Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2016) 17(2), 236–246 DOI: 10.1111/mpp.12276

Surface polysaccharides and quorum sensing are involved in the attachment and survival of *Xanthomonas albilineans* on sugarcane leaves

IMENE MENSI[1], JEAN-HEINRICH DAUGROIS[1], ISABELLE PIERETTI[1], DANIEL GARGANI[1], LAURA A. FLEITES[2], JULIE NOELL[1], FRANCOIS BONNOT[1], DEAN W. GABRIEL[2] AND PHILIPPE ROTT[1,2,*,†]

[1]*UMR BGPI, CIRAD, F-34398 Montpellier, France*
[2]*Plant Pathology Department, University of Florida, Gainesville, 32611, USA*

SUMMARY

Xanthomonas albilineans, the causal agent of sugarcane leaf scald, is a bacterial plant pathogen that is mainly spread by infected cuttings and contaminated harvesting tools. However, some strains of this pathogen are known to be spread by aerial means and are able to colonize the phyllosphere of sugarcane before entering the host plant and causing disease. The objective of this study was to identify the molecular factors involved in the survival or growth of *X. albilineans* on sugarcane leaves. We developed a bioassay to test for the attachment of *X. albilineans* on sugarcane leaves using tissue-cultured plantlets grown *in vitro*. Six mutants of strain XaFL07-1 affected in surface polysaccharide production completely lost their capacity to survive on the sugarcane leaf surface. These mutants produced more biofilm *in vitro* and accumulated more cellular poly-β-hydroxybutyrate than the wild-type strain. A mutant affected in the production of small molecules (including potential biosurfactants) synthesized by non-ribosomal peptide synthetases (NRPSs) attached to the sugarcane leaves as well as the wild-type strain. Surprisingly, the attachment of bacteria on sugarcane leaves varied among mutants of the *rpf* gene cluster involved in bacterial quorum sensing. Therefore, quorum sensing may affect polysaccharide production, or both polysaccharides and quorum sensing may be involved in the survival or growth of *X. albilineans* on sugarcane leaves.

Keywords: biofilm, NRPS, quorum sensing, *rpf* genes, sugarcane, surface polysaccharides, *Xanthomonas albilineans*.

INTRODUCTION

Prior to entry into plant tissues via wounds or natural openings, such as stomata and hydathodes, many plant-pathogenic bacteria are able to survive as epiphytes on the aerial parts of plants (also called the phyllosphere) without causing any visible symptoms (Hirano and Upper, 2000; Vorholt, 2012). The success of this early phase of foliar colonization is crucial to these pathogens as the occurrence and development of a foliar disease is related to the epiphytic population size of the pathogen (Akhavan *et al*., 2013; Champoiseau *et al*., 2009; Wilson *et al*., 1999). Epiphytic populations can also provide inoculum for subsequent infection of plants or for outbreaks of diseases under appropriate conditions (Hirano and Upper, 2000). Because of the frequent and rapid alteration of environmental conditions on leaf surfaces, the phyllosphere is considered as a harsh habitat in which epiphytic bacteria are confronted by multiple biotic and abiotic stresses, including nutrient limitation, desiccation, UV irradiation and antimicrobial compounds (Hirano and Upper, 2000; Lindow and Brandl, 2003).

Epiphytic bacteria employ common and species-specific strategies that allow them to resist these stresses and adapt to the phyllosphere (Beattie and Lindow, 1999; Lindow and Brandl, 2003; Vorholt, 2012). Traits carried on plasmids confer selective phyllosphere fitness to strains of *Pseudomonas fluorescens* during the growing season (Lindow and Brandl, 2003). Beattie and Lindow (1999) suggested that some species of phyllobacteria develop large external populations, whereas others develop only small external populations. Specific effectors of *P. syringae* pv. *syringae* str. B728a are involved in the development of aggregates on leaf surfaces that are host dependent (Vorholt, 2012). Aggregation in a structured biofilm is a widely conserved strategy among epiphytic bacteria and is produced by many pathogens for protection against desiccation and other stresses (Danhorn and Fuqua, 2007; Jacques *et al*., 2005; Monier and Lindow, 2003, 2004; Rigano *et al*., 2007). Biofilms are formed when microorganisms attach to a surface and to each other, and together form a matrix of exopolysaccharides (EPSs), lipopolysaccharides (LPSs), adhesins and DNA in which they are embedded (Danhorn and Fuqua, 2007; Li and Wang, 2011a). Quorum sensing has been shown to be involved in the triggering of biofilm formation and, consequently, in epiphytic survival of foliar pathogens (Guo *et al*., 2012; Li and Wang, 2011a; Quinones *et al*., 2005).

**Correspondence*: Email: pcrott@ufl.edu
†*Present address*: Everglades Research and Education Center, University of Florida, 3200 East Palm Beach Road, Belle Glade, FL 33430, USA.

Several genes involved in epiphytic survival have been identified in bacterial plant pathogens. Mutants of the gene *gumB* (coding for EPS production) of *Xanthomonas axonopodis* pv. *citri* are no longer able to form biofilms or to survive on citrus leaves (Rigano *et al*., 2007). The *rpf* gene cluster is involved in the regulation of quorum sensing in xanthomonads (Dow, 2008; Ryan and Dow, 2011). Biofilm formation and dispersal, as well as EPS biosynthesis, are controlled by this system, which is also directly involved in the epiphytic survival of *Xanthomonas* species (Dow *et al*., 2003; Torres *et al*., 2007). In *X. campestris* pv. *campestris*, the *rpf* signalling system also regulates the production of a small molecule which is necessary to overcome stomatal closure, a reaction of the plant against invasion of the pathogen (Gudesblat *et al*., 2009).

Historically, sugarcane leaf scald caused by *X. albilineans* was known to be transmitted mechanically by cutting implements or by planting infected cuttings from symptomless plants (Rott and Davis, 2000). Sordi and Tokeshi (1986) reported the occurrence of *X. albilineans* in guttation droplets of infected sugarcane and sweetcorn leaves, demonstrating that the pathogen was also present on the leaf surface of the host plant. Unusual symptoms caused by *X. albilineans* were observed in a nursery plot in Guadeloupe (FWI) in 1993 (Daugrois *et al*., 2012) and reported from Mauritius in 1995 (Autrey *et al*., 1995) and from Florida, USA in 2001 (Comstock, 2001). These unusual symptoms were attributed to aerial transmission of the pathogen (Comstock, 2001; Daugrois *et al*., 2003). Furthermore, outbreaks of sugarcane leaf scald that occurred in the late 1980s in Florida were also attributed to a genotype of the pathogen that had the propensity to be spread aerially (Davis *et al*., 1997).

Studies performed by Daugrois *et al*. (2003) and Champoiseau *et al*. (2009) in Guadeloupe clearly showed that *X. albilineans* colonizes the sugarcane canopy before entering the leaves and causing disease symptoms. The molecular mechanisms involved in the establishment of the pathogen on the sugarcane canopy are, however, largely unknown. In addition, *X. albilineans* lacks *gum* genes and therefore does not produce the xanthan gum that is involved in the mucoid phenotype of bacterial colonies and biofilm formation by other xanthomonads (Pieretti *et al*., 2012). However, *X. albilineans* is able to synthesize several small non-ribosomal peptides, including the toxin albicidin, which is involved in pathogenicity and is encoded by three large non-ribosomal peptide synthetases (NRPSs) (Birch, 2001; Royer *et al*., 2013). This type of enzyme is also involved in the biosynthesis of biosurfactants playing a role in surface attachment (Das *et al*., 2008). The leaf scald pathogen also possesses the *rpf* quorum sensing system that is involved in the regulation of sugarcane stalk colonization (Rott *et al*., 2013). The objective of this study was therefore to identify the molecular factors involved in the attachment or survival of *X. albilineans* on sugarcane leaves. A simplified bioassay using tissue-cultured plants of sugarcane was developed to reproduce survival/growth on leaves of *X. albilineans* wild-type strains. This bioassay was then used to test the extent of leaf attachment (ELA) of several mutants of the pathogen impaired in the capacity to cause disease symptoms or to multiply in the sugarcane stalk (Rott *et al*., 2011).

RESULTS

A bioassay to study the attachment of *X. albilineans* on sugarcane leaves

In preliminary experiments, 4-week-old tissue-cultured plantlets of cultivar CP68-1026 were immersed in bacterial suspensions at 10^6, 10^7 and 10^8 colony-forming units (CFU)/mL of *X. albilineans* strains XaFL07-1 (from Florida) and GPE PC73 (from Guadeloupe) (Table 1). Foliar imprints of the plantlets were performed on selective growth medium 14 days after inoculation. ELA, which was based on the growth density in the imprint area, varied in the range 50–59 (on a 0–100 scale) (Fig. 1). No significant difference was found between the two strains of the pathogen at the three inoculum densities. *Xanthomonas albilineans* strain XaFL07-1 and an inoculum density of 10^7 CFU/mL were chosen for all subsequent experiments.

