Biofilm Formation, Epiphytic Fitness, and Canker Development in *Xanthomonas axonopodis* pv. *citri*

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The phytopathogenic bacterium *Xanthomonas axonopodis* pv. *citri* is responsible for the canker disease affecting citrus plants throughout the world. Here, we have evaluated the role of bacterial attachment and biofilm formation in leaf colonization during canker development on lemon leaves. Crystal violet staining and confocal laser scanning microscopy analysis of *X. axonopodis* pv. *citri* strains expressing the green fluorescent protein were used to evaluate attachment and biofilm formation on abiotic and biotic (leaf) surfaces. Wild-type *X. axonopodis* pv. *citri* attached to and formed a complex, structured biofilm on glass in minimal medium containing glucose. Similar attachment and structured biofilm formation also were seen on lemon leaves. An *X. axonopodis* pv. *citri* *gumB* mutant strain, defective in production of the extracellular polysaccharide xanthan, did not form a structured biofilm on either abiotic or biotic surfaces. In addition, the *X. axonopodis* pv. *citri* *gumB* showed reduced growth and survival on leaf surfaces and reduced disease symptoms. These findings suggest an important role for formation of biofilms in the epiphytic survival of *X. axonopodis* pv. *citri* prior to development of canker disease.

Additional keywords: *Citrus limon*, EPS, GFP-labeled *X. axonopodis* pv. *citri*.

In natural ecosystems, microorganisms grow preferentially attached to surfaces, forming matrix-enclosed biofilms (Kolter and Greenberg 2006; Parsek and Fuqua 2004; Ramey et al. 2004; Stoodley et al. 2002). Microbial biofilms generally are defined as conglomerates of cells immobilized in an organic polymer matrix of microbial origin, with extracellular polysaccharides (EPSs) as the major constituent, along with proteins, lipids, and nucleic acids (Branda et al. 2005; Southey-Pillig et al. 2005; Steinberger and Holden 2005; Sutherland 2001).

Bacteria in biofilm have a different physiology from that of free-living (planktonic) cells. They generally live under nutrient limitation, which may promote the exchange of genetic

material and generate more diversity than planktonic populations (Boles et al. 2004). Biofilms also are known to protect bacteria from environmental stresses, host defense mechanisms, and antimicrobial compounds (Branda et al. 2005; Camilli and Bassler 2006; Leid et al. 2005; Parsek and Fuqua 2004).

An association between biofilm formation and virulence has been reported for a variety of chronic bacterial infections in humans (Costerton et al. 1999; Parsek and Singh 2003). However, few studies have examined the role of biofilm formation in the development of bacterial diseases in plants (Crossman and Dow 2004; Kang et al. 1999; Koutsoudis et al. 2006; Osiro et al. 2004; Ramey et al. 2004). The ability to produce EPS is critical for biofilm formation in a number of pathogenic bacteria. Attachment and survival of bacteria on host tissue requires biofilm formation; as such, it is not surprising that EPS production is correlated with virulence in numerous bacteria (Dow et al. 2003; Fujishige et al. 2006; Laue et al. 2006).

Xanthan, the EPS produced by xanthomonads, is a polymer of repeating pentasaccharide units with the mannose-(β -1,4)-glucuronic acid-(β -1,2)-mannose-(α -1,3)-cellobiose structure (Jansson et al. 1975). Production of xanthan is regulated in part by a cell-to-cell signaling system encoded by a cluster of regulation of pathogenicity factors (*rpf*) genes and the diffusible signal factor (DSF) (Crossman and Dow 2004; Dow et al. 2003). In *Xanthomonas campestris* pv. *campestris*, the biosynthesis of xanthan gum is directed by a cluster of 12 genes (*gumB* to *-M*); the function of most of the protein products has now been assigned (Katzen et al. 1998; Vojnov et al. 1998, 2002). Functional *gumB* and *gumC* genes are necessary for the production of mature extracellular xanthan from pentasaccharide intermediates (Vojnov et al. 1998).

X. axonopodis pv. *citri* is the bacterial pathogen responsible for citrus canker—the citrus disease with greatest worldwide impact. *X. axonopodis* pv. *citri* invasion of host mesophyll is accomplished through natural openings, such as stomata, and through lesions (Brunings and Gabriel 2003). Canker symptoms on leaves and fruit are characterized by surface-penetrating necrotic lesions surrounded by oily, water-soaked margins and yellow chlorotic rings (Brunings and Gabriel 2003). The canker itself, along with surviving populations of bacteria, constitutes the principal sources of disease spread (Cubero and Graham 2004).

Most phytopathogenic bacteria exist as epiphytes on healthy plants prior to colonization, and the probability of disease occur-

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rence is related directly to the epiphytic population size of the pathogen (Hirano and Upper 1983). In this study, we have investigated biofilm formation by *X. axonopodis* pv. *citri* and the possible role of biofilm in epiphytic fitness and canker development. We show that *X. axonopodis* pv. *citri* forms structured biofilms both in vitro on glass slides and in vivo on citrus leaves. In contrast, an *X. axonopodis* pv. *citri* *gum* mutant strain defective in xanthan synthesis was unable to form a structured biofilm either in vitro or in vivo and was compromised for epiphytic survival and disease development in plants. These findings point to the importance of biofilm formation in the epiphytic phase of canker disease.

