et al., 1975; Melton et al., 1976).

Most of the biochemical details of the biosynthesis of xanthan (Ielpi et al., 1993) and the genes that encode for the enzymes involved in the transfer of the sugars and of the nonglycosidic substituents have been described (Vanderslice et al., 1990). Xanthan-deficient mutants of *Xcc* have been derived from wild-type strain 8004 by Tn*5* mutagenesis (Barrère et al., 1986). The structural analysis

[1] This work was supported by the Agencia de Promoción Científicas y Tecnológica (PICT–02 no. 08–10740) and Concejo Nacional de Investigaciones Científicas y Técnicas (to A.A.V.), and by the Conseil de Recherche en Science Naturelles et Génie du Canada, the Fondation Canadienne pour l'Innovation, and the Université de Sherbrooke (to K.B.).

[2] These authors contributed equally to the paper.

* Corresponding author; e-mail avojnov@leloir.org.ar; fax 011541152387501.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Adrián A. Vojnov (avojnov@leloir.org.ar).

[OA] Open Access articles can be viewed online without a subscription.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.074542.

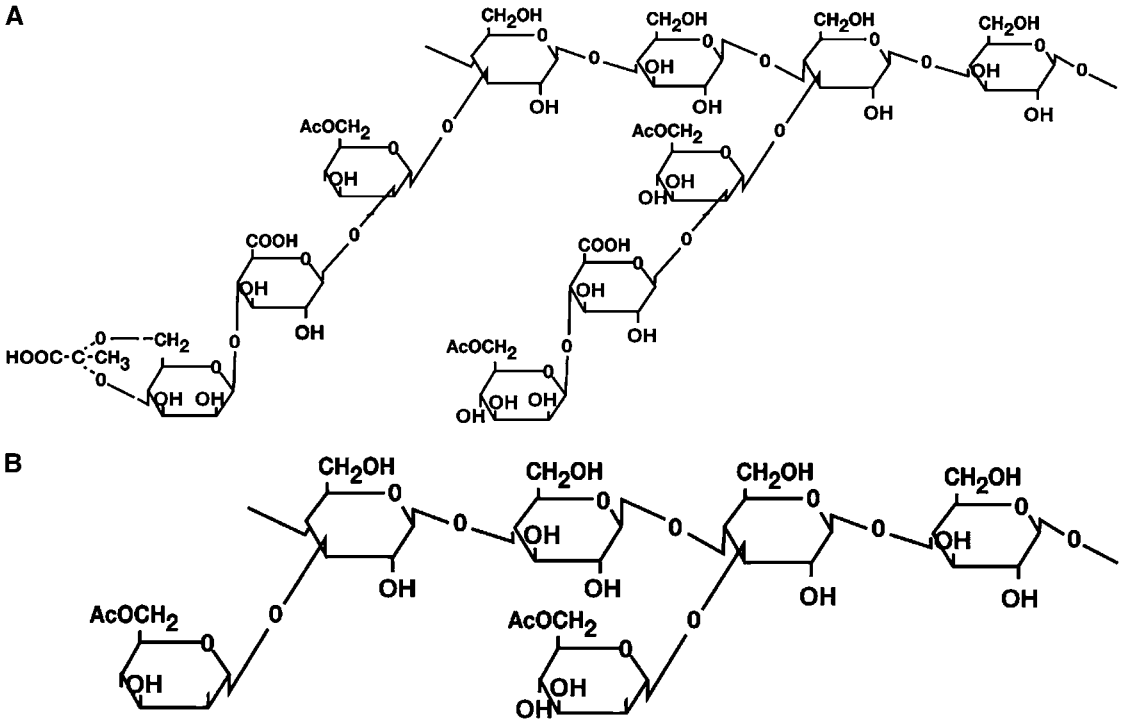

Figure 1. Structures of the exopolysaccharides produced by *Xcc*. A, Xanthan produced by 8004 strain. B, Truncated xanthan produced by 8396 strain.

of the polysaccharide produced by one of such Tn*5* mutants, the 8396 strain, has been described. The new polymer contains Glc and Man in a molar ratio of 2.0:1.0 instead of Glc, Man, and GlcUA (2.0:2.0:1.0) as in xanthan (Fig. 1B; Heyraud et al., 1998; Vojnov et al., 2002). Genetic and biochemical analysis of another mutant, the 8397 strain, provided evidence for a role for *gumB* and *gumC* in the formation of high-molecular-mass xanthan from its pentasaccharide repeating unit (Vojnov et al., 1998). This mutant does not produce xanthan and became unable to grow on turnip (*Brassica campestris*; Newman et al., 1994). Both 8397 and 8396 strains were complemented by recombinant plasmid pIZD15-261 that contains the whole gum cluster (Vojnov et al., 1998, 2002).

The production of xanthan polymers by phytopathogenic bacteria has been implicated in several symptoms, including the wilting induced by vascular pathogens and the water soaking associated with foliar pathogens (Denny, 1995). In addition, the virulence of numerous phytopathogenic bacteria, including *R.* (*Pseudomonas*) *solanacearum*, *Erwinia amilovora*, *Erwinia stewartii*, and *X. campestris*, have been correlated with their ability to produce exopolysaccharide polymers in planta (Dolph et al., 1988; Kao et al., 1992; Geier and Geider, 1993; Saile et al., 1997; Yu et al., 1999; Newman et al., 1994; Vojnov et al., 2001).

Suppression of host defenses is emerging as a key pathogenesis-related mechanism. A *Pseudomonas syringae* type III effector has been implicated in suppression of cell wall-based extracellular defense in Arabidopsis (*Arabidopsis thaliana*) plants (Hauck et al., 2003). In addition to type III effectors, the tomato and Arabidopsis pathogen *P. syringae* pv *tomato* (DC3000 strain) also produces the phytotoxin coronatine, which is required for full virulence in Arabidopsis (Mittal and Davis, 1995; Bender et al., 1999). In their study of *X. campestris* pv *Vesicatoria*, Keshavarzi et al. (2004) show that wild-type bacteria suppresses cell wall alterations, including callose deposition, that constitute a basal form of resistance to bacterial colonization.

Despite the extensive data available on exopolysaccharides of Xanthomonas, little is known of its role in host-pathogen interaction. In this report, we evaluate the role of xanthan in the pathogenicity of *Xcc* in Arabidopsis and *Nicotiana benthamiana*, the latter being more amenable to in planta studies. The involvement of xanthan and its structural features in the plant defense response were analyzed by comparing the population dynamics and symptom development of wild-type and xanthan-deficient strains. Our results suggest that xanthan specifically suppresses local plant defense by the inhibition of callose deposition, and that the biological function of this exopolysaccharide depends on its chemical composition.