Surface polysaccharide mutants of *X. albilineans* are not able to survive on sugarcane leaves, but overproduce biofilm and accumulate poly-β-hydroxybutyrate (PHB)

Six mutants of *X. albilineans* unable to produce surface polysaccharides after transposon mutagenesis were identified *in vitro* (Table 1; Rott *et al*., 2011). Mutations in each of these mutants were located in different genes, including *xanB*, *gmd* and *rmd* (Table 1). Genes *gmd* and *rmd*, as well as open reading frames (ORFs) XALc_2705 (coding for a glycosyltransferase of operon *gmd-rmd*) and XALc_2709 (coding for an ABC transporter), were expected to be involved in LPS production as these genes are bordered by genes *etfA* and *metB* (Lu *et al*., 2008; Pieretti *et al*., 2012). The texture of colonies of these mutants on modified Wilbrink's (MW) medium was dry and rough, whereas the texture of colonies of the wild-type strain was slightly viscous and easily identified when bacteria were subcultured on fresh medium with a transfer loop (Fig. 2).

Wild-type strain XaFL07-1 of *X. albilineans* and the six polysaccharide mutants were inoculated on tissue-cultured plantlets of sugarcane. Fourteen days after inoculation, the mean ELA score was 50 for the wild-type strain and only 0.5–5 for the six polysaccharide mutants (Table 2). The loss of leaf attachment of polysaccharide mutant M1116 was further investigated by washing and homogenizing leaves after inoculation of tissue-cultured plantlets with this mutant and wild-type strain XaFL07-1. One hour after inoculation, both the wild-type and mutant strains were each

Table 1 Plasmids and bacterial strains used in this study.

Plasmid/strain	Relevant characteristics*	Reference/source
Plasmids		
pALB204	pUFR043 containing a 33-kb insert from Xa23R1 including *rmd* gene, Gm^r, Kn^r	Rott *et al*. (1996)
pUFR043	IncW Mob1 LacZa Gm^r Nm^r Cos	De Feyter and Gabriel (1991)
pUFR047	IncW, Mob +, *lac*Zα+, Par+, Gm^r, Amp^r	De Feyter *et al*. (1993)
pPR017	18.3 kb; 9.7-kb *Bam*HI-digested *rpfB-prfB* fragment from pPYOAAB9CC10 cloned into pUFR047, Gm^r, Amp^r	Rott *et al*. (2013)
pPR017Met	pPR017 transformed into chemically competent cells of *Escherichia coli* containing pY0AAB27CH12, Cm^r	Rott *et al*. (2013)
Escherichia coli strains		
DH5α	F-, *endA1*, *hsdR17*(rk-,mk+), *supE44*, λ-*thi-1*, *recA1*, *gyrA96*, *relA1*, *phoA*, Φ80*lacZ*ΔM15, Δ(*lacZYA-argF*)U169	Gibco-BRL, Gaithersburg, MD, USA
DH5α pRK2073	*E. coli* containing pRK2013 derivative, Kms (*npt*::Tn*7*) Sp^r Tra1, helper plasmid	Leong *et al*. (1982)
Xanthomonas albilineans strains		
GPE PC73	Wild-type strain from Guadeloupe	Pieretti *et al*. (2009)
XaFL07-1	Wild-type strain from Florida	Rott *et al*. (2011)
Xanthomonas albilineans derivatives		
M427	*xanB*::Tn*5* derivative of strain XaFL07-1, defective in biosynthesis of surface polysaccharides, Kn^r	Rott *et al*. (2011)
M448	Glycosyl transferase of the galU operon::Tn*5* derivative of strain XaFL07-1, defective in biosynthesis of surface polysaccharides, Kn^r	P. Rott *et al*. (unpublished data)
M541	ABC transporter ATP adhesion protein::Tn*5* derivative of strain XaFL07-1, defective in biosynthesis of surface polysaccharides, Kn^r	P. Rott *et al*. (unpublished data)
M903	XALc_2705::Tn*5* derivative of strain XaFL07-1, defective in biosynthesis of surface polysaccharides, Kn^r	Rott *et al*. (2011)
M967	*gmd*::Tn*5* derivative of strain XaFL07-1, defective in biosynthesis of surface polysaccharides, Kn^r	Rott *et al*. (2011)
M1116	*rmd*::Tn*5* derivative of strain XaFL07-1, defective in biosynthesis of surface polysaccharides, Kn^r	Rott *et al*. (2011)
Δ*albXXI* M8	Δ*albXXI*, defective in production of albicidin and other non-ribosomal peptide synthetase (NRPS) molecules	Rott *et al*. (2011)
Δ*rpfF* M7	Δ*rpfF*, defective in production of diffusible signal factor (DSF)	Rott *et al*. (2013)
Δ*rpfG* M6	Δ*rpfG*, defective in DSF receptor protein RpfG	Rott *et al*. (2013)
Δ*rpfC* M29	Δ*rpfC*, defective in DSF receptor protein RpfC	Rott *et al*. (2013)
rpfGCF M15	Δ*rpfF*, Δ*rpfG*, Δ*rpfC*, defective in DSF production and receptor proteins RpfG and RpfC	Rott *et al*. (2013)

*Gm^r, Nm^r, Amp^r, Cm^r, Sp^r and Kn^r indicate resistance to gentamycin, neomycin, ampicillin, chloramphenicol, spectinomycin and kanamycin, respectively.

Table 2 Attachment on sugarcane leaves of six mutants of surface polysaccharides of *Xanthomonas albilineans* strain XaFL07-1.

Strain of *X. albilineans*	Extent of leaf attachment (ELA)*	Biofilm amount†
XaFL07-1 (wild-type)	50 b	0.71 a
M427	2 a	4.07 d
M448	4 a	2.52 c
M541	5 a	1.63 b
M903	3 a	3.91 d
M967	2 a	1.53 a,b
M1116	0.5 a	3.86 d

*On a 0–100 scale (see Experimental procedures and Fig. 1). ELA data are the means of 18 sugarcane plantlets per strain and two to four leaves per plantlet (data from three independent experiments were combined). Values followed by the same letter are not significantly different according to the Tukey test ($P < 0.05$).

†Absorption at 570 nm of solubilized crystal violet stain recovered from the glass tube sides (see Fig. 3). Data are the means of two independent experiments with five replicates per strain and experiment. Values followed by the same letter are not significantly different according to the Tukey test ($P < 0.05$).

recovered from all 20 inoculated leaves by leaf washing (Table 3). The mean CFU was 6.3×10^3 CFU/leaf for XaFL07-1 and 3.2×10^3 CFU/leaf for M1116, suggesting successful initial attachment of both strains to the sugarcane leaves. Seven and 14 days after inoculation, the wild-type strain was recovered by washing from 16 of 20 and 11 of 20 leaves, respectively, and population means of the pathogen were no different at either date [$(3.2–4.0) \times 10^3$ CFU/leaf]. In contrast, mutant M1116 was not recovered by leaf washing from any of 20 leaves 7 days after inoculation, and only from one leaf 14 days after inoculation. Strain XaFL07-1 was also recovered by leaf homogenization from all 20 leaves and 16 of 20 leaves, 7 and 14 days after inoculation, respectively (Table 3). In contrast, mutant M1116 was recovered by leaf homogenization from only three of 20 and one of 20 leaves, 7 and 14 days after inoculation, respectively. Mean bacterial populations recovered from XaFL07-1-positive leaves (630 CFU/leaf) were also more than 10 times higher than the mean populations recovered from M1116-positive leaves (40 CFU/leaf), 7 days after inoculation. These data suggest

that M1116 is affected with regard to survival on the leaf surface rather than to attachment *per se*.

The wild-type strain of *X. albilineans* produced biofilm that adhered to glass (Fig. 3). All polysaccharide mutants were also able to produce this biofilm, and some to a greater extent than the wild-type strain. The biofilm amount, measured by the intensity of crystal violet coloration, was significantly higher for five of the six mutants (Table 2 and Fig. 3). The capacity for growth in rich and minimal media of the six mutants was no different or only slightly lower than that of the wild-type (Fig. 4). No difference in appearance of the external membrane was found between polysaccharide mutant M1116 (Tn*5* insertion in gene *rmd*; Table 1) and the wild-type parental strain when cells were observed by transmission electron microscopy (Fig. 5). However, mutant M1116 showed many more inclusion bodies expected to contain PHB than did the wild-type strain (Fig. 5).