RESULTS

Isolation of xanthan-deficient mutant in *X. axonopodis* pv. *citri*.

The work in this article investigates biofilm formation in *X. axonopodis* pv. *citri* and the role of xanthan in the various steps leading to a mature biofilm. In order to investigate the role of xanthan in biofilm formation, it was necessary to create a mutant strain of *X. axonopodis* pv. *citri* specifically deficient in the production of the polysaccharide. Genome comparison of *X. axonopodis* pv. *citri* (strain 306) (da Silva et al. 2002) and *X. campestris* pv. *campestris* (strain 8004) (Qian et al. 2005) revealed that the *gum* gene cluster, involved in *X. campestris* pv. *campestris* xanthan biosynthesis, is largely conserved in both bacteria in terms of size and gene order, indicating that *X. axonopodis* pv. *citri* also may be able to produce xanthan. *X. axonopodis* pv. *citri* GumB protein (GenBank accession number NP_642899) shares 92% amino acid identity with *X. campestris* pv. *campestris* GumB protein (GenBank accession number. YP_242744). Previously, it was shown that mutation of the *gumB* gene in *X. campestris* pv. *campestris* has a polar effect on *gumB* and *gumC* expression and that both gene products are required for the formation of xanthan from the pentasaccharide repeating unit (Vojnov et al. 1998).

We generated the *X. axonopodis* pv. *citri* *gum* mutant strain through allelic exchange mutagenesis, in which the functional *gumB* gene was replaced through homologous recombination with a copy containing a kanamycin-resistant (Km^r) (Ω) cassette. Disruption of the *gumB* gene in the mutant strain was confirmed by polymerase chain reaction (PCR) and Southern blot (data not shown). Extracellular polysaccharides were isolated from wild-type (WT) *X. axonopodis* pv. *citri* and *gumB* strains grown in peptone yeast and malt extract (PYM) medium supplemented with 2% glucose and quantified as described by Vojnov and associates (1998). Like *X. campestris* pv. *campestris*, *X. axonopodis* pv. *citri* synthesizes the EPS, xanthan, and releases it into the growth medium as a soluble polysaccharide. Large differences in EPS production were observed between WT *X. axonopodis* pv. *citri* strains (8.2 g liter^{-1}) and the *gumB* mutants ($0.15 \text{ g liter}^{-1}$) after ethanol precipitation from the culture supernatant. Complementation of the *gum* mutant with the recombinant plasmid pIZD15-261, carrying the entire *gum* cluster of *X. campestris* pv. *campestris* (Vojnov et al. 1998, 2002), restores xanthan production to essentially the same level as in the *X. axonopodis* pv. *citri* WT (9.1 g liter^{-1}). Cultures of WT *X. axonopodis* pv. *citri* and the *gumB* mutant exhibit the same growth kinetics in PYM medium supplemented with 1% glucose (data not shown); thus, the mutant showed no loss of viability compared with WT *X. axonopodis* pv. *citri*.

Bacterial attachment to abiotic and biotic surfaces and the role of xanthan.

In a preliminary assay, bacterial adhesion to polystyrene microtiter plates was analyzed by crystal violet staining. For

these experiments, bacteria were grown overnight in PYM medium and then diluted into Y minimal medium. This assay detected no significant differences in the initial adhesion (1 to 3 h) between the WT and *gumB* mutant (Fig. 1A). However, at 12 h or later, the culture of the *gumB* strain showed significantly less crystal violet staining than the WT (Fig. 1A). This suggests that EPS does play a role in biofilm development, particularly in later stages.

Bacterial adhesion to lemon leaves also was studied. Leaf disks were cut from healthy leaves and mounted onto petri dishes, adaxial side down. The disks were inoculated with *X. axonopodis* pv. *citri* WT and the *gumB* mutant strains and incubated at 28°C. After 1, 3, 15, and 24 h of incubation, the

Fig. 1. Adhesion of *Xanthomonas axonopodis* pv. *citri* strains to abiotic and biotic surfaces. **A**, Bacterial adhesion and biofilm formation on inert plastic surface were assessed by the use of crystal violet at 1, 3, 12, 24, and 72 h of incubation. Values are expressed as means $\pm$ standard deviation, $n = 36$. Data set marked with an asterisk is significantly different as assessed by the Student's *t* test, $P < 0.05$. **B**, Bacterial adhesion and biofilm formation on abaxial surfaces of lemon leaves were assessed by the use of crystal violet at 1, 3, 15, and 24 h of incubation. The stains attached cells to the leaf surface were analyzed macroscopically by the intensity of the blue dye. YMM = Y minimal medium.

attached bacteria were revealed by crystal violet staining. The number of attached bacteria differed significantly after 15 h of incubation (Fig. 1B). No differential attachment was observed when growth of *X. axonopodis* pv. *citri* WT was compared with that of the *MgumB* mutant complemented by the entire *gum* cluster cloned into pIZD15-261 (data not shown).