RESULTS

Xanthan Is Required for *Xcc* Virulence and Necrosis Development in *N. benthamiana* and Arabidopsis

Two *X. campestris* mutants defective in xanthan production (strains 8397 and 8396) were used for testing

the importance of xanthan in plant-Xanthomonas interaction. Both 8397 and 8396 were shown to grow similarly in PYM (peptone, yeast, and malt extracts) liquid media, indicating that lack of xanthan production did not impair their in vitro growth (data not shown). Symptoms and growth in *N. benthamiana* and Arabidopsis plants were checked after inoculating the leaves with the wild-type strain and the 8397 and 8396 mutants (Vojnov et al., 1998, 2002). To assess bacterial development in *N. benthamiana*, leaf discs were bored from the infiltrated area, ground in 10 mM $MgCl_2$, and serially diluted to measure bacterial numbers (Bouarab et al., 2002). Arabidopsis plants were submerged upside down in the bacterial solution for 30 s and then covered with a transparent lid for 2 d to allow bacteria to penetrate the leaf and the first measure of bacterial population was carried out (Tornero and Dangl, 2001). In contrast to the wild type, both mutant strains produced almost no symptoms in *N. benthamiana* and Arabidopsis plants (Fig. 2,A and B). Growth of the mutants was reduced in *N. benthamiana* and Arabidopsis. For the wild-type strain, populations increased more than three orders of magnitude over the 4 d monitoring period. In contrast, the strain 8397 showed a 25- to 30-fold lower final concentration, after the same infection period, in both *N. benthamiana* (Fig. 2C) and Arabidopsis (Fig. 2D). A similar growth profile has been shown for this mutant in turnip plants (Newman et al., 1994). Finally the strain

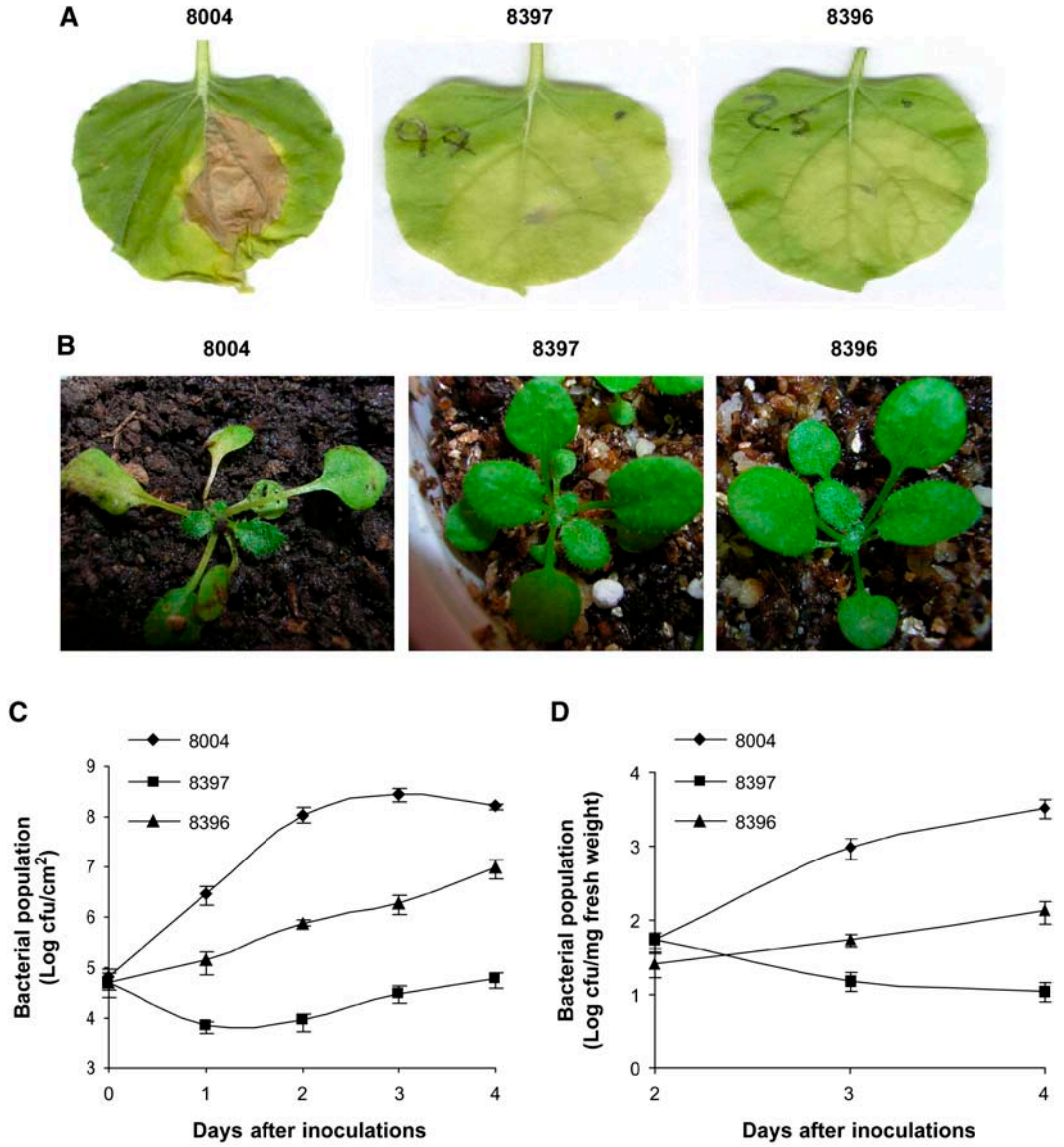

Figure 2. Infection of *N. benthamiana* and Arabidopsis (*Arabidopsis thaliana*) with *Xcc* strains. Symptoms in *N. benthamiana* leaves (A) and Arabidopsis plants (B) 4 days after inoculation with either wild-type *Xcc* strain 8004 and *Xcc* xanthan mutant strains 8397 or 8396 (10^7 cfu/mL). Photos of disease symptoms were taken 8 dpi. Bacterial population of *Xcc* strains in *N. benthamiana* leaves (C) and Arabidopsis plants (D). The mean and standard deviation of three independent experiments of bacterial numbers are given.

8396 showed an intermediate but significant lesser growth in both *N. benthamiana* (Fig. 2C) and Arabidopsis (Fig. 2D) compared with the wild type.

Xanthan Induces Susceptibility to *Xcc* in *N. benthamiana* and Arabidopsis

To investigate the effect of xanthan in Xanthomonas-plant interactions, *N. benthamiana* and Arabidopsis leaves were preinfiltrated with purified xanthan 24 h before inoculation with *Xcc* strains (Fig. 3). Interestingly, leaves of both *N. benthamiana* and Arabidopsis preinfiltrated with xanthan showed disease symptoms in response to *Xcc* strains 8397 and 8396 (Fig. 3, A and B), whereas control leaves that had been preinfiltrated with water showed no symptoms of disease restoration (data not shown). Bacterial populations were assessed 4 d after inoculation. For the strain 8397, the number of bacteria recovered from leaves of *N. benthamiana* and Arabidopsis plants pretreated with xanthan was approximately 30- or 25-fold higher than one of the plants pretreated with water, and it reached the same levels of infection as the wild-type strain (Fig. 3, C and D). Similarly, the bacteria number of the strain 8396 was approximately 15-fold higher than the one from water-treated plants (Fig. 3, C and D).