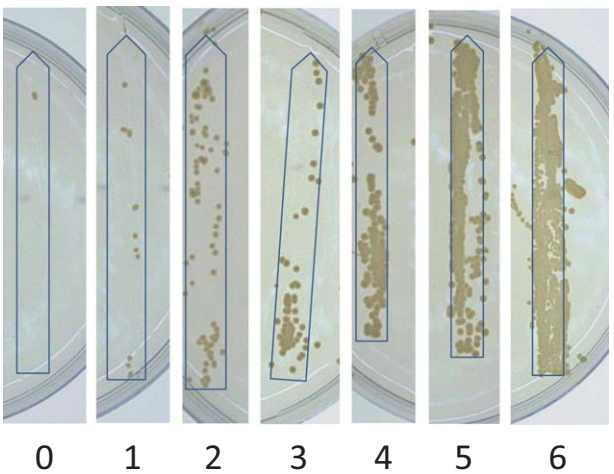

Fig. 1 Growth of *Xanthomonas albilineans* XaFL07-1 in imprints of sugarcane leaves on selective medium 2 weeks after immersion of leaves in a bacterial suspension at 1×10^7 colony-forming units (CFU)/mL. 0, 0–5 colonies in the leaf imprint; 1, 6–50 colonies in the leaf imprint; 2, more than 50 colonies and no confluent growth of bacteria in the leaf imprint; 3, confluent growth of bacteria in less than 10% of the leaf imprint; 4, confluent growth of bacteria in 10%–40% of the leaf imprint; 5, confluent growth of bacteria in 41%–80% of the leaf imprint; 6, confluent growth of bacteria in 81%–100% of the leaf imprint. Boxed areas represent leaf imprint areas.

Plasmid pALB204 harbouring wild-type gene *rmd* [encoding a putative nucleoside diphosphate (NDP)-hexose oxidoreductase] was introduced into mutant M1116 and tested for its capacity to restore leaf attachment of this mutant. Two weeks after inoculation of six sugarcane plantlets per strain, the mean ELA score of mutant M1116 complemented with this plasmid was 20, whereas the mean ELA scores of the mutant and the wild-type strain containing the empty vector (pUFR043) were 0.6 and 50, respectively. Plasmid pALB204 therefore partially restored the capacity of epiphytic survival of mutant M1116. The texture of the colonies of the complemented mutant was also slightly viscous, similar to the wild-type, whereas the texture of mutant M1116 containing the empty vector was dry and easily identified when bacteria were subcultured with a transfer loop.

A mutation of *X. albilineans* affecting the production of small NRPS molecules, including albicidin, is unaffected in attachment to sugarcane leaves

Gene *albXXI* (= *xabA*) encodes a phosphopantetheinyl transferase, an enzyme involved in post-translational activation of

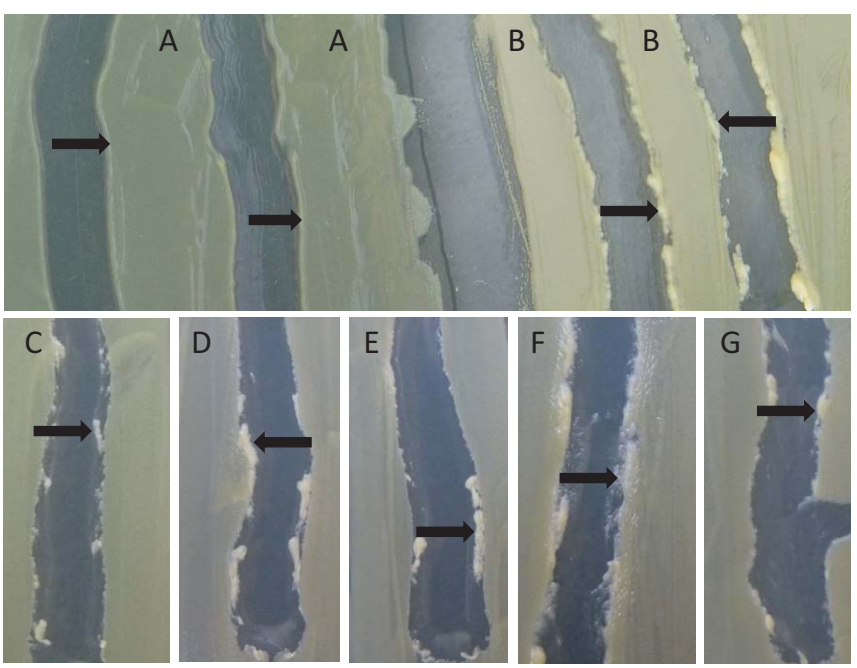

Fig. 2 Aspect of cultures of *Xanthomonas albilineans* XaFL07-1 (A) and polysaccharide mutants M1116 (B), M427 (C), M448 (D), M541 (E), M903 (F) and M967 (G) after passage with a transfer loop: the edges of the wild-type culture appear viscous and homogeneous (arrows), whereas the edges of the mutant cultures are irregular and rough (arrows).

Table 3 Recovery of *Xanthomonas albilineans* from inoculated sugarcane leaves after leaf washing and leaf homogenization.

	Bacterial recovery after leaf washing*						Bacterial recovery after leaf homogenization*			
	Number of positive leaves after			Mean bacterial population [log_{10}(CFU/positive leaf)] after			Number of positive leaves after		Mean bacterial population [log_{10}(CFU/positive leaf)] after	
Strain of *X. albilineans*	1 h	7 days	14 days	1 h	7 days	14 days	7 days	14 days	7 days	14 days
XaFL07-1 (wild type)	20	16	11	3.8	2.5	2.6	20	16	2.8	3.0
M1116	20	0	1	3.5	–	2.2	3	1	1.6	2.5

*Data are for 20 leaves and combined from two separate experiments of five plantlets each (two leaves sampled per inoculated sugarcane plantlet).

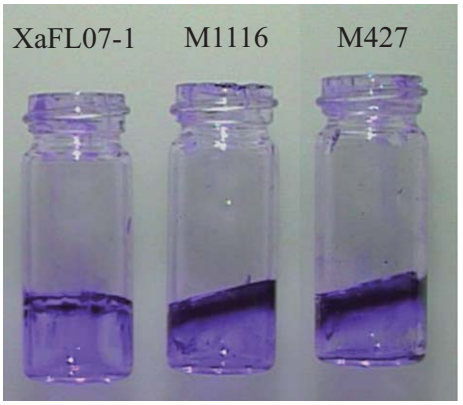

Fig. 3 Formation of biofilm *in vitro* by wild-type strain XaFL07-1 and two polysaccharide mutants (M427 and M1116) of *Xanthomonas albilineans*. Biofilm attached to the glass tubes was stained with crystal violet after 14 days of stationary culture in modified Wilbrink's medium + 5 g/L yeast extract at 28 °C (see Table 2 for biofilm amount of all tested strains).

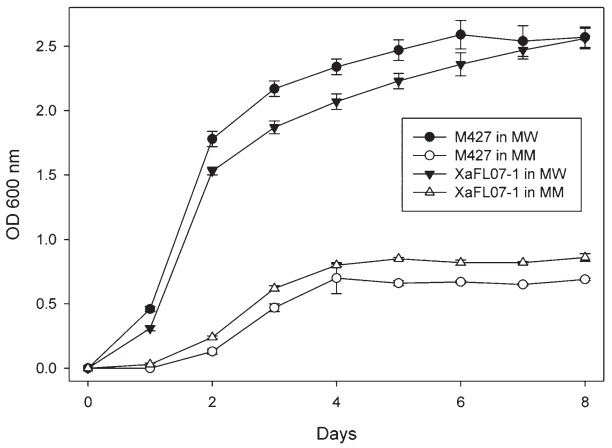

Fig. 4 Growth of *Xanthomonas albilineans* XaFL07-1 (wild-type) and polysaccharide mutant M427 in modified liquid Wilbrink's medium (MW) and liquid minimal medium (MM). Growth measured for 8 days after inoculation of each medium is expressed as the optical density at 600 nm (OD 600 nm), and each point represents the mean of four different tubes per strain. Bars represent standard deviation. Growth of the other five polysaccharide mutants (M448, M541, M903, M967, M1116) was identical to growth of the wild-type strain or mutant M427 in MW and MM (data not shown).

Table 4 Attachment on sugarcane leaves of a mutant of small non-ribosomal peptide synthetase (NRPS) molecules and four mutants of quorum sensing of *Xanthomonas albilineans* strain XaFL07-1.

Strain of *X. albilineans*	Extent of leaf attachment (ELA)*
XaFL07-1 (wild-type)	57 b
Δ*albXXI* M8	57 b
Δ*rpfF* M7	50 a,b
Δ*rpfG* M6	62 b
Δ*rpfC* M29	63 b
Δ*rpfGCF* M15	35 a

*On a 0–100 scale (see Experimental procedures); ELA data are the means of 18 sugarcane plantlets per strain and two to four leaves per plantlet (data from three different experiments were combined). Values followed by the same letter are not significantly different according to the Tukey test ($P < 0.05$).