The *X. axonopodis* pv. *citri* WT strain forms structured biofilms in vitro that require xanthan production.

In further experiments, we used inverted confocal laser scanning microscopy (CLSM) to analyze the biofilm structure developed by *X. axonopodis* pv. *citri* in vitro over a 4-day time course experiment in chambered cover slides. A green fluorescent protein (GFP)-labeled strain of *X. axonopodis* pv. *citri* was used to observe different stages of biofilm development at the bottom of chambered cover slides. A flat lawn of bacteria without any apparent microcolony or organized structure was observed in static cultures over the course of the experiment in PYM medium (data not shown). However, clear stages of bacterial biofilm development were observed for the *X. axonopodis* pv. *citri* WT strain grown on Y minimal medium containing 1% glucose. During initial attachment, bacteria often contacted the glass surface via one cell pole (Fig. 2, day 1). Attachment was followed by bacterial growth, likely accomplished through both cell division and cell migration. By days 3 and 4 of culture, isolated bacteria and single microcolonies were well established, and more complex structures had begun to form. These structures consisted of multiple layers of cells in contact with each other, predominantly through lateral interactions, though pole-to-pole interactions also were observed (Fig. 2). The architecture of the biofilm consisted of interconnected complex cell clustering similar to that of a honeycomb (Fig. 2, day 3).

In contrast, the *gumB* mutant labeled with GFP and grown on Y minimal medium containing 1% glucose failed to form microcolonies and complex structures after 4 days (Fig. 2). This distinct growth pattern was reproduced in four independent experiments. The whole *gum* cluster cloned in pIZD15-261 restored normal xanthan production in the *gum* mutant, as well as the mutant's ability to develop microcolonies, cell aggregates,

and three-dimensional biofilm on the base of the chamber in static cultures (data not shown). These observations strongly suggest that biofilm formation in *X. axonopodis* pv. *citri* requires synthesis of xanthan.

Biofilm and epiphytic fitness during *X. axonopodis* pv. *citri*–lemon plant interaction.

Formation of canker disease pustules may depend on how well bacteria attach and form biofilms on leaf surfaces. To examine the role of biofilm formation in canker disease, we examined the ability of *Xanthomonas* spp. to develop a biofilm on lemon leaves. Biofilm formation was monitored by CLSM over a 25-day period. GFP fluorescence was detected 24 h after spray inoculation on epidermal cells, and structures similar to the biofilms observed in vitro could be distinguished as early as 6 days postinoculation (dpi). *X. axonopodis* pv. *citri* cells were attached perpendicularly and horizontally and also were found distributed randomly on the lemon leaves (Fig. 3A). As we observed in the in vitro experiments, the xanthan-deficient mutant grew as single cells and did not form microcolonies on lemon leaves during the length of the experiment, indicating that xanthan affects aggregate formation.

To evaluate the influence of biofilm formation on bacterial viability on plant leaves, we monitored the cell number on the leaf surfaces of *Citrus limon* for 10 dpi. By 24 h after inoculation, the epiphytic population of the WT had increased significantly and was nearly 100 times higher than for the *gumB* mutant (Fig. 3B). From that point forward, the population size of the WT remained approximately constant, whereas the numbers of *gumB* mutant bacteria declined slowly, so that, at 7 dpi, the difference in population size was three orders of magnitude (Fig. 3B).

Canker development on lemon leaves is associated with biofilm formation.

To assess the importance of the ability to form biofilms on canker development, leaves were inoculated with bacterial suspensions of the WT and *gumB* mutant at a concentration of 10^7 CFU ml⁻¹ by three methods: spraying, nicking and spraying,

Fig. 2. Biofilm in wild-type and *gumB* mutant *Xanthomonas axonopodis* pv. *citri* strains. Green fluorescent protein (GFP)-labeled cells grown on chambered cover slides were visualized at different stages of the biofilm formation under confocal laser scanning microscopy for 4 days after inoculation. The panels (left to right) show cell attachment to the glass surface at 1.5 μ m from the base glass. The top inset for day 3 shows a detail of a typical wild-type *X. axonopodis* pv. *citri* (*Xac*) microcolony honeycomb structure and on the bottom, the biofilm on *x-z* planes. Magnifications, $\times 1,000$; zooms (inserts), $\times 4,000$. Scale bars, 20 μ m. A minimum of four independent experiments was carried out for each strain, with essentially the same outcome.

and pressure infiltration. Canker symptoms on lemon leaves 30 dpi are shown in Figure 4A. The *gumB* mutant was found to be severely compromised in its ability to cause canker disease in citrus using all three inoculation methods; although, in leaves subjected to pressure infiltration, the mutant showed slight water soaking around the infiltration site. Plants inoculated with $MgCl_2$ showed no symptoms and the *MgumB* with the entire *gum* cluster cloned in pIZD15-261 restored the WT phenotype (Fig. 4A).