The xanthan effect was dose dependent. The minimal concentration of xanthan required for restoring disease symptoms in 8397 strain was 50 μg/mL (Fig. 4A). Surprisingly, coinoculation of xanthan with this strain is sufficient to permit the mutant to grow in *N. benthamiana* leaves (Fig. 4B). Thus, in 8397 mutant strain, the restoration of infection by xanthan does not require an induction period to be effective. The same results were obtained in the Arabidopsis experiment (data not shown).

Truncated Xanthan Is Compromised in Its Ability to Induce Susceptibility to *Xcc* 8396 and 8397 Strains

We extended our experiments to understand whether the structure of xanthan is essential for the susceptibility recovery of *Xcc* mutants shown in *N. benthamiana* and Arabidopsis. Truncated xanthan produced by the 8396 strain lacks the negatively charged GlcUA and ketal-pyruvate residues (Fig. 1B). We then tested whether this truncated xanthan is able to restore the disease to *Xcc* as pure xanthan did. Pretreatment of *N. benthamiana* and Arabidopsis leaves with the truncated exopolysaccharide failed to induce susceptibility to *Xcc* 8396 or 8397 strains (Fig. 5, A and B). Bacterial populations in the zone of infiltration were assessed 4 d after

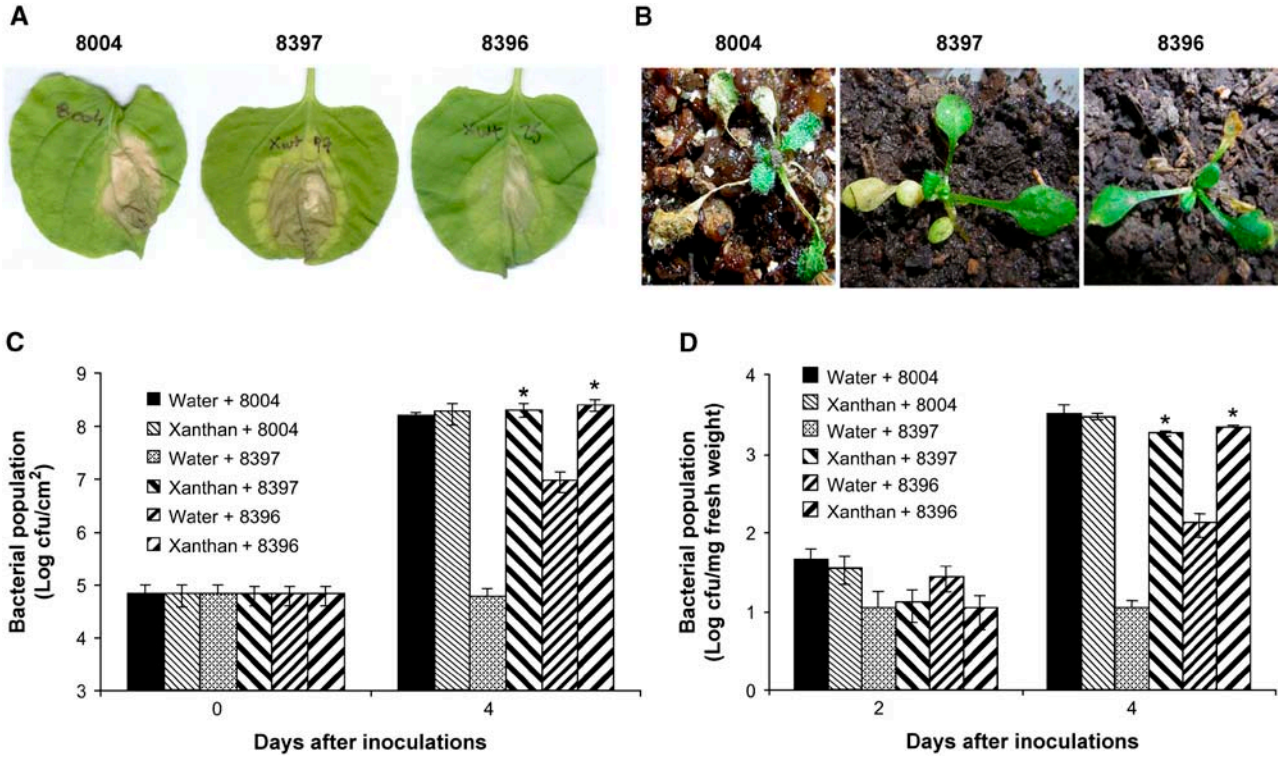

Figure 3. Xanthan induces susceptibility to 8397 and 8396 strains in *N. benthamiana* and Arabidopsis. Symptoms in *N. benthamiana* leaves (A) and Arabidopsis plants (B) treated with xanthan (150 μg/mL) and then inoculated with 8004, 8397, and 8396 strains (10^7 cfu/mL as in Fig. 2) 24 h after treatment. Photos of disease symptoms were taken 8 dpi. Bacterial populations were accounted at 0 and 4 dpi of *N. benthamiana* (C) and Arabidopsis (D). The mean and SD of three separate measurements of bacterial numbers are given. Data sets marked with an asterisk are significantly different from control (water-pretreated leaves) as assessed by the Student's *t* test: $^*P < 0.001$.