NRPSs, and is required for albicidin biosynthesis (Huang *et al*., 2000; Royer *et al*., 2004). Mutant Δ*albXXI* M8 does not produce the toxin albicidin and other small NRPS molecules activated by *albXXI* (Rott *et al*., 2011). Two weeks after inoculation of sugarcane plantlets, the mean ELA scores of this mutant and of the wild-type were identical (Table 4).

The *rpf* gene cluster is involved in the attachment of *X. albilineans* on sugarcane leaves

The mean ELA score of Δ*rpfF* mutant M7, which no longer produces the diffusible signal factor (DSF) involved in quorum sensing (Rott *et al*., 2013), was 50, which was not significantly different from the mean ELA score of 57 of the wild-type parental strain (Table 4). Similarly, the mean ELA scores of Δ*rpfC* and Δ*rpfG* (which are defective in DSF receptor proteins) were also not significantly different from the mean ELA score of XaFL07-1 (Table 4). However, the mean ELA score of the triple mutant Δ*rpfGCF* M15, which lacks simultaneously *rpfF*, *rpfG* and *rpfC*, was significantly lower than the mean ELA score of the wild-type strain, scoring 35 and 57, respectively. Mutant Δ*rpfGCF* also produced visibly less biofilm than the wild-type strain (data not shown).

Plasmid pPR017Met, harbouring wild-type genes *rpfGCF* (Rott *et al*., 2013), was introduced into mutant Δ*rpfGCF* M15 and tested for its capacity to restore epiphytic survival. In a first experiment, the ELA score of the complemented mutant (Δ*rpfGCF* M15 +

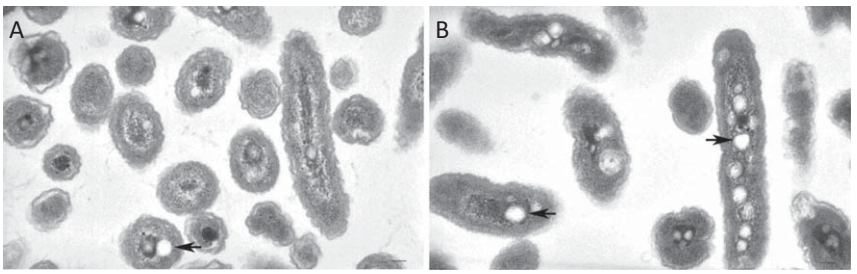

Fig. 5 Cells of *Xanthomonas albilineans* observed by transmission electron microscopy (TEM). (A) XaFL07-1 wild-type strain. (B) Mutant M1116 (gene *rmd*) deficient in surface polysaccharide production. Arrows indicate inclusion bodies with poly-β-hydroxybutyrate. Bars, 100 nm.

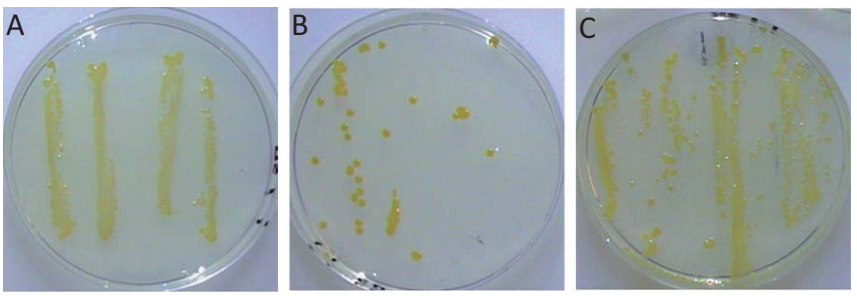

Fig. 6 Growth of *Xanthomonas albilineans* on selective medium after imprinting sugarcane leaves (upper and lower surfaces), 2 weeks after immersion of leaves in a bacterial suspension at 1×10^7 colony-forming units (CFU)/mL. (A) XaFL07-1-pUFR047 (wild-type strain XaFL07-1 containing empty vector pUFR047). (B) *ΔrpfGCF* M15-pUFR047 (quorum sensing mutant *ΔrpfGCF* M15 containing empty vector pUFR047). (C) *ΔrpfGCF* M15-pPR017Met (mutant *ΔrpfGCF* M15 containing complementing plasmid pPR017Met).

pPR017Met) was no different from the ELA score of the wild-type strain containing the empty vector (pUFR047) for three of the six inoculated plantlets. In a second experiment, the ELA score of the complemented mutant was no different from that of the wild-type strain containing pUFR047 for each of the six inoculated plantlets (Fig. 6). Plasmid pPR017Met therefore fully restored the capacity for leaf attachment of mutant Δ*rpfGCF* M15 on most inoculated sugarcane plantlets. Plasmid pPR017Met also restored and even increased the biofilm production of mutant Δ*rpfGCF*, as measured by the intensity of crystal violet coloration (Fig. 7). No significant differences in growth in liquid media were observed between the wild-type strain containing empty vector pUFR047, mutant Δ*rpfGCF* containing empty vector pUFR047 and mutant Δ*rpfGCF* containing complementing plasmid pPR017Met (Fig. 8).

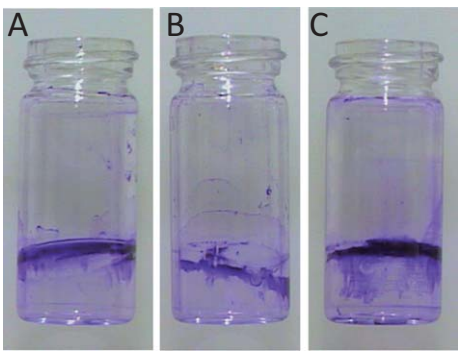

Fig. 7 Formation of biofilm *in vitro* by *Xanthomonas albilineans* strain XaFL07-1 containing empty shuttle vector pUFR047 (A), quorum sensing mutant Δ*rpfGCF* M15 containing pUFR047 (B) and mutant Δ*rpfGCF* M15 complemented with plasmid pPR017Met (C). Biofilm attached to the glass tubes was stained with crystal violet after 14 days of stationary culture in modified Wilbrink's medium + 5 g/L yeast extract at 28 °C. Data below the tubes refer to the mean amount of crystal violet that stayed on the tube sides. Values are the means of two independent experiments with five replicates per strain and experiment. Values followed by the same letter are not significantly different according to the Tukey test ($P < 0.05$).

DISCUSSION AND CONCLUSION

Bacterial surface components, such as polysaccharides, play a crucial role in epiphytic survival and/or colonization of aerially disseminated pathogens (Bogino *et al*., 2013; Li and Wang, 2012; Rigano *et al*., 2007). Attachment to plant surfaces and biofilm formation are critical mechanisms in these processes (Bogino *et al*., 2013). The six different polysaccharide-deficient mutants of *X. albilineans* were all greatly impaired in their capacity to survive on sugarcane leaves, whether the mutations were monofunctional (involved in EPS or LPS biosynthesis) or bifunctional (involved in EPS and LPS biosynthesis). In numerous bacteria, EPSs are released as an extracellular slime. Because of their highly hydrated and anionic consistency, EPSs help to maintain a hydrated microenvironment surrounding the bacteria, protecting them from desiccation and thus increasing their survival (Chang *et al*., 2007). *Xanthomonas* species are known to produce a characteristic EPS, called xanthan gum, which is encoded by the *gum* gene cluster (*gumB* to *gumM*) (Vorhölter *et al*., 2008). A *gumB* mutant strain

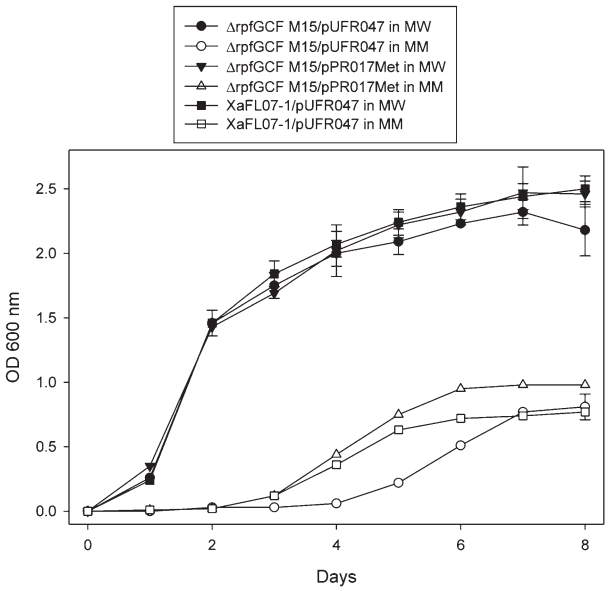

Fig. 8 Growth of *Xanthomonas albilineans* (squares) and quorum sensing mutant Δ*rpfGCF* M15 [with empty vector pUFR047 (circles) or complementing plasmid pPR017Met (triangles)] in modified liquid Wilbrink's medium (MW; filled symbols) and liquid minimal medium (MM; open symbols). Growth measured for 8 days after inoculation of each medium is expressed as the optical density at 600 nm (OD 600 nm), and each point represents the mean of four different tubes per strain. Bars represent standard deviation.