Comparison of the bacterial numbers in planta following inoculation via spraying revealed no significant differences between the WT and *gumB* mutant growth until 14 dpi, after which the population size of the *gumB* mutant growth showed no further increase. After 21 dpi, the population of the *gumB* mutant began to decline and no mutant bacteria could be recovered after 5 weeks. In contrast, growth of the WT continued, so that

the population increased by more than six orders of magnitude over the 5-week monitoring period (Fig. 4B). Similar differences in behavior of WT and mutant bacteria were seen after pressure infiltration, with the exception that infiltration introduced a larger number of bacteria into the leaves than spraying (data not shown). Spray inoculation resembles natural inoculation (e.g., rain splash) more closely than either nicking or infiltrating.

We used CLSM with GFP-labeled *X. axonopodis* pv. *citri* cells to investigate whether bacteria were present within a biofilm structure during these canker developments on lemon leaves. The bacteria were monitored by CLSM over a 25-day period. In this analysis (Fig. 5), we were able to visualize aggregated bacteria within the developed canker. These aggregates of bacteria showed a three-dimensional structure with extensive water spaces redolent of the in vitro structure observed by CLSM analysis. Higher magnification of *X. axonopodis* pv. *citri* biofilms showed bacterial cells apparently embedded within an extracellular polymeric matrix, and aggregates appeared to be connected by this matrix. These aggregates were not seen with the *gumB* mutant bacteria, suggesting that EPS contributes to the structured architecture within cankers, which may provide an optimal environment for canker development.

Fig. 3. Biofilm development and epiphytic growth on superficial panel leaf. **A**, Confocal laser scanning microscopy analysis of wild-type and *gumB* mutant. Bacterial suspensions were inoculated onto leaves surfaces using the spray method. XY, ZX, and ZY are the XY, ZX, and ZY axis projected images, respectively. Scale bars, 20 μ m. **B**, Growth in vivo of epiphytic population of *Xanthomonas axonopodis* pv. *citri* (*Xac*) and the *MgumB* mutant on lemon leaves. Bacterial populations were determined by homogenizing the leaves in $MgCl_2$ followed by dilution plating. Values are expressed as means $\pm$ standard deviation.

Fig. 4. Symptom development induced by *Xanthomonas axonopodis* pv. *citri* strains in *Citrus limon*. **A**, Macroscopic symptoms on lower surfaces of lemon leaves 30 days postinoculation (d.p.i.) with *gumB* mutant, the wild-type (WT) strains of *Xanthomonas axonopodis* pv. *citri*, and *MgumB*, with the entire *gum* cluster gene cloned in pIZD15-261 (*MgumB* + pIZD15-261). Bacterial suspensions were prepared in 10 mM $MgCl_2$ solution and inoculated onto leaves surfaces using three different methods: spray, nick and spray; and pressure infiltration. **B**, Growth in vivo of *X. axonopodis* pv. *citri* strains on *C. limon* leaves. Values are expressed as means $\pm$ standard deviation.

DISCUSSION

We have analyzed the structure of *X. axonopodis* pv. *citri*-produced biofilms on different solid surfaces to gain insight into the dynamics of *X. axonopodis* pv. *citri*-host interactions in the development of citrus canker disease. Three stages of biofilm development were recognized: bacterial attachment, formation of aggregates or microcolonies, and finally the formation of structured biofilms within colonized leaves.

We have shown that the *X. axonopodis* pv. *citri* xanthan is essential for the formation of microcolonies and the subsequent development of more complex structures, in which bacteria are tightly packed in hexagonal arrays separated by water-filled channels (Figs. 2, 3, and 5). Such complex structures are found both in vitro and in planta. Similar phenotypes have been reported for the soil bacteria *Rhizobium leguminosarum* (Russo et al. 2006). These structures are remarkably different from the growth mode proposed for other species, including *Pseudomonas aeruginosa*, *Rhodococcus ruber*, and *Proteus mirabilis*, in which clonal growth occurs in the initial phase (mushroom stalks), followed by aggregation in later stages (mushroom caps) (Jones et al. 2007; Klausen et al. 2003; Sivan et al. 2006). Presumably, the diversity of biofilm architectures reflects the adaptation to specific microenvironments. Complex biofilm structure was not observed when *X. axonopodis* pv. *citri* bacteria were grown in the rich PYM medium (data not shown), suggesting that nutrient status is a critical factor in biofilm development.