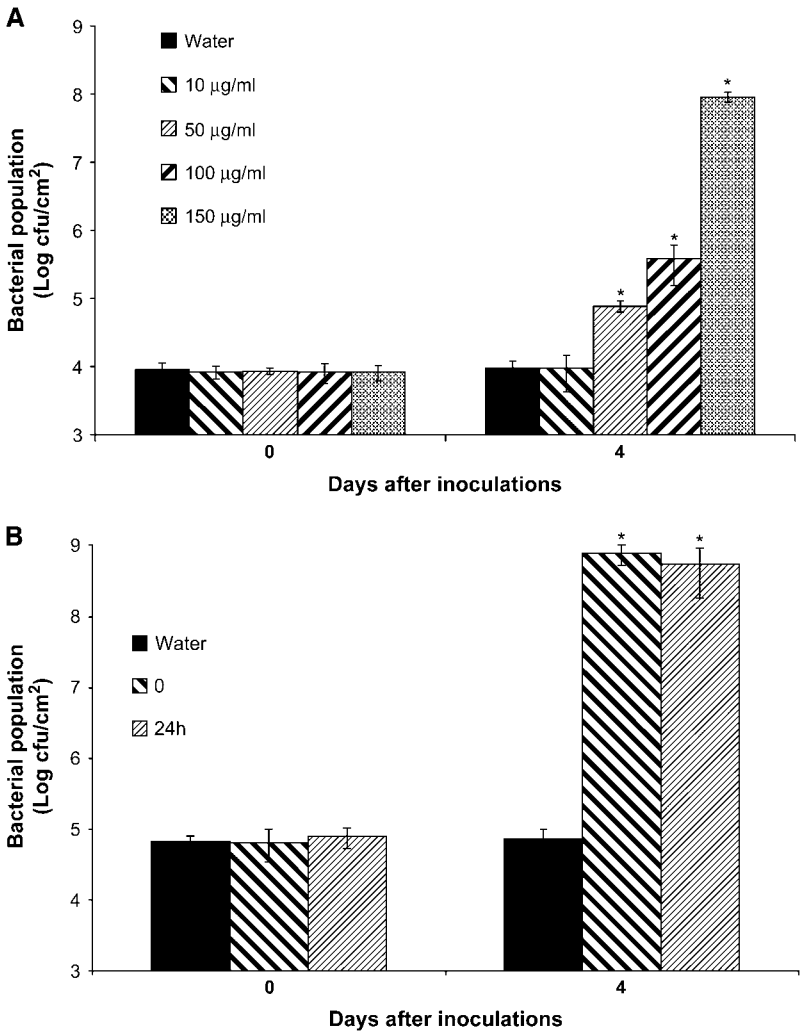

Figure 4. The susceptibility to *Xcc* induced by xanthan is dose dependent. A, Leaves of 4-week-old plants were preinfiltrated with either different concentrations of *Xcc* xanthan or water and 24 h later pretreated leaves were inoculated with the *Xcc* mutant strain 8397 (10^7 cfu/mL). B, Immediate establishment of susceptibility to *Xcc* 8397 strain by the xanthan suppressor. Leaves were preinfiltrated with *Xcc* xanthan (150 μg/mL) 0 and 24 h before inoculation (0 and 24), or water 24 h before inoculation (water) and then pretreated leaves were infected with the *Xcc* mutant strain 8397 as before. Numbers of bacteria were assessed immediately upon infection and 4 d later. The mean and SD of three separate measurements of bacterial numbers are given. Data sets marked with an asterisk are significantly different from control (water-pretreated leaves) as assessed by the Student's *t* test: $*P < 0.001$.

inoculation. The number of bacteria population recovered from leaves that had been pretreated with truncated xanthan was not affected by this pretreatment. Truncated xanthan could not recover bacterial population number of 8396 or 8397 strains at wild-type levels as xanthan pretreatment did (Fig. 5, C and D). These results suggest that the negatively charged GlcUA and ketal-pyruvate residues of xanthan are required for the biological activity of the polymer, and the modification of its structure abolishes the effect on the defense response of *N. benthamiana* and Arabidopsis.

Xanthan Suppresses Callose Deposition

Callose is a β-1,3 glucan with (1,6) modifications that are usually localized in pollen grains and tubes, dead elements of the phloem, plasmodesmatas, and tracheids. Callose synthesis can be induced by mechanical wound, physiological stress, and phytopathogen infection (Stone and Clarke, 1992).

To determine whether the callose deposition is one of the mechanisms involved in reducing the growth of 8397 and 8396 mutants in planta, *N. benthamiana* leaves were inoculated with *Xcc* strains 8004, 8397, and 8396 and callose deposition was monitored. Twenty-four hours after infection, the inoculated leaves were stained for callose with aniline blue and cytological observations were performed at the sites of infection with UV-fluorescence microscopy. Callose depositions can be identified as bright-green points in leaves or veins (Fig. 6). The callose depositions were quantified with IMAGE PRO PLUS software (Media Cybernetics). More than 10 adjacent fields of view along the length of the leaf were analyzed and averaged. The values provided are the average and SDs of more than four independent leaves for each replicate. The leaves inoculated with either 8397 or 8396 strains had a higher level (approximately 10-fold) of defense-associated callose deposition in the host cell wall than the leaves inoculated with *Xcc* 8004 strain (Fig. 6, A and B). The same results were obtained in Arabidopsis (data not shown). Thus, reduced virulence associated with the lack of xanthan or the modification of its structure correlates with a severe diminution of the callose deposition.

To investigate if xanthan is the molecule responsible of the inhibition of callose accumulation after infection,

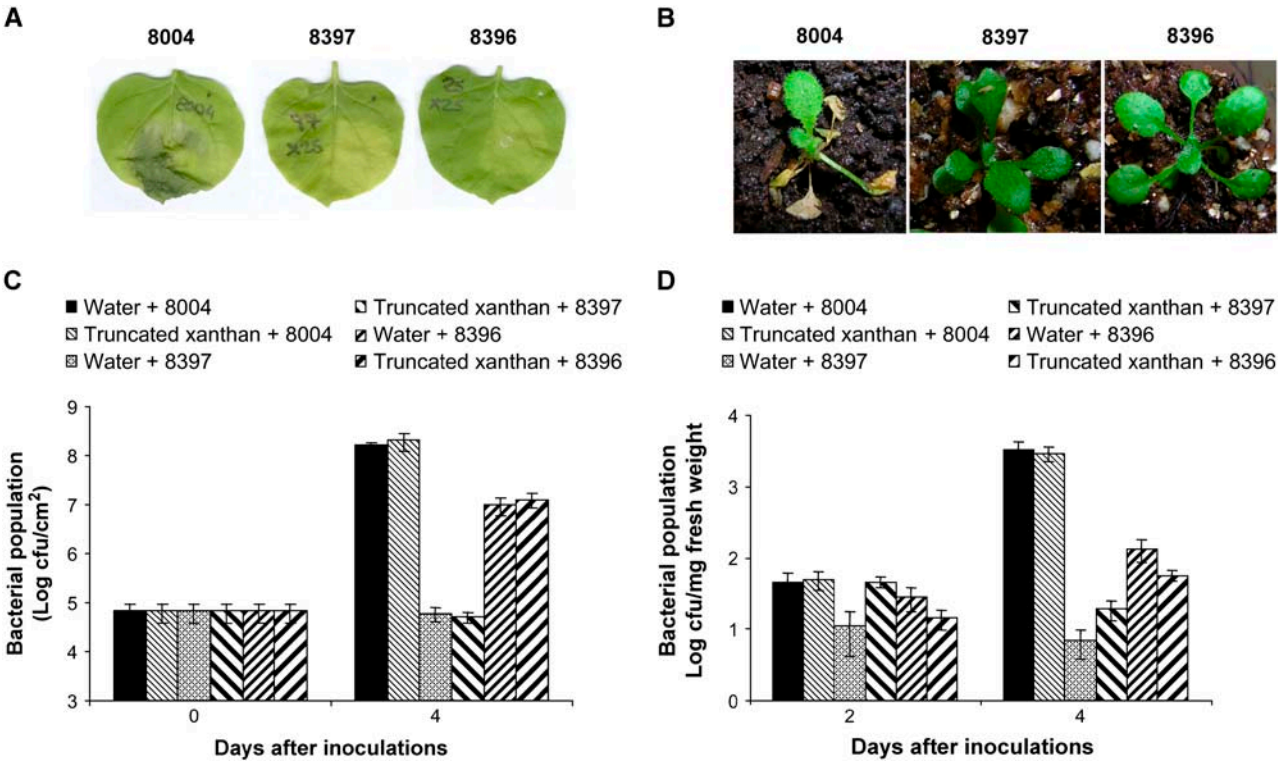

Figure 5. The structure of xanthan is essential for its effect in *N. benthamiana* and Arabidopsis. Symptoms in *N. benthamiana* leaves (A) and Arabidopsis plants (B) treated with the truncated xanthan (150 μg/mL) produced by 8396 mutant strain, and inoculated with 8004, 8397, or 8396 strains 24 h after treatment as before. Photos of disease symptoms were taken 8 dpi. Bacterial populations were accounted at 0 and 4 dpi of *N. benthamiana* (C) or Arabidopsis (D). The mean and SD of three separate measurements of bacterial numbers are given.