(defective in EPS synthesis) of *X. axonopodis* pv. *citri* is unable to form structured biofilms, and shows compromised survival on leaf surfaces (Rigano *et al*., 2007). A *gumD* mutant of the same pathogen, which is also altered in EPS production, displays impaired survival under oxidative stress during stationary phase, as well as impaired epiphytic survival on citrus leaves (Dunger *et al*., 2007).

Xanthan gum is found in almost all *Xanthomonas* species isolated from plants (Gumus *et al*., 2010); however, it is not found in *X. albilineans*. *Xanthomonas albilineans* is non-mucoid in culture and all *gum* genes are absent from its genome (Pieretti *et al*., 2009, 2012). Nevertheless, *X. albilineans* possesses genes *xanA*, *xanB*, *pgi*, *galU* and *ugd* which direct xanthan gum precursor biosynthesis, and *in planta* production of a xanthan-like EPS by *X. albilineans* has been reported (Blanch *et al*., 2008). Therefore, the chemical nature of EPS involved in leaf attachment and produced by *X. albilineans* remains to be determined.

Among the six surface polysaccharide mutants impaired in survival on sugarcane leaves, four mutants reported herein are predicted to encode LPS. LPS is the major polysaccharide component on the cell surface of Gram-negative bacteria and is essential for protecting the cell from hostile environments (Newman *et al*., 2001). In plant-pathogenic bacteria, LPS is an important virulence factor that has been increasingly recognized as a major pathogen-associated molecular pattern (PAMP) factor (Desaki *et al*., 2006; Dow *et al*., 2000; Keshavarzi *et al*., 2004). The involvement of LPS in biofilm formation and pathogenicity has been demonstrated in animal-, human- and recently also in plant-pathogenic bacteria (Kingsley *et al*., 1993; Lau *et al*., 2009; Li and Wang, 2011b, 2012; Yethon *et al*., 2000). For example, in *X. axonopodis* pv. *citri*, LPS has been shown to play a role in biofilm formation and in epiphytic survival (Li and Wang, 2011b, 2012). Furthermore, an *X. campestris* pv. *campestris* mutant altered in LPS synthesis is strongly affected in its capacity to colonize hydathodes (Hugouvieux *et al*., 1998). The precise role of LPS and EPS in attachment or survival of *X. albilineans* on sugarcane leaves, as well as in virulence in a broad sense, requires further investigation, especially as LPS genes show great variation among strains of the sugarcane leaf scald pathogen (I. Pieretti *et al*., unpublished data).

Surprisingly, and in contrast with other xanthomonads defective in surface polysaccharide production, such as EPS xanthan or LPS (for example, Guo *et al*., 2010; Li and Wang, 2011b; Rigano *et al*., 2007), mutants of *X. albilineans* lacking surface polysaccharides always produced more biofilm than the wild-type strain *in vitro*. Strong attachment of surface polysaccharide mutants to abiotic surfaces is not characteristic of the phenotype observed on the sugarcane phyllosphere. *Xanthomonas albilineans* may employ different mechanisms to adhere to these two types of surface. The genome of this pathogen contains genes that encode fimbrial and non-fimbrial adhesion proteins that could be involved in attachment to inert surfaces (Pieretti *et al*., 2012).

Enhanced attachment to abiotic surfaces compared with the wild-type was reported for LPS mutants of *Escherichia coli*, and this phenomenon was attributed to strong cell surface hydrophobicity (Nakao *et al*., 2012). Furthermore, the absence of LPS or EPS may allow other molecules present on the cell surface to be exposed and favour aggregation of bacteria (Bogino *et al*., 2013). The increased biofilm formation of polysaccharide mutants of *X. albilineans* might also be a stress response related to increased vulnerability of mutated bacterial cells. Mutant M1116 accumulated more inclusion bodies with PHB than did the wild-type parental strain, and PHBs, which are intracellular energy and storage compounds, are known to be produced under nutrient-imbalanced or otherwise stressful conditions (Kadouri *et al*., 2003; Wang and Yu, 2007).

Interestingly, small molecules produced by NRPSs do not seem to be involved in the attachment of *X. albilineans* to the leaf. Microbial biosurfactants are surface-active metabolites synthesized by NRPS enzymes (Das *et al*., 2008). Three NRPS genes are essential for cyclic lipopeptide (CLP) antibiotic synthesis in *P. fluorescens*, and CLP production plays a key role in biofilm formation in this bacterial species (De Bruijn *et al*., 2007). The genome of *X. albilineans* contains several NRPS genes, including those involved in the biosynthesis of the phytotoxin and antibiotic albicidin (Pieretti *et al*., 2009, 2012). Therefore, if not involved in adhesion, NRPS-synthesized molecules of *X. albilineans*, such as those belonging to the albicidin family, may play an essential role

in the capacity of this pathogen to colonize the phyllosphere, and to progress within an environment containing other leaf-colonizing bacteria (Birch, 2001).

Biofilm development and the interactions between plants and colonizing bacteria often require a cell-to-cell crosstalk mechanism among the bacteria, known as quorum sensing. It has been shown that quorum sensing plays a pivotal role in the epiphytic survival of several phytopathogenic bacteria, including *P. syringae* pv. *syringae*, *X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* (Dow *et al*., 2003; Guo *et al*., 2012; He *et al*., 2006; Poplawsky and Chun, 1998; Poplawsky *et al*., 1998; Quinones *et al*., 2004, 2005). Quorum sensing is adopted by these pathogens to coordinate and regulate the expression of virulence factors, including those required for phyllosphere colonization (i.e. biofilm formation and dispersal, EPS production, motility and chemotaxis).

Quorum sensing involving DSF regulates the production of several pathogenicity factors in xanthomonads (Ryan and Dow, 2011). In *X. albilineans* strain XaFL07-1, DSF appears to play a limited role in pathogenicity (Rott *et al*., 2013). In contrast, DSF receptor proteins are involved in the control of swimming motility and the spread of XaFL07-1 in the sugarcane stalk (Rott *et al*., 2013). Similarly, the absence of DSF production did not significantly impair the capacity of *X. albilineans* to attach on sugarcane leaves. However, when the DSF synthase and the receptor genes were all deleted in a single mutant, the pathogen was significantly affected in its capacity to attach to sugarcane leaves. Whether the *rpf* genes only control biofilm production or also other mechanisms involved in surface attachment of *X. albilineans* remains to be determined. In *X. axonopodis* ssp. *citri*, *rpfF*, *rpfC* and *rpfG* control a core group of genes, but also unique genes, suggesting broader roles in gene regulation other than the transduction of DSF signals (Guo *et al*., 2012). A similar phenomenon may occur in *X. albilineans*.

Surface polysaccharides were found to be crucial for the establishment of *X. albilineans* on sugarcane leaves. As these genes are also essential for *in planta* multiplication and invasion (Rott *et al*., 2011), polysaccharides may be potential targets for the screening of antimicrobial compounds for leaf scald control. The DSF quorum sensing system may also be a potential target for control purposes as this system contributes in multiple ways to successful and optimum pathogenicity by *X. albilineans* of sugarcane.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and culture conditions

The primary characteristics of the bacterial strains and plasmids used in this study are summarized in Table 1. *Xanthomonas albilineans* strains were cultured routinely at 28 °C on MW agar medium containing sucrose (10 g), peptone (5 g), $K_2HPO_4.3H_2O$ (0.50 g), $MgSO_4.7H_2O$ (0.25 g), Na_2SO_3 (0.05 g), agar (15 g), distilled water (1 L), pH 6.8–7.0 (Davis *et al*., 1994). Derivatives of strain XaFL07-1 (from Florida) were produced and cultured on the same medium supplemented with appropriate antibiotics as described below. All strains and mutants of *X. albilineans* were stored at −80 °C as turbid water suspensions and retrieved before plantlet inoculation by plating on MW agar medium supplemented with appropriate antibiotics. *Escherichia coli* strains were grown on Luria–Bertani (LB) agar or in LB broth at 37 °C, and were used and stored according to standard protocols (Sambrook *et al*., 1989). Antibiotics were used at the following concentrations: ampicillin, 50 μg/mL; gentamicin, 3 μg/mL; kanamycin, 20 or 50 μg/mL for *X. albilineans* and *E. coli* strains, respectively.