Many biofilm matrix polysaccharide components of gram-negative bacteria have been reported to play important roles in biofilm physiology and persistence, such as colanic acid in *Escherichia coli* K-12 (Danese et al. 2000); alginate, Pel, and PIs in *Pseudomonas aeruginosa* (Jackson et al. 2004; Wozniak et al. 2003); alginate and levan in *P. syringae* (Laue et al. 2006); cellulose in *Salmonella* spp. and *E. coli* (Solano et al. 2002; Ude et al., 2006); and acidic EPS in *Rhizobium leguminosarum* (Russo et al. 2006). Furthermore, *X. campestris* pv. *campestris* xanthan has been shown to be involved in biofilm formation in liquid medium (Crossman and Dow 2004; Dow et al. 2003). These EPSs are key elements that shape and provide structural support for bacterial biofilms.

The synthesis of EPS in many bacteria is under complex regulatory control. It has been demonstrated that synthesis of EPS is regulated by cell-to-cell signaling or quorum sensing in several phytopathogenic bacteria, including *Pantoea stewartii* (Koutsoudis et al. 2006), *Pseudomonas syringae* (Quinones et al. 2005), and *Ralstonia solanacearum* (Schell 2000), as well as *X. campestris* pv. *campestris* (Dow et al. 2000; Slater et al. 2000; Vojnov et al. 2001). Quorum sensing also can regulate expression of other factors or traits involved in virulence and epiphytic fitness (Chatterjee et al. 2007; Karamanoli and Lindow 2006; Quinones et al. 2004; Slater et al. 2000). In *X. campestris* pv. *campestris*, cell-to-cell signaling mediated by the diffusible signal factor controls biofilm dynamics, the synthesis of EPS, and the synthesis of extracellular enzymes (Crossman and Dow 2004; Dow et al. 2003; Slater et al. 2000). The synthesis of DSF depends upon RpfB and RpfF (Barber et al. 1997). DSF perception and signal transduction is believed to involve the sensor kinase RpfC and cognate response regulator RpfG and is linked to the degradation of the intracellular signaling molecule, cyclic di-GMP, through the HD-GYP domain of RpfG (Ryan et al. 2006). Recently, we provided evidence that *rpf/DSF* system also is involved in controlling virulence factor synthesis in *X. axonopodis* pv. *citri* (Siciliano et al. 2006), suggesting that cell-to-cell signaling plays a role in canker pathogenicity. The mechanism or mechanisms through which cyclic di-GMP influences the synthesis of xanthan is currently obscure. The *rpf/DSF* system appears to regulate the expression of the *gum* operon at the transcriptional level (Vojnov et al. 2001). Although this suggests that cyclic di-GMP may influence transcription, we cannot exclude the possibility that the nucleotide exerts post-translational effects; for example, on the activity of GumB or other proteins involved in xanthan synthesis.

The presence of plant-associated biofilms is correlated with *X. axonopodis* pv. *citri* pathogenicity, which was severely attenuated by the disruption of a single gene involved in the biosynthesis of EPS, *gumB* (Fig. 4). Xanthan-deficient mutants of various xanthomonads show reduced virulence compared with WT bacteria in numerous studies (Chou et al. 1997; Dharmapuri and Sonti 1999; Katzen et al. 1998; Kemp et al. 2004; Li et al.

Fig. 5. Real-time imaging of the canker development on lemon leaves. **A**, Green fluorescent protein (GFP) fluorescence from wild-type (WT) *Xanthomonas axonopodis* pv. *citri* strain within a canker pustule was detected by confocal laser scanning microscopy. Each picture shows the analyzed canker and the distribution of GFP-labeled bacteria within the pustule at **B**, $\times 100$, **C**, $\times 1,000$, and **D**, $\times 4,000$. Scale bars are **B**, 200 μm , **C**, 20 μm , and **D**, 5 μm . Areas within the dotted red lines of panels **A** and **C** are shown enlarged in panels **B** and **D**, respectively. **E**, WT GFP-labeled *X. axonopodis* pv. *citri* in a canker pustule showed at different distances from the base. Scale bars, 20 μm .

2001; Newman et al. 1994; Yun et al. 2006). In contrast, a recent report indicates that the *X. axonopodis* pv. *citri* *gumD* gene, although contributing to epiphytic survival, is not involved in pathogenesis (Dunger et al. 2007), challenging previous findings and arguing against the role of xanthan in the virulence of *X. axonopodis* pv. *citri*. Differences in experimental approach perhaps could account for these divergent conclusions about the role of xanthan in *X. axonopodis* pv. *citri*–*Citrus* spp. interactions. Such differences occur in the plant host (*C. sinensis* versus *C. limon*) and WT isolates of *X. axonopodis* pv. *citri* used, in the mutations used to generate the xanthan-minus *X. axonopodis* pv. *citri* strains, and in the inoculation procedures.