leaves of *N. benthamiana* were pretreated with xanthan or water (control) 24 h before inoculation with the *Xcc* 8397 or 8396 strains. Control leaves showed an accumulation of callose deposition while the leaves pretreated with xanthan failed to accumulate callose in response to both mutant strains (Fig. 6, A and B). Remarkably the truncated xanthan was not able to suppress the callose deposition (Fig. 6, A and B). The same results were obtained in Arabidopsis (data not shown). This suggested that xanthan induces susceptibility of *N. benthamiana* to *Xcc* by suppressing the accumulation of callose deposition. This suppression effect is dependent on the structure of the xanthan.

Inhibition of Callose Deposition by 2-Deoxy-D-Glc Allows the Xanthan-Deficient Mutants to Grow in *N. benthamiana*

To examine whether the callose defense response observed with the nonvirulent *Xcc* strains was relevant to the plant resistance against the pathogen, *N. benthamiana* infection experiments were performed 24 h after the administration of 2-deoxy-D-Glc (2 DDG), a callose synthesis inhibitor (Jaffe and Leopold, 1984). Callose deposition was evaluated 48 h postinoculation as before. As expected, no callose deposition was observed after 2 DDG treated leaves (Fig. 7, B and C). Interestingly, when leaves were pretreated with 250 μM, but not 25 μM, of this inhibitor, both *Xcc* mutant strains 8397 and 8396 were unable to induce the callose deposition response and recovered the ability to cause infection, represented by the appearance of foliar symptoms as well as by the increase in bacterial populations of both strains (Fig. 7, A and D). The 2 DDG inhibitor solution did not produce any symptoms or foliar changes when infiltrated alone (data not shown). The experiment has also been done using Glc (25 and 250 μM) as a control, and the same result as for water pretreated leaves was obtained for both concentrations (data not shown). The controls with the *Xcc* 8004 strain (wild type) were done for each experiment described in Figure 7. As shown in Figure 6, the wild-type bacteria did not induce callose deposition and no effect was observed when pretreatment was done with 2 DGG (data not shown). The data presented here shows direct correlation between the absence of callose deposition and the presence of infection symptoms, suggesting a major role of this mechanism in the defense response to *Xcc*.

DISCUSSION

Exopolysaccharides have been implicated in plant-pathogen interaction as important virulence factors. Several results reported that exopolysaccharides production

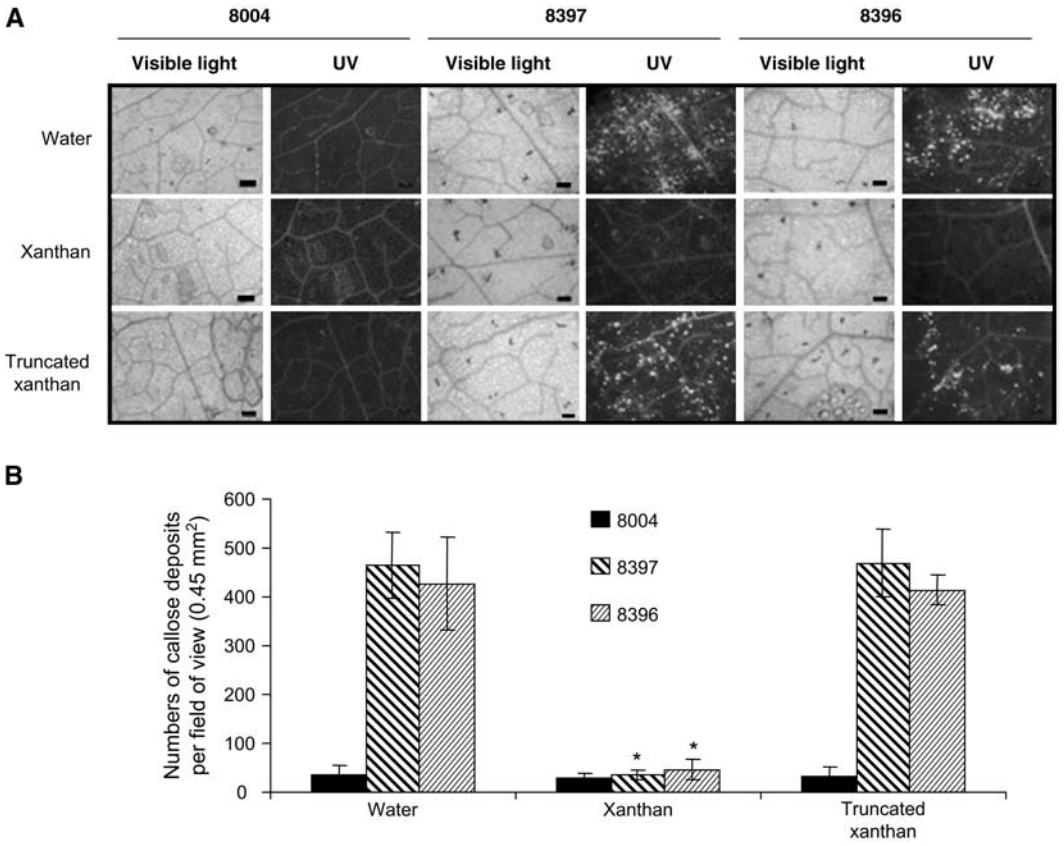

Figure 6. Callose deposition in *N. benthamiana* leaves is associated with resistance and is suppressed by the *Xcc* exopolysaccharide xanthan. *N. benthamiana* leaves were pretreated with water, xanthan, or truncated xanthan and inoculated with strains of *Xcc* as before. A, The leaves were then stained for callose deposits 24 h post inoculation (white dots) and observed by light (left, light sections) and fluorescence microscopy (right, dark sections). Scale bars = 200 μm. B, Average numbers of callose deposits per field of view (0.45 mm^2) are displayed. Error bars represent the SDS from three leaves of each plant and three independent experiments. Data sets marked with an asterisk are significantly different from the control (water-treated leaves followed by 8004 infection and water-pretreated leaves followed by 8397 or 8396 infections) as assessed by the Student's *t* test.