Leaf attachment determined by leaf imprinting

Tissue-cultured plantlets of sugarcane cultivar CP68-1026, exhibiting two to four fully expanded leaves, were immersed for 30–40 s in a suspension of *X. albilineans* at 10^7 CFU/mL in sterile distilled water. After immersion, plantlets were placed in 200 mm × 20 mm test tubes containing an appropriate nutritive medium (Rott and Chagvardieff, 1984), and incubated in a growth chamber at 28 °C with 12 h of light. Two weeks after inoculation, the upper and lower surfaces of leaves were imprinted on Wilbrink selective Davis (WSD) medium (Davis *et al*., 1994). The plates were examined for the presence or absence of bacterial colonies of *X. albilineans* after 5 days of incubation at 28 °C.

The imprint area of the leaf was determined by trans-illumination using day or artificial light. Leaf attachment was estimated using a scale of 0–6: 0, 0–5 colonies in the leaf imprint; 1, 6–50 colonies in the leaf imprint; 2, more than 50 colonies and no confluent growth of bacteria in the leaf imprint; 3, confluent growth of bacteria in less than 10% of the leaf imprint; 4, confluent growth of bacteria in 10%–40% of the leaf imprint; 5, confluent growth of bacteria in 41%–80% of the leaf imprint; 6, confluent growth of bacteria in 81%–100% of the leaf imprint (Fig. 1). The area of confluent growth was estimated visually using a template with 10% leaf area increments. ELA was expressed as $ELA = 100[(1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5 + 6 \times N_6)/(6 \times N_T)]$, where N_i is the number of leaf imprints with score i and N_T is the total number of leaf imprints per plantlet (Fleites *et al*., 2013).

Plantlets were inoculated with all strains of *X. albilineans* listed in Table 1 and sterile distilled water as a control. In each experiment, six plantlets were inoculated per strain and randomly distributed in the growth chamber. Experiments were performed independently at least twice.

Leaf attachment determined by leaf washing and leaf homogenization

Tissue-cultured plantlets of sugarcane cultivar CP68-1026 exhibiting two to four fully expanded leaves were inoculated by immersing the foliage for 3 min in a water suspension of *X. albilineans* at 10^7 CFU/mL. After inoculation, plantlets were placed into the glass tubes used for growth and incubated in a growth chamber as described above. Plantlets were removed from the tubes for the determination of bacterial leaf adhesion after incubation for 1 h, 7 or 14 days. Two leaves were sampled per plantlet. A 7-cm-long fragment from each leaf was individually immersed in a 1.5-mL Eppendorf tube containing 1 mL of sterile distilled water with

0.005% Tween-20. Leaf fragments were washed by vigorously vortexing the tubes for 10 s. One hundred microlitres were used for dilution and four 20-μL drops of each undiluted, 1:10 and 1:100 dilutions of the washing water were deposited on WSD medium. The leaves were then homogenized in the water (820 μL) remaining in the washing tube using a pestle made for 1.5-mL microcentrifuge tubes. Four 20-μL drops of each undiluted, 1:10 and 1:100 dilutions of the homogenized tissue were deposited on WSD medium. For both methods, colonies of *X. albilineans* were counted after 6 days of growth, and bacterial populations were expressed as the number of CFU per leaf section.

Biofilm formation assay

To analyse the potential role of genes involved in leaf attachment on biofilm formation, we performed a colorimetric assay using crystal violet staining of the biofilm. *Xanthomonas albilineans* strain XaFL07-1 and mutants affected in leaf attachment (polysaccharide mutants and mutant Δ*rpfGCF* M15) were each grown in MW medium at 28 °C to mid-exponential growth phase (optical density at 600 nm of 0.3–0.4). Bacteria (300 μL at 10^9 CFU/mL) were then inoculated into 10-mL glass vials containing 3 mL of MW liquid medium supplemented with 5 g/L of yeast extract. Stationary cultures (five replications per strain) were incubated at 30 °C for 14 days. Then, liquid cultures were eliminated from the vials and the remaining biofilm adherent to the glass was stained with 3 mL of a 1% (w/v) crystal violet solution. After 15 min of incubation, crystal violet solution was discarded and vials were gently rinsed by the addition of 10 mL of distilled sterile water three times. Vials were examined visually for the presence of a crystal violet-stained ring which was indicative of biofilm formation on the walls of the vials. The crystal violet in each vial was then solubilized by adding 3 mL of ethanol (70%). The biofilm cell-associated dye was measured by absorption at 570 nm using a biophotometer after 1:10 dilution of the crystal violet solution.

In vitro growth of *X. albilineans*

The growth kinetics of *X. albilineans* XaFL07-1 and mutants of this strain were determined using 50-mL Corning® tubes (VWR, Fontenay-sous-Bois, France) containing 10 mL of either MW medium or minimal medium (MM). The latter medium contained sucrose (10 g), L-methionine (0.1 g), $K_2HPO_4.3H_2O$ (3 g), NaH_2PO_4 (1 g), NH_4Cl (1 g), $MgSO_4.7H_2O$ (0.3 g), distilled water (1 L), pH 6.8–7.0 (Birch and Patil, 1985). Four tubes per strain were inoculated with 100 μL of a bacterial suspension at 10^9 CFU/mL sterile distilled water and incubated at 28 °C on an orbital shaker at 150 rpm. Culture samples of 100 μL were taken from each tube every day for 6–8 days, and the optical density at 600 nm of the bacterial suspensions was measured using a biophotometer.

Complementation of *rmd* mutant M1116 of *X. albilineans*

Plasmid pALB204 harbouring gene *rmd* of *X. albilineans* was retrieved from the cosmid library of strain Xa23R1 (Rott *et al*., 1996). The DNA insert carried by this plasmid corresponds to genome positions 3 198 858 to 3 232 397 of the genome sequence of *X. albilineans* GPE PC73 that was fully sequenced (Pieretti *et al*., 2009). DNA transfer of pALB204 (or empty shuttle vector pUFR043) between *E. coli* donor and recipient wild-type strain or mutant M1116 of *X. albilineans* (*rmd*::Tn*5* derivative of strain XaFL07-1) was accomplished by triparental transconjugation with plasmid pRK2073 as the helper, as described by Rott *et al*. (1996).

Complementation of *rpf* mutant Δ*rpfGCF* M15

Plasmid pPR017Met harbouring wild-type genes *rpfGCF* was produced and transferred by electroporation into *rpf* mutant Δ*rpfGCF* M15, as described by Rott *et al*. (2013). Plasmid vector pUFR047 was used as a negative control.

Transmission electron microscopy

Bacteria grown for 2–3 days on Wilbrink's medium were fixed in a 4% glutaraldehyde, 0.1 M cacodylate buffer (pH 7.2), post-fixed in 1% osmium tetroxide, dehydrated using a series of acetone washes and embedded in TAAB 812 epon resin. Ultrathin sections (60 nm) were mounted on collodion carbon-coated copper grids, contrasted using uranyl acetate and lead citrate, and examined at 80 kV with a transmission electron microscope (Jeol 100CX II, Tokyo, Japan).

Statistical methods

Analyses of variance and statistical comparisons of biofilm and epiphytic survival means were performed using the statistical software package R, version 2.14.1 (R Development Core Team, 2011). Multiple comparisons of means were carried out using Tukey's honest significant difference method (Miller, 1981) employing the function TukeyHSD of the standard R package stats.

ACKNOWLEDGEMENTS

We thank Marie-Josée Darroussat for excellent technical assistance and Monique Royer for critical review of the manuscript. Imène Mensi was supported by a PhD fellowship from Cirad.

REFERENCES

Akhavan, A., Bahar, M., Askarian, H., Lak, M.R., Nazemi, A. and Zamani, Z. (2013) Bean common bacterial blight: pathogen epiphytic life and effect of irrigation practices. *Springerplus*, **2**, 41.

Autrey, L.J.C., Saumtally, S., Dookun, A., Sullivan, S. and Dhayan, S. (1995) Aerial transmission of the leaf scald pathogen, *Xanthomonas albilineans*. *Proc. Int. Soc. Sugar Cane Technol.* **21**, 508–526.

Beattie, G.A. and Lindow, S.E. (1999) Bacterial colonization of leaves: a spectrum of strategies. *Phytopathology*, **89**, 353–359.

Birch, R.G. (2001) *Xanthomonas albilineans* and the antipathogenesis approach to disease control. *Mol. Plant Pathol.* **2**, 1–11.

Birch, R.G. and Patil, S.S. (1985) Preliminary characterization of an antibiotic produced by *Xanthomonas albilineans* which inhibits DNA synthesis in *Escherichia coli*. *J. Gen. Microbiol.* **131**, 1069–1075.

Blanch, M., Legaz, M.-E. and Vicente, C. (2008) Xanthan production by *Xanthomonas albilineans* infecting sugarcane stalks. *J. Plant Physiol.* **165**, 366–374.

Bogino, P.C., de las Mercedes Oliva, M., Sorroche, F.G. and Giordano, W. (2013) The role of bacterial biofilms and surface components in plant–bacterial associations. *Int. J. Mol. Sci.* **14**, 15 838–15 859.