It is difficult to determine whether the difference in aggressiveness of *X. axonopodis* pv. *citri* strains is affected by the strategy used to generate xanthan deficiency. Dunger and associates (2007) generated a xanthan-deficient mutant by a deletion part of the *gumD* gene, which encodes a glycosyltransferase involved in synthesis of the first xanthan lipid intermediate. The *X. axonopodis* pv. *manihotis* *gumD* gene was shown to be essential for EPS production and pathogenicity and contributes to epiphytic fitness on the host plant (Kemp et al. 2004). In contrast, our study generated a xanthan-deficient strain through mutation of the *gumB* gene. The *gumB* protein polymerizes a pentasaccharide intermediate into mature xanthan (Vojnov et al. 1998). In *X. campestris* pv. *campestris*, *gumB* mutants produce no xanthan, show reduced virulence (Chou et al. 1997; Katzen et al. 1998; Li et al. 2001), and fail to grow when inoculated into leaves of *Brassica campestris*, *Nicotiana benthamiana*, and *Arabidopsis thaliana* (Newman et al. 1994; Yun et al. 2006). One difference between the use of *gumB* and *gumD* mutants would be in the accumulation of the pentasaccharide lipid intermediate in the former compared with the lipid alone in the latter. Although it is possible that this pentasaccharide lipid has some negative effects on additional cellular functions that contribute to virulence, we consider this unlikely because the *gumB* mutation had no effect on *X. axonopodis* pv. *citri* growth, and studies in *X. campestris* pv. *campestris* have shown that the level of the pentasaccharide lipid intermediate is much lower in the *gumB* mutant than in the WT (Vojnov et al. 1998). Intriguingly, in *Xylella fastidiosa*, the disruption of the *gumB* gene does not significantly alter production of the EPS fastidian, but mutants do show a reduced capacity to form biofilms (Souza et al. 2006).

A second significant experimental difference is in the inoculation methods. In our experiments, the loss of pathogenicity was especially evident following spray and nick-spray infection, in which the *gumB* mutant failed to induce any symptoms. Spray and nick-spray methods mimic *Xanthomonas axonopodis* pv. *citri* entry in vivo, in which invasion of host mesophyll is accomplished through natural openings such as stomata and through lesions. In contrast, the findings of Dunger and associates (2007) are based on two methods that do not mimic natural processes. The first method, pressure infiltration, serves to mechanically destroy the primary physical barriers in plant defense: the cuticle and cell wall. For this reason, *X. axonopodis* pv. *citri* *gumB* mutants that are pressure infiltrated are more capable of causing canker symptoms than mutants inoculated via spray or nick and spray (Fig. 4A). The second method—spray-inoculation of bacteria in the presence of Silwet L-77—is likely to have the same effect as pressure infiltration. Silwet L-77 is a surface-active agent that lowers surface tension. It is used to facilitate penetration of bacteria into plant tissues (Wu et al. 2003), but likely allows for partial infiltration of the leaf. Finally, the discrepancy between our results and those of Dunger and associates (2007) could reflect differences in the species of *Citrus* employed as a host and in the WT *X. axonopodis* pv. *citri* strains used. Perhaps the contribution of differ-

ent factors contributing to virulence varies quantitatively in different *X. axonopodis* pv. *citri*–*Citrus* spp. interactions.

Based upon our results, the ability of *X. axonopodis* pv. *citri* to develop a biofilm that induces canker disease in citrus suggests that a biofilm lifestyle is a natural environment for *X. axonopodis* pv. *citri* and that extracellular components are particularly relevant. Living within a biofilm could offer many advantages to *X. axonopodis* pv. *citri* in comparison with the planktonic state; the biofilm can act as a defense or survival mechanism, or it can facilitate cell-to-cell communication and growth in the poor nutrient-leaf environment. In addition, the growth of the bacteria within biofilms has been shown to generate a large amount of genetic diversity (Boles et al. 2004), which would allow the bacterial community to adapt to sudden environmental changes better than if the populations were genetically homogenous.

Our results indicate that xanthan plays an important role in the formation of biofilms and in bacterial survival on the leaf. Without xanthan, the bacteria are unable to retain water and cannot withstand abiotic stress and, thus, cannot survive on the leaf surface. Bacterial colonization also is required for expression of virulence-associated genes that induce plant defenses (Marco et al. 2005). The role of xanthan in both pathogen virulence and host defense may explain why smaller epiphytic bacterial populations were observed for the mutant compared with WT (Fig. 3B). However, other roles played by xanthan in disease development should not be ruled out. For example, EPS has been shown to mediate suppression of plant defense reactions (D’Haeze and Holsters 2004; Yun et al. 2006), and EPS can act as a specific recognition signal in human cells (Bylund et al. 2006).

Further studies on the temporal and spatial regulation of xanthan production in canker development, as well as studies to understand other regulatory mechanisms of gene expression involved in biofilm formation, should advance our understanding of the adaptation of bacteria to parasitic life within plants. Such knowledge may provide a rational basis for the development of crop protection methods based on interference with key processes in the epiphytic and disease phases of bacterial pathogenesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

The WT strain of *X. axonopodis* pv. *citri* was described previously (Siciliano et al. 2006). *Xanthomonas* strains were grown at 28°C in PYM nutrient medium (Siciliano et al. 2006). For analysis of biofilm development, bacteria were grown in Y minimal medium containing glucose (1% wt vol⁻¹) as the carbon source (Sherwood 1970). *E. coli* was grown at 37°C in Luria-Bertani medium (Sambrook et al. 1989). Bacterial growth was measured in a Spectronic 20Genesys spectrophotometer (Thermo Electron Corporation, Boston) at 600 nm. When required, the antibiotics kanamycin and gentamicin were added to the growth media at concentrations of 50 and 10 µg ml⁻¹, respectively.