facilitates the dissemination of phytopathogenic bacteria such as *E. stewartii* and *P. syringae* pv *syringae* (Braun, 1990; Saile et al., 1997; Yu et al., 1999). Also, its presence has been implicated in the production of infection symptoms, as the wilting induced by vascular pathogens and the water soaking associated with foliar pathogens (Denny, 1995). Here we used a genetic approach to evaluate the role of the exopolysaccharide xanthan in the pathogenicity of *Xcc* by comparing the wild-type effect (8004 strain) with two xanthan-deficient mutants. One of these mutants, the 8397 strain, was obtained by transposon insertion and eliminates the production of xanthan almost completely (Vojnov et al., 1998). The other mutant, the 8396 strain, produces a neutral polysaccharide through the polymerization of a truncated repeating unit, resulting in a polytrisaccharide instead of a polypentasaccharide as xanthan is (Vojnov et al., 2002). We have shown that both xanthan-defective mutants are unable to cause disease in *N. benthamiana* and Arabidopsis leaves, and that pretreatment of the plant with xanthan restores the pathogenicity in both strains. Our data is consistent with callose accumulation inhibiting the growth of the pathogen in the plant, and with xanthan being involved in the suppression of this plant defense mechanism (Fig. 6). Thus we have shown that xanthan induces host susceptibility to 8397 and 8396 strains and we have identified one of the defense mechanisms (callose synthesis) that is altered by this exopolysaccharide. On the other hand, the inhibition of callose synthetase by 2 DDG was enough to allow bacterial populations and symptom developments of both 8397 and 8396 strains in *N. benthamiana*. These results suggest that plant cell wall-associated defense seems to be the main initial barrier against Xanthomonas infection in *N. benthamiana*.

The suppression of plant defense mechanisms plays a crucial role in causing plant diseases (Bouarab et al., 2002; Hauck et al., 2003; Nomura et al., 2005). Several virulence factors, which function as avirulence determinants that activate the plant defense responses triggered by recognition of their specific R proteins, act as suppressors of plant innate immunity in the absence of their specific R proteins (for review, see Abramovitch

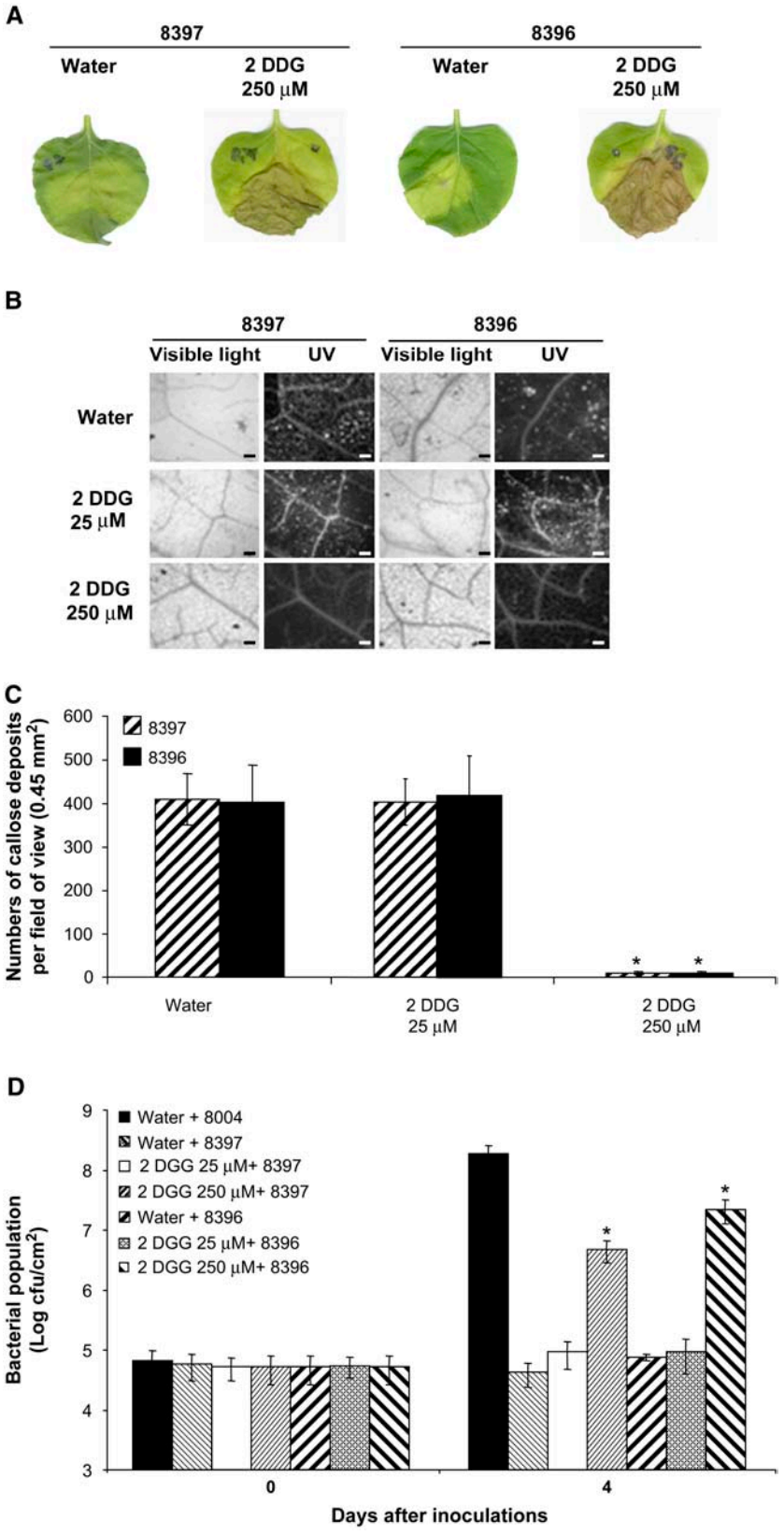

Figure 7. Inhibition of callose synthetase by 2 DDG restores mutant strain symptoms and bacterial populations. A, Disease symptoms on *N. benthamiana* leaves preinfiltrated with water, 25 or 250 μM of 2 DDG, and subsequently infected with *Xcc* mutant strains 8397 or 8396 (10^7 cfu/mL). Photos of disease symptoms were taken 8 dpi. B, Microscopy pictures of callose deposition from leaves treated with 2 DDG followed by infection with 8397 and 8396 strains as in A. C, Average numbers of callose deposits per field of view (0.45 mm^2) are displayed and data with asterisk are significantly different from the control (water-pretreated leaves followed by 8397 or 8396 infections) as assessed by the Student's *t* test. D, For every treatment bacterial populations were calculated at days 0 and 4, after inoculation as before. Error bars represent the SDs and data sets marked with an asterisk are significantly different from the control (water-pretreated leaves followed by 8397 or 8396 infections) as assessed by the Student's *t* test.

and Martin, 2004, 2005; Alfano and Collmer, 2004; Nomura et al., 2005). It has recently been shown that the *P. syringae* type III secretion system down-regulates the expression of a set of Arabidopsis genes encoding putatively secreted cell wall and defense proteins in a salicylic acid-independent manner (Hauck et al., 2003). Transgenic expression of the avirulence gene AvrPto represses a similar set of host genes in susceptible Arabidopsis plants, compromises defense-related callose deposition in the host cell wall, and permits substantial multiplication of a bacterial *hrp* (hypersensitive response and pathogenicity) mutant (Hauck et al., 2003). In addition, DebRoy and collaborators (2004) identified a key group of conserved type III effectors in plant-pathogenic bacteria that target salicylic acid-dependent basal immunity, including callose deposition, and promote disease necrosis in plants. In this study, we have shown that xanthan production induces plant disease by suppressing callose deposition. We have also demonstrated that the truncated xanthan fails to suppress this defense mechanism and therefore the resistance of *N. benthamiana* to 8397 and 8396 strains. This result suggests that the presence of the negatively charged GlcUA and ketal-pyruvate residues in the xanthan might be essential for its biological function during bacterial-plant interaction.

