Identification of New Candidate Pathogenicity Factors in the Xylem-Invading Pathogen *Xanthomonas albilineans* by Transposon Mutagenesis

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Submitted 9 July 2010. Accepted 16 December 2010.

Xanthomonas albilineans is a xylem-invading pathogen that produces the toxin albicidin that blocks chloroplast differentiation, resulting in disease symptoms of sugarcane leaf scald. In contrast to other xanthomonads, *X. albilineans* does not possess a hypersensitive response and pathogenicity type III secretion system and does not produce xanthan gum. Albicidin is the only previously known pathogenicity factor in *X. albilineans*, yet albicidin-deficient mutant strains are still able to efficiently colonize sugarcane. To identify additional host adaptation or pathogenicity factors, sugarcane ‘CP80-1743’ was inoculated with 1,216 independently derived Tn5 insertions in *X. albilineans* XaFL07-1 from Florida. Sixty-one Tn5 mutants were affected in development of leaf symptoms or in stalk colonization. The Tn5 insertion sites of these mutants were determined and the interrupted genes were identified using the recently available genomic DNA sequence of *X. albilineans* GPE PC73 from Guadeloupe. Several pathogenicity-related loci that were not previously reported in *Xanthomonas* spp. were identified, including loci encoding hypothetical proteins, a membrane fusion protein conferring resistance to novobiocin, transport proteins, TonB-dependent outer-membrane transporters, and an OmpA family outer-membrane protein.

Xanthomonas albilineans is a gram-negative bacterium that causes leaf scald, a lethal disease of sugarcane (Rott and Davis 2000). This pathogen multiplies in the xylem and systemically colonizes the entire host plant (Champoiseau et al. 2006b; Klett and Rott 1994). It is transmitted by infected cuttings, contaminated harvesting tools, and aerial means. Disease symptoms vary from a single, white, narrow, sharply defined leaf stripe to complete wilting and necrosis of infected leaves, leading to plant death. Sugarcane leaves may also emerge with extensive white chlorosis from which the pathogen is sometimes absent. However, even if leaf scald symptoms result from changes in the chlorenchyma cells, *X. albilineans* is constrained within the xylem (Birch 2001).

Most plant-pathogenic bacteria possess a hypersensitive response and pathogenicity (Hrp) type III secretion system (T3SS) to deliver effector proteins or virulence factors from the bacterial cytoplasm into the host cell. The complete genome sequence of *X. albilineans* revealed that, in contrast to other pathogenic xanthomonads (Büttner and Bonas 2010), *X. albilineans* does not have an Hrp T3SS to cause disease, although it does possess an

animal pathogen-associated T3SS of the SPI-1 injectisome family (Pieretti et al. 2009). However, the role of this SPI-1 T3SS is unknown, and mutants in this secretion system are still able to efficiently colonize the sugarcane stalk and to cause leaf symptoms (Marguerettaz et al. 2011). Additionally, *X. albilineans* experienced a reductive genome evolution and is phylogenetically close to *Xylella fastidiosa*, another xylem-invading and xylem-limited pathogen lacking an Hrp T3SS (Pieretti et al. 2009). However, both species possess other secretion systems that could deliver virulence determinants into the plant cell or to the bacterial environment. For example, *Xylella fastidiosa* produces enzymes that degrade the pit membranes, allowing bacteria to move into previously uncolonized xylem vessels, most likely thanks to a type II secretion system (Perez-Donoso et al. 2010; Vojnov et al. 2010). Other virulence factors of *Xylella fastidiosa* and xanthomonads include motility, cell attachment by cell-surface adhesins, and formation of biofilms. Biofilms are structures that allow the bacteria to attach to a surface, and genes involved in the synthesis of pili play an important role in this adhesion process (Li et al. 2007; Vojnov et al. 2010).

In 1985, Birch and Patil first showed that a potent phytotoxin and antibiotic, called albicidin, was produced by *X. albilineans* (Birch and Patil 1985a and b). Albicidin causes foliar symptoms of leaf scald in sugarcane by inhibiting the replication of proplastic DNA and, consequently, blocking the differentiation of chloroplasts (Birch and Patil 1987a and b). On a molecular level, this toxin is a potent DNA gyrase inhibitor (Hashimi et al. 2007). Albicidin is the only previously known pathogenicity factor in *X. albilineans*, yet albicidin-deficient mutant strains are still able to efficiently colonize sugarcane (Birch 2001; Champoiseau et al. 2006a).

Variation in pathogenicity among strains of *X. albilineans* is known to occur, and published data support the possible existence of different races in Mauritius (Autrey et al. 1995). The outbreak of leaf scald in Florida in the late 1980s was closely associated with the appearance of a genetically new strain of the pathogen (Davis et al. 1997). The capacity of *X. albilineans* to colonize sugarcane stalks or cause symptoms varies according to the strain of the pathogen, indicating the existence of different pathotypes within the species (Champoiseau et al. 2006a; Daugrois et al. 2003; Huerta-Lara et al. 2009; Mohamed et al. 1996). High variation in pathogenicity was shown to exist in closely related strains of *X. albilineans* in Guadeloupe (Champoiseau et al. 2006b). Albicidin production in vitro varies also according to the strain of the pathogen. However, no relationship was found between the amount of albicidin produced in vitro and the pathotypes or genetic diversity of the pathogen (Renier et al. 2007). Therefore, albicidin is necessary but not sufficient

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for leaf scald development, and it appears to be one virulence factor acting coordinately with other virulence factors.

In this study, we used transposome mutagenesis to produce mutants of *X. albilineans* affected in capacity to produce foliar symptoms or to multiply and spread in the sugarcane stalk. We report the identification of new candidate pathogenicity factors in this pathogen, including some that were not previously reported in the genus *Xanthomonas*. Additionally, in contrast to previously reported data, we show that albicidin is not always necessary for expression of leaf scald symptoms caused by *X. albilineans*.

RESULTS

Transposon mutagenesis and screening of mutants of *X. albilineans* for reduced pathogenicity.

Following electroporation of the transposase-Tn5 DNA synaptic complexes into *X. albilineans* XaFL07-1, several thousand kanamycin-resistant colonies were obtained. The transposition efficiency was approximately 1.5 to 3×10^4 Tn5 mutants per 20 ng of transposome. Sequence analysis of the DNA regions flanking the insertion sites in a first set of 10 mutants indicated that the transposon inserted randomly in the *X. albilineans* genome and, therefore, Tn5 mutants could be screened for pathogenicity. Sugarcane 'CP80-1743' was inoculated with a total of 1,216 mutants, and approximately 200 mutants were screened in each single inoculation experiment. Leaf scald symptoms were recorded on three to four emerging leaves 1 month after inoculation, and stalk colonization by the pathogen was determined 2 months after inoculation. Mutants that did not produce disease symptoms or did not efficiently colonize the sugarcane stalk were selected for a second round of plant inoculation (three to five sugarcane plants inoculated per mutant). Mutants that again caused significantly less leaf symptoms or showed reduced colonization of the sugarcane stalk compared with the wild-type strain XaFL07-1 were used to determine the insertion site of the transposon in the chromosome of *X. albilineans*.

Genomic distribution and predicted products of transposon-tagged pathogenicity-related genes.

Seventeen pathogenicity-reduced mutants carried Tn5 insertions in genes involved in the ribosome machinery (including *rpsA*, *rRNA-5S*, *rRNA-16S*, and *rRNA-23S*), and these mutants were not further investigated. Forty-four additional mutants carried insertions in 33 loci whose interruption resulted in reduced pathogenicity (Table 1). Single Tn5 insertions were obtained for 28 of these loci. Multiple independent insertions were obtained for five loci, including XALc_0007 (two mutants), XALc_0557 (five mutants), XALc_0624 or gene *fabH* (three mutants), XALc_2682 (two mutants), and XALc_2705 (four mutants) (Table 1). Interestingly, some pathogenicity mutants affected contiguous loci that were previously not known to be related in terms of pathogenicity function, including XALc_0885 and XALc_0886; and XALc_2704, XALc_2705, XALc_2706 (*gmd*), and XALc_2707 (*rmd*).

Pathogenicity-related loci identified in the genome of *X. albilineans* were predicted to encode proteins involved in a variety of functions, including exopolysaccharide (EPS) and lipopolysaccharide (LPS) biosynthesis (six loci), fatty acid biosynthesis (two loci), amino acid biosynthesis (one locus), purine biosynthesis (one locus), carbohydrate metabolism (one locus), nitrogen