Site-directed gene replacement of *X. axonopodis* pv. *citri*.

The molecular techniques used in this study were based on protocols described previously (Sambrook et al. 1989). Genomic DNA from the *X. axonopodis* pv. *citri* strain was extracted according to Chen and Kuo (1993). From the region encoding the WT *X. axonopodis* pv. *citri* *gumB* and *gumC* genes, 1,457-bp fragments were PCR amplified using the primer pairs AGTTA CGTGTGGCGATCTTGGC (sense) and AATGGCGTGCGG TCTCTTTC (antisense), designed from *X. axonopodis* pv. *citri* sequences available in GenBank (AE008923) (da Silva et al.

2002). Each 50- μ l PCR reaction consisted of 0.2 μ M each primer, 1 $\times$ PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 75 ng DNA μ l⁻¹, and 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.). PCR amplifications were performed with a Mastercycler (Eppendorf, Hamburg, Germany) using the following conditions (35 cycles): 95°C for 60 s, 63°C for 30 s, and 72°C for 90 s. All PCR reactions were started with an initial denaturation step of 10 min at 95°C and a single final extension cycle at 72°C for 10 min. The PCR products were resolved in a 0.8% agarose gel electrophoresis (Sambrook et al. 1989). The corresponding band was excised and gel purified using the Qiaex II gel extraction kit (Qiagen, Valencia, CA, U.S.A.) and cloned into pGEM-Teasy vector (Promega, Madison, WI, U.S.A.) to produce pLR-XB. The *gumB*-C fragment contains a cleavage site for restriction enzyme *Bam*HI. The 2-kb fragment containing the Km^r (Ω) cassette from pAZ(Ω) (kindly provided by Dr. Angeles Zorreguieta, Fundación Instituto Leloir, Buenos Aires, Argentina) was inserted in the *gumB* cleavage site, *Bam*HI, generating a disrupted copy of the *gumB* gene. These constructs were digested with *Not*I and subcloned into the suicide vector, pJQ200KS (Muller et al. 1993). The generating pSac-EPS plasmid was transformed into WT *X. axonopodis* pv. *citri* by electroporation (Guilhbert et al. 2001). Transformed bacteria were selected on PYM nutrient medium solidified with 1.5% (wt/vol) agar, kanamycin at 50 μ g ml⁻¹, and 5% saccharose (Muller et al. 1993). Disruption of the *gumB* locus in the mutant strain *gumB::Km^r* was confirmed by PCR and Southern blot (data not shown). Isolated mutant was screened for EPS synthesis by precipitation of xanthan from the culture supernatant (Vojnov et al. 1998, 2002).

Assay for xanthan production.

For measurements of xanthan production, strains were grown in PYM medium supplemented with 2% D-glucose at 28°C for 24 h with shaking. EPS was precipitated from culture supernatants by ethanol, dried, and weighed as described by Vojnov and associates (1998).

Plant preparation and pathogenicity assays.

Lemon plants (*C. limon* cv. Eureka) were grown as previously reported (Siciliano et al. 2006). All plant inoculations involved a minimum of three leaves from each plant and three plants were inoculated for each strain of *Xanthomonas* tested. Inoculations of bacteria into the lamina of young lemon leaves were done according to published methods (Siciliano et al. 2006). Bacterial suspensions of *X. axonopodis* pv. *citri*, *X. axonopodis* pv. *citri* mutant, and *MgumB* + pIZD15-261 (10⁷ CFU ml⁻¹ in 10 mM MgCl₂) were inoculated by pressure infiltration, nick and spray, and spray alone. MgCl₂ was used as control of noninfected leaves. Inoculated plants were maintained for 25 to 30 days in a growth cabinet, with temperatures ranging from 25 to 28°C, high humidity, a photoperiod of 16 h of light, and a light intensity of 150 to 200 μ E sm⁻². Disease progression was monitored phenotypically in three separate biological assays and through analysis of bacterial growth curves.

Isolation of bacterial strains.

Bacterial populations were isolated from the leaves according to the method described by Siciliano and associates (2006). Three leaf disks of 1 cm² were selected randomly from the inoculated leaves. The disks were immersed in 500 μ l of 10 mM MgCl₂ in Eppendorf microfuge tubes. Bacterial cells were collected by homogenization of tissue with a plastic pestle. The suspension was stirred at room temperature for 5 min and serial dilutions of this suspension were plated on PYM agar plates to estimate the size of the total bacterial population. Leaf samples were taken once a week to determine bacterial population sizes

on the leaves. At each time point, samples were determined from three separate experiments and each experiment was measured in triplicate.