The polysaccharide pair mannuronate-guluronate was previously shown to be the preferred binding site for bivalent calcium ions in the bacterial alginate (Lattner et al., 2003). Moreover, xanthan, through its negatively charged GlcUA and ketal-pyruvate residues, is adsorbed significantly by magnesium carbonate, aluminum hydroxide, zinc oxide, and calcium carbonate, demonstrating its capacity to interact with suspended solids (Tempio and Zatz, 1981). Callose deposition has been considered to reflect local changes of the plant cell membrane, which leads to an increased Ca^{2+} concentration, activating membrane-bound callose synthase (Kauss, 1991; Amor et al., 1995; Delmer and Amor, 1995). In addition, local increase in Ca^{2+} ions could directly activate the callose synthase enzyme and initiate callose formation (Köhle et al., 1985). This suggests that one mechanism by which xanthan could act to suppress cell wall-based plant defense would be the binding of extracellular calcium ions, through its negative charge, which consequently interferes with signal transduction linked to callose synthetase activation.

In conclusion, we show in this article that callose is required for resistance to *Xcc* and xanthan induces susceptibility to *Xcc* in *N. benthamiana* and Arabidopsis by suppressing the callose deposition. This suppression effect depends on the chemical structure of the exopolysaccharide. Our data present an important conceptual stride forward in understanding the role of exopolysaccharides produced by pathogens in plant disease establishment. An exciting future challenge will be the biochemical and genetic elucidation of this suppression effect, which may have implications for our understanding of *Xcc* pathogenesis and to develop innovative disease control methods.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Xcc strains 8004 (wild type), 8397 (*gum*::Tn*5lac*; *EPS* minus), and 8396 (*gum*::Tn*5lac*; truncated *EPS*) were described previously (Cadmus et al., 1976; Daniels et al., 1984; Tang et al., 1991; Slater et al., 2000) and characterized (Vojnov et al., 1998, 2002). *Xcc* strains were cultured in a 28°C shaker in PYM medium as reported by Cadmus and collaborators (1976). Bacterial populations were measured in a U-3200 spectrophotometer (Hitachi Instruments) or an Ultrospec 1000 Pharmacia Biotech UV/visible spectrophotometer, at 600 nm. The antibiotics kanamycin (50 μg/mL) and rifampicin (25 μg/mL) were added when appropriate.

Xanthan Preparations

Xanthan or truncated xanthan were purified from culture of *Xcc* as previously described (Vojnov et al., 1998).

Plant Material

Nicotiana benthamiana and Arabidopsis (*Arabidopsis thaliana*; Columbia ecotype) seeds were germinated on 0.8% agar. Two-leaved seedlings were then grown on soil in growth chamber at 22°C with 70% relative humidity and a 12-h light/12-h dark cycle.

N. benthamiana Inoculations

All plant inoculations involved a minimum of three leaves from each of three plants, and each experiment was carried out at least three times. Leaves of 30-d-old plants were inoculated, by infiltration, with *Xcc* strains (10^7 cfu/mL in water) or water only as previously described (Newman et al., 1994). For the infiltration experiments leaves were preinfiltrated with either water, purified xanthan, or truncated xanthan (150 μg/mL), and then inoculated with *Xcc* 8004, 8397, or 8396 bacterial suspensions 24 h later. Inoculation was performed according to published methods (Newman et al., 1994). Bacteria were hand infiltrated into plant leaves at the abaxial surface by using a 1-mL syringe without needle. After inoculation, observations were performed every 6 h, assessing the foliar symptoms and taking samples for different assays. When necessary, treatment with 2-deoxy-D-Glc (Sigma-Aldrich) was carried out 24 h before inoculations, in concentrations of 25 or 250 μM as used previously (Ton and Mauch-Mani, 2004). To assess bacterial development in planta, six 0.6 cm^2 discs from each leaf were taken at 0, 1, 2, 3, and 4 d postinoculation (dpi) and bacterial populations monitored as described (Newman et al., 1994).

Arabidopsis Inoculations

Plant infections were performed as previously described (Tornero and Dangl, 2001) but adaptations were made for the infection with *Xcc*. Bacterial suspensions (OD_{600} = 0.05) were prepared from 24 h/28°C PYM-liquid *Xcc* cultures (Cadmus et al., 1976) with 10 mM $MgCl_2$, and Silwet L-77 was added (200 μL/L). Plant pots (nine seedlings each) were submerged upside down in the bacterial solution for 30 s and then covered with a transparent lid. Forty-eight hours after inoculation the lid was removed and samples were taken (days 2, 3, and 4). Bacterial populations were measured as previously described (Tornero and Dangl, 2001).

Callose Deposition Assay

Callose staining was performed 24 h after bacterial inoculation as described previously (Hauck et al., 2003). Leaves were examined with a Zeiss Axiophot D-7082 photomicroscope with an A3 fluorescence cube. The callose depositions were quantified with IMAGE PRO PLUS software (Media Cybernetics).

ACKNOWLEDGMENTS

We thank our lab colleagues for fruitful discussions and for critical reading of the manuscript. Atilio Castagnaro, Maria Rosa Marano, Marcelo Dankert, and Adrian Vojnov are members of the Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas.

Received November 23, 2005; revised February 11, 2006; accepted February 16, 2006; published March 10, 2006.

LITERATURE CITED

Abramovitch RB, Martin GB (2004) Strategies used by bacterial pathogens to suppress plant defenses. Curr Opin Plant Biol **7:** 356–364

Abramovitch RB, Martin GB (2005) AvrPtoB: a bacterial type III effector that both elicits and suppresses programmed cell death associated with plant immunity. FEMS Microbiol Lett **245:** 1–8

Alfano JR, Collmer A (2004) Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Annu Rev Phytopathol **42:** 385–414

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proc Natl Acad Sci USA **92:** 9353–9357

Barrère GC, Barber CE, Daniels MJ (1986) Molecular cloning of genes involved in the production of the extracellular polysaccharide xanthan by *Xanthomonas* campestris pv *campestris.* Int J Biol Macromol **8:** 372–374

Bender CL, Alarcon-Chaidez F, Gross DC (1999) *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol Mol Biol Rev **63:** 266–292

Bouarab K, Melton R, Peart J, Baulcombe D, Osbourn A (2002) A saponin-detoxifying enzyme mediates suppression of plant defenses. Nature **418:** 889–892

Braun EJ (1990) Colonization of resistant and susceptible maize plants by *Erwinia stewartii* strains differing in exopolysaccharide production. Physiol Mol Plant Pathol **36:** 363–379

Cadmus M, Rojovin SP, Burton KA, Pittsley JE, Knutson CA, Jeanes A (1976) Colonial variation in *Xanthomonas campestris* NRRL B-1459 and characterization of the polysaccharide from a variant strain. Can J Microbiol **22:** 942–948

Daniels MJ, Barber CE, Turner PC, Sawczyc MK, Byrde RJW, Fielding AH (1984) Cloning of genes involved in pathogenicity of *Xanthomonas campestris pv. campestris* using the broad host range cosmid pLAFR1. EMBO J **3:** 3323–3328

DebRoy S, Thilmony R, Kwack YB, Nomura K, He SY (2004) A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. Proc Natl Acad Sci USA **101:** 9927–9932

Delmer DP, Amor Y (1995) Cellulose biosynthesis. Plant Cell **7:** 987–1000

Denny TP (1995) Involvement of bacterial polysaccharide in plant pathogenesis. Annu Rev Phytopathol **33:** 173–197

Dolph PJ, Majerczak DR, Coplin DL (1988) Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii.* J Bacteriol **170:** 865–871

Dow JM, Daniels MJ (2000) *Xylella* genomics and bacterial pathogenicity to plants. Yeast **17:** 263–271

Geier G, Geider K (1993) Characterization and influence on virulence of the levansucrase gene from the fireblight pathogen *Erwinia amylovora.* Physiol Mol Plant Pathol **42:** 387–404

Hauck P, Thilmony R, He SY (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. Proc Natl Acad Sci USA **100:** 8577–8582

Heyraud A, Sayah B, Vojnov A, Colin-Morel P, Gey C, Geremia RA, Dankert M (1998) Structure of an extracellular mannosylated cellulose produced by a mutant strain of Xanthomonas campestris. Cell Mol Biol (Noisy-le-grand) **44:** 447–454

Ielpi L, Couso RO, Dankert MA (1993) Sequential assembly and polymerization of the polyprenol-linked pentasaccharide repeating unit of the xanthan polysaccharide in *Xanthomonas campestris.* J Bacteriol **175:** 2490–2500

Jaffe MJ, Leopold AC (1984) Callose deposition during gravitropism of *Zea mays* and *Pisum sativum* and its inhibition by 2-deoxy-D-glucose. Planta **161:** 20–26

Jansson PE, Kenne L, Lindberg B (1975) Structure of extracellular polysaccharide from *Xanthomonas campestris.* Carbohydr Res **45:** 274–282

Kang Y, Saile E, Schell MA, Denny TP (1999) Quantitative immunofluorescence of regulated eps gene expression in single cells of *Ralstonia solanacearum.* Appl Environ Microbiol **65:** 2356–2362

Kao CC, Barlow E, Sequeira L (1992) Extracellular polysaccharide is required for wild-type virulence of *Pseudomonas solanacearum.* J Bacteriol **174:** 1068–1071

Kauss H (1991). The role of the plasma membrane in host-pathogen interactions. *In* C Larsson, IM Moller, eds, The Plant Plasma Membrane. Springer-Verlag, Berlin, pp 320–350

Keshavarzi M, Soylu S, Brown I, Bonas U, Nicole M, Rossiter J, Mansfield J (2004) Basal defenses induced in pepper by lipopolysaccharides are suppressed by *Xanthomonas campestris pv. Vesicatoria.* Mol Plant Microbe Interact **17:** 805–815

Köhle H, Jeblick W, Poten F, Blaschek W, Kauss H (1985) Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+}-dependent process. Plant Physiol **77:** 544–551

Lattner D, Flemmingb HC, Mayera C (2003) ^{13}C-NMR study of the interaction of bacterial alginate with bivalent cations. Int J Biol Macromol **33:** 81–88

Melton LD, Mindt L, Rees DA, Sanderson GR (1976) Covalent structure of the extracellular polysaccharide from *Xanthomonas campestris*: evidence from partial hydrolysis studies. Carbohydr Res **46:** 245–257

Mittal S, Davis KR (1995) Role of the phytotoxin coronatine in the infection of Arabidopsis thaliana by *Pseudomonas syringae* pv *tomato.* Mol Plant Microbe Interact **8:** 165–171

Newman MA, Conrads-Strauch J, Scofield G, Daniels MJ, Dow JM (1994) Defense-related gene induction in *Brassica campestris* in response to defined mutants of *Xanthomonas campestris* with altered pathogenicity. Mol Plant Microbe Interact **7:** 553–563

Nomura K, Melotto M, He SY (2005) Suppression of host defense in compatible plant-*Pseudomonas syringae* interactions. Curr Opin Plant Biol **8:** 361–368

Saile E, McGarvey JA, Schell MA, Denny TP (1997) Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum.* Phytopathology **87:** 1264–1271

Slater H, Alvarez-Morales A, Barber CE, Daniels MJ, Dow JM (2000) A two-component system involving an HD-GYP domain protein links cell-cell signalling to pathogenicity gene expression in *Xanthomonas campestris.* Mol Microbiol **38:** 986–1003

Stone BA, Clarke AE (1992) Chemistry and Biology of (1→3)-β-D-Glucans. La Trobe University Press, Victoria, Australia

Tang JL, Liu YN, Barber CE, Dow JM, Wootton JC, Daniels MJ (1991) Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris pathovar campestris.* Mol Gen Genet **226:** 409–417

Tempio JS, Zatz JL (1981) Interaction of xanthan gum with suspended solids. J Pharm Sci **70:** 554–558

Ton J, Mauch-Mani B (2004) β-Amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J **38:** 119–130

Tornero P, Dangl JL (2001) A high-throughput method for quantifying growth of phytopathogenic bacteria in *Arabidopsis thaliana.* Plant J **28:** 475–481

Vanderslice RW, Doherty DH, Capage MA, Betlach MR, Hassler RA, Henderson NM, Ryan-Graniero J, Tecklenburg M (1990) Biomedical and Biotechnological Advances in Industrial Polysaccharides. Gordon and Breach Science Publishers, New York, pp 145–156

Vojnov AA, Bassi DE, Daniels MJ, Dankert MA (2002) Biosynthesis of a substituted cellulose from a mutant strain of *Xanthomonas campestris.* Carbohydr Res **337:** 315–326

Vojnov AA, Slater H, Daniels MJ, Dow JM (2001) Expression of the *gum* operon directing xanthan biosynthesis in *Xanthomonas campestris* and its regulation in planta. Mol Plant Microbe Interact **14:** 768–774

Vojnov AA, Zorreguieta A, Dow JM, Daniels MJ, Dankert MA (1998) Evidence for a role for the *gumB* and *gumC* gene products in the formation of xanthan from its pentasaccharide repeating unit by *Xanthomonas campestris.* Microbiology **144:** 1487–1493

Yu J, Peñaloza-Vázquez A, Chakrabarty AM, Bender CL (1999) Involvement of the exopolysaccharide alginate in the virulence and epiphytic fitness of *Pseudomonas syringae pv. syringae.* Mol Microbiol **33:** 712–720