Champoiseau, P., Rott, P. and Daugrois, J.H. (2009) Epiphytic populations of *Xanthomonas albilineans* and subsequent sugarcane stalk infection are linked to rainfall in Guadeloupe. *Plant Dis.* **93**, 339–346.

Chang, W.S., van de Mortel, M., Nielsen, L., Nino de Guzman, G., Li, X. and Halverson, L.J. (2007) Alginate production by *Pseudomonas putida* creates a hydrated microenvironment and contributes to biofilm architecture and stress tolerance under water-limiting conditions. *J. Bacteriol.* **189**, 8290–8299.

Comstock, J.C. (2001) Foliar symptoms of sugarcane leaf scald. *Sugar J.* **64**, 23–32.

Danhorn, T. and Fuqua, C. (2007) Biofilm formation by plant-associated bacteria. *Annu. Rev. Microbiol.* **61**, 401–422.

Das, P., Mukherjee, S. and Sen, R. (2008) Genetic regulations of the biosynthesis of microbial surfactants: an overview. *Biotechnol. Genet. Eng. Rev.* **25**, 165–186.

Daugrois, J.-H., Dumont, V., Champoiseau, P., Costet, L., Boisne-Noc, R. and Rott, P. (2003) Aerial contamination of sugarcane in Guadeloupe by two strains of *Xanthomonas albilineans*. *Eur. J. Plant Pathol.* **109**, 445–458.

Daugrois, J.-H., Boisne-Noc, R., Champoiseau, P. and Rott, P. (2012) The revisited infection cycle of *Xanthomonas albilineans*, the causal agent of leaf scald of sugarcane. *Funct. Plant Sci. Biotechnol.* **6** (Special issue 2), 91–97.

Davis, M.J., Rott, P., Baudin, P. and Dean, J.L. (1994) Evaluation of selective media and immunoassays for detection of *Xanthomonas albilineans*, causal agent of sugarcane leaf scald disease. *Plant Dis.* **78**, 78–82.

Davis, M.J., Rott, P., Warmuth, C.J., Chatenet, M. and Baudin, P. (1997) Intraspecific genomic variation within *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. *Phytopathology*, **87**, 316–324.

De Bruijn, I., de Kock, M.J.D., Yang, M., de Waard, P., van Beek, T.A. and Raaijmakers, J.M. (2007) Genome-based discovery, structure prediction and functional analysis of cyclic lipopeptide antibiotics in *Pseudomonas* species. *Mol. Microbiol.* **63**, 417–428.

De Feyter, R. and Gabriel, D.W. (1991) Use of cloned DNA methylase genes to increase the frequency of transfer of foreign genes into *Xanthomonas campestris* pv. *malvacearum*. *J. Bacteriol.* **173**, 6421–6427.

De Feyter, R., Yang, Y. and Gabriel, D.W. (1993) Gene-for-gene interactions between cotton *R* genes and *Xanthomonas campestris* pv. *malvacearum avr* genes. *Mol. Plant–Microbe Interact.* **6**, 225–237.

Desaki, Y., Miya, A., Venkatesh, B., Tsuyumu, S., Yamane, H., Kaku, H., Minami, E. and Shibuya, N. (2006) Bacterial lipopolysaccharides induce defense responses associated with programmed cell death in rice cells. *Plant Cell Physiol.* **47**, 1530–1540.

Dow, J.M., Crossman, L., Findlay, K., He, Y.-Q., Feng, J.-X. and Tang, J.-L. (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell–cell signalling and is required for full virulence to plants. *Proc. Natl. Acad. Sci. USA*, **100**, 10 995–11 000.

Dow, M. (2008) Diversification of the function of cell-to-cell signalling in regulation of virulence within plant pathogenic xanthomonads. *Sci. Signal.* **1**, pe23.

Dow, M., Newman, M.A. and von Roepenack, E. (2000) The induction and modulation of plant defense responses by bacterial lipopolysaccharides. *Annu. Rev. Phytopathol.* **38**, 241–261.

Dunger, G., Relling, V.M., Tondo, M.L., Barreras, M., Ielpi, L., Orellano, E.G. and Ottado, J. (2007) Xanthan is not essential for pathogenicity in citrus canker but contributes to *Xanthomonas* epiphytic survival. *Arch. Microbiol.* **188**, 127–135.

Fleites, L.A., Mensi, I., Gargani, D., Zhang, S., Rott, P. and Gabriel, D.W. (2013) *Xanthomonas albilineans* OmpA1 appears to be functionally modular and both the OMC and C-like domains are necessary for leaf scald disease of sugarcane. *Mol. Plant–Microbe Interact.* **26**, 1200–1210.

Gudesblat, E.G., Pablo, S.T. and Adrián, A.V. (2009) *Xanthomonas campestris* overcomes *Arabidopsis* stomatal innate immunity through a DSF cell-to-cell signal-regulated virulence factor. *Plant Physiol.* **149**, 1017–1027.

Gumus, T., Demirci, A.S., Mirik, M., Arici, M. and Aysan, Y. (2010) Xanthan gum production of *Xanthomonas* spp. isolated from different plants. *Food Sci. Biotechnol.* **19**, 201–203.

Guo, Y., Sagaram, U.S., Kim, J. and Wang, N. (2010) Requirement of the *galU* gene for polysaccharide production by and pathogenicity and growth *in planta* of *Xanthomonas citri* subsp. *citri*. *Appl. Environ. Microbiol.* **76**, 2234–2242.

Guo, Y., Zhang, Y., Li, J.-L. and Wang, N. (2012) Diffusible signal factor-mediated quorum sensing plays a central role in coordinating gene expression of *Xanthomonas citri* subsp. *citri*. *Mol. Plant–Microbe Interact.* **25**, 165–179.

He, Y.-W., Xu, M., Lin, K., Ng, Y.-J.A., Wen, C.-M., Wang, L.-H., Liu, Z.-D., Zhang, H.-B., Dong, Y.-H., Dow, J.M. and Zhang, L.-H. (2006) Genome scale analysis of diffusible signal factor regulon in *Xanthomonas campestris* pv. *campestris*: identification of novel cell–cell communication-dependent genes and functions. *Mol. Microbiol.* **59**, 610–622.

Hirano, S.S. and Upper, C.D. (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*—a pathogen, ice nucleus, and epiphyte. *Microbiol. Mol. Biol. Rev.* **64**, 624–653.

Huang, G.Z., Zhang, L.H. and Birch, R.G. (2000) Albicidin antibiotic and phytotoxin biosynthesis in *Xanthomonas albilineans* requires a phosphopantetheinyl transferase gene. *Gene*, **258**, 193–199.

Hugouvieux, V., Barber, C.E. and Daniels, M.J. (1998) Entry of *Xanthomonas campestris* pv. *campestris* into hydathodes of *Arabidopsis thaliana* leaves: a system for studying early infection events in bacterial pathogenesis. *Mol. Plant–Microbe Interact.* **11**, 537–543.

Jacques, M., Josi, K., Darrasse, A. and Samson, R. (2005) *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* is aggregated in stable biofilm population sizes in the phyllosphere of field-grown beans. *Appl. Environ. Microbiol.* **71**, 2008–2015.

Kadouri, D., Jurkevitch, E. and Okon, Y. (2003) Involvement of the reserve material poly-β-hydroxybutyrate in *Azospirillum brasilense* stress endurance and root colonization. *Appl. Environ. Microbiol.* **69**, 3244–3250.

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

Kingsley, M.T., Gabriel, D.W., Marlow, G.C. and Roberts, P.D. (1993) The opsX locus of *Xanthomonas campestris* affects host range and biosynthesis of lipopolysaccharide and extracellular polysaccharide. *J. Bacteriol.* **175**, 5839–5850.

Lau, P.C.Y., Lindhout, T., Beveridge, T.J., Dutcher, J.R. and Lam, J.S. (2009) Differential lipopolysaccharide core capping leads to quantitative and correlated modifications of mechanical and structural properties in *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* **191**, 6618–6631.

Leong, S.A., Ditta, G.S. and Helinski, D.R. (1982) Heme biosynthesis in *Rhizobium*: identification of a cloned gene coding for aminolevulinic acid synthetase from *Rhizobium meliloti*. *J. Biol. Chem.* **257**, 8724–8730.

Li, J. and Wang, N. (2011a) Genome-wide mutagenesis of *Xanthomonas axonopodis* pv. *citri* reveals novel genetic determinants and regulation mechanisms of biofilm formation. *PLoS ONE*, **6**, e21804.

Li, J. and Wang, N. (2011b) The *wxacO* gene of *Xanthomonas citri* ssp. *citri* encodes a protein with a role in lipopolysaccharide biosynthesis, biofilm formation, stress tolerance and virulence. *Mol. Plant Pathol.* **12**, 381–396.