For determination of epiphytic survival, bacterial populations were isolated from the leaves according to the method described by Morris and associates (1998). Epiphytic bacterial numbers were determined as above, but leaf disks were stirred gently at room temperature for 2 min to release bacteria on the surface of the leaves without liberating bacteria within the cellular apoplast. The experiment was repeated once a day for 1 week to determine epiphytic survival on lemon leaves. At each time point, samples were taken from three separate plants and each experiment was measured in triplicate.

Microscopy and image of the dynamic of biofilm development.

Inverted CLSM was used for the study of biofilms both in vitro and in vivo. For the in vitro study, biofilm formation assays in chambered coverglass slides containing a borosilicate glass 1 μ m thick (no. 155411; Lab-Tek, Nunc, Naperville, IL, U.S.A.) were performed as described previously (Russo et al. 2006). Each strain was transformed by triparental mating, with plasmid pMP2444 expressing the GFP (Stuurman et al. 2000). The *gfp*-expressing bacterial strains were grown overnight on Y minimal medium supplemented with 1% glucose containing the appropriate antibiotics at 28°C (Sherwood 1970). Bacterial cultures were diluted 1:1000 and grown in chambers without shaking at 28°C for 7 days. To prevent desiccation, the chambers were incubated in a humid sterile petri dish. Briefly, for the in vivo study, leaves of *C. limon* were infected by spray, as described above, with GFP-labeled bacterial suspension of *X. axonopodis* pv. *citri* WT and the mutant strain *gumB::Km^r*. Plants were incubated in the same conditions described above until canker formation. Areas of approximately 1 cm² were cut from the leaves and mounted on the adaxial leaf surface under glass coverslips.

Bacterial growth on both abiotic and biotic surfaces were examined with an inverted confocal microscope (LSM510-Axiovert 100M; Carl Zeiss, Oberkochen, Germany). Simulated three-dimensional images and sections were generated by using the Zeiss LSM Image Browser, version 3.2.0. Canker development by bacteria carrying the plasmid pMP2444 was similar to *X. axonopodis* pv. *citri*. No difference in growth or biofilm formation could be detected using (nonfluorescence) microscopy of biofilms formed by bacteria containing or lacking the GFP plasmid.

Bacterial adhesion study.

To analyze the function of EPS production on initial attachment and biofilm formation, we used the microplate colorimetric assay involving crystal violet staining of biofilms (Davey and O'Toole 2000). *X. axonopodis* pv. *citri* WT and the mutant strain *gumB::Km^r* were grown in wells of polystyrene 96-well flat-bottom tissue culture plates (Corning Incorporated, Corning, NY, U.S.A.) as follows. Bacteria were grown overnight in PYM medium, then inoculated into Y medium at a 1:1000 dilution (Sherwood 1970). Once the *X. axonopodis* pv. *citri* cultures reached an optical density at 600 nm of 0.1 in Y minimal medium, aliquots of 100 μ l were placed into the wells of microtiter plates and incubated at 28°C for different lengths of times (Fig. 1A). After incubation, the medium was gently removed using a pipette, microtiter plate wells were washed three times gently with 150 μ l of sterile distilled water, and the bacteria attached to the surface was incubated at 60°C for 20 min. The amount of surface-attached biofilm was determined using 150 μ l of 0.30% (wt vol⁻¹) crystal violet (Acros Organics, Geel, Belgium) (Davey and O'Toole 2000) for 45 min at room

temperature. The unbound crystal violet stain was removed using a pipette, and the wells were washed gently three times with 150 μ l of sterile distilled water. The crystal violet in each well was solubilized by adding 100 μ l of acetone-ethanol (20% acetone). The biofilm cell-associated dye was measured by absorption at 570 nm using a microtiter plate reader (PowerWave XS; Bio-Tek, Winooski, VT, U.S.A.). Readings from 36 independent experiments were averaged. To compare the adherence between strains, we used the statistic analysis Student's *t* test ($P < 0.05$).

Bacterial adhesion also was studied on lemon leaves. *X. axonopodis* pv. *citri* WT and the mutant strain *gumB::Km^r* were grown in the same conditions described for the microplate colorimetric assay. Leaf disks of approximately 35 mm² were cut from healthy leaves and mounted onto petri dishes, adaxial side down. Leaf disks were inoculated with 3 ml of the bacterial suspension at a concentration of 10⁷ CFU ml⁻¹ and incubated at 28°C. After 1, 3, 15, and 24 h of incubation, the leaf disks were rinsed three times with distilled water. The adhered bacteria on the leaf surface were warmed at 60°C for 20 min. The amount of surface-attached biofilm *in vivo* was determined using 3 ml of 0.30% crystal violet for 45 min at room temperature. The unbound crystal violet stain was removed and the leaf disks were washed gently three times with distilled water. The stains attached cells to the leaf surface that were analyzed macroscopically by the intensity of the blue dye.

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