Li, J. and Wang, N. (2012) The *gpsX gene* encoding a glycosyltransferase is important for polysaccharide production and required for full virulence in *Xanthomonas citri* subsp. *citri*. *BMC Microbiol.* **12**, 381.

Lindow, S.E. and Brandl, M.T. (2003) Microbiology of the phyllosphere. *Appl. Environ. Microbiol.* **69**, 1875–1883.

Lu, H., Patil, P., Van Sluys, M.-A., White, F.F., Ryan, R.P., Dow, J.M., Rabinowicz, P., Salzberg, S.L., Leach, J.E., Sonti, R., Brendel, V. and Bogdanove, A.J. (2008) Acquisition and evolution of plant pathogenesis-associated gene clusters and candidate determinants of tissue-specificity in *Xanthomonas*. *PLoS ONE*, **3**, e3828.

Miller, R.G. (1981) *Simultaneous Statistical Inference*, 2nd edn. New York: Springer-Verlag.

Monier, J.M. and Lindow, S.E. (2003) Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces. *Proc. Natl. Acad. Sci. USA*, **100**, 15 977–15 982.

Monier, J.M. and Lindow, S.E. (2004) Frequency, size, and localization of bacterial aggregates on bean leaf surfaces. *Appl. Environ. Microbiol.* **70**, 346–355.

Nakao, R., Ramstedt, M., Wai, S.N. and Uhlin, B.E. (2012) Enhanced biofilm formation by *Escherichia coli* LPS mutants defective in Hep biosynthesis. *PLoS ONE*, **7**, e51241.

Newman, M.A., Dow, J.M. and Daniels, M.J. (2001) Bacterial lipopolysaccharides and plant–pathogen interactions. *Eur. J. Plant Pathol.* **107**, 95–102.

Pieretti, I., Royer, M., Barbe, V., Carrere, S., Koebnik, R., Cociancich, S., Couloux, A., Darrasse, A., Gouzy, J., Jacques, M.A., Lauber, E., Manceau, C., Mangenot, S., Poussier, S., Segurens, B., Szurek, B., Verdier, V., Arlat, M. and Rott, P. (2009) The complete genome sequence of *Xanthomonas albilineans* provides new insights into the reductive genome evolution of the xylem-limited *Xanthomonadaceae*. *BMC Genomics*, **10**, 616.

Pieretti, I., Royer, M., Barbe, V., Carrere, S., Koebnik, R., Couloux, A., Darrasse, A., Gouzy, J., Jacques, M.A., Lauber, E., Manceau, C., Mangenot, S., Poussier, S., Segurens, B., Szurek, B., Verdier, V., Arlat, M., Gabriel, D.W., Rott, P. and Cociancich, S. (2012) Genomic insights into strategies used by *Xanthomonas*

albilineans with its reduced artillery to spread within sugarcane xylem vessels. *BMC Genomics*, **13**, 658.

Poplawsky, A.R. and Chun, W. (1998) *Xanthomonas campestris* pv. *campestris* requires a functional *pigB* for epiphytic survival and host infection. *Mol. Plant–Microbe Interact.* **11**, 466–475.

Poplawsky, A.R., Chun, W., Slater, H., Daniels, M.J. and Dow, J.M. (1998) Synthesis of extracellular enzymes, and xanthomonadin in *Xanthomonas campestris*: evidence for the involvement of two intercellular regulatory signals. *Mol. Plant–Microbe Interact.* **11**, 68–70.

Quinones, B., Pujol, C.J. and Lindow, S.E. (2004) Regulation of AHL production and its contribution to epiphytic fitness in *Pseudomonas syringae*. *Mol. Plant–Microbe Interact.* **17**, 521–531.

Quinones, B., Dulla, G. and Lindow, S.E. (2005) Quorum sensing regulates exopolysaccharide production, motility, and virulence in *Pseudomonas syringae*. *Mol. Plant–Microbe Interact.* **18**, 682–693.

R Development Core Team (2011) *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rigano, L.A., Siciliano, F., Enrique, R., Sendín, L., Filippone, P., Torres, P.S., Qüesta, J., Dow, J.M., Castagnaro, A.P., Vojnov, A.A. and Marano, M.R. (2007) Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv. *citri*. *Mol. Plant–Microbe Interact.* **20**, 1222–1230.

Rott, P. and Chagvardieff, P. (1984) Inoculation de la canne à sucre (*Saccharum sp.*) cultivée *in vitro* avec *Xanthomonas albilineans* (Ashby) Dowson. *L'Agron. Trop.* **39**, 89–92.

Rott, P. and Davis, M.J. (2000) Leaf scald. In: *A Guide to Sugarcane Diseases* (Rott, P., Bailey, R.A., Comstock, J.C., Croft, B.J. and Saumtally, A.S., eds), pp. 38–44. Montpellier: La Librairie du Cirad.

Rott, P., Costet, L., Davis, M.J., Frutos, R. and Gabriel, D.W. (1996) At least two separate gene clusters are involved in albicidin production by *Xanthomonas albilineans*. *J. Bacteriol.* **178**, 4590–4596.

Rott, P., Fleites, L., Marlow, G., Royer, M. and Gabriel, D.W. (2011) Identification of new candidate pathogenicity factors in the xylem-invading pathogen *Xanthomonas albilineans* by transposon mutagenesis. *Mol. Plant–Microbe Interact.* **24**, 594–605.

Rott, P., Fleites, L., Mensi, I., Sheppard, L., Daugrois, J.-H., Dow, J.M. and Gabriel, D.W. (2013) The RpfCG two-component system negatively regulates the colonization of sugarcane stalks by *Xanthomonas albilineans*. *Microbiology*, **159**, 1149–1159.

Royer, M., Costet, L., Vivien, E., Bes, M., Cousin, A., Pieretti, I., Savin, A., Megessier, S., Viard, M., Frutos, R., Gabriel, D.W. and Rott, P.C. (2004) Albicidin pathotoxin produced by *Xanthomonas albilineans* is encoded by three large PKS and NRPS genes present in a gene cluster containing also several putative modifying, regulatory and resistance genes. *Mol. Plant–Microbe Interact.* **17**, 414–427.

Royer, M., Koebnik, R., Marguerettaz, M., Barbe, V., Robin, G.P., Brin, C., Carrere, S., Gomez, C., Hügelland, M., Völler, G.H., Noëll, J., Pieretti, I., Rausch, S., Verdier, V., Poussier, S., Rott, P., Süssmuth, R. and Cociancich, S. (2013) Genome mining reveals the genus *Xanthomonas* to be a promising reservoir for new bioactive non-ribosomally synthesized peptides. *BMC Genomics*, **14**, 658.

Ryan, R.P. and Dow, J.M. (2011) Communication with a growing family: diffusible signal factor (DSF) signaling in bacteria. *Trends Microbiol.* **19**, 145–152.

Sambrook, J.C., Fritsch, E. and Manihatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sordi, R.A. and Tokeshi, H. (1986) Presence of *Xanthomonas albilineans* in guttation droplets of sugarcane and sweet corn leaves showing leaf scald disease symptoms. *Soc. Téc. Açucar. Brasil* **4**, 60–63.

Torres, P.S., Malamud, F., Rigano, L.A., Russo, D.M., Marano, M.R., Castagnaro, A.P., Zorreguieta, A., Bouarab, K., Dow, J.M. and Vojnov, A.A. (2007) Controlled synthesis of the DSF cell–cell signal is required for biofilm formation and virulence in *Xanthomonas campestris*. *Environ. Microbiol.* **9**, 2101–2109.

Vorholt, J.A. (2012) Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* **10**, 828–840.

Vorhölter, F.-J., Schneiker, S., Goesmann, A., Krause, L., Bekel, T., Kaiser, O., Linke, B., Patschkowski, T., Rückert, C., Schmid, J., Sidhu, V.K., Sieber, V., Tauch, A., Watt, S.A., Weisshaar, B., Becker, A., Niehaus, K. and Pühler, A. (2008) The genome of *Xanthomonas campestris* pv. *campestris* B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. *J. Biotechnol.* **134**, 33–45.

Wang, J. and Yu, H.-Q. (2007) Biosynthesis of polyhydroxybutyrate (PHB) and extracellular polymeric substances (EPS) by *Ralstonia eutropha* ATCC 17699 in batch cultures. *Appl. Microbiol. Biotechnol.* **75**, 871–878.

Wilson, M., Hirano, S.S. and Lindow, S.E. (1999) Location and survival of leaf-associated bacteria in relation to pathogenicity and potential for growth within the leaf. *Appl. Environ. Microbiol.* **65**, 1435–1443.

Yethon, J.A., Gunn, J.S., Ernst, R.K., Miller, S.I., Laroche, L., Malo, D. and Whitfield, C. (2000) *Salmonella enterica* serovar Typhimurium *waaP* mutants show increased susceptibility to polymyxin and loss of virulence *in vivo*. *Infect. Immun.* **68**, 4485–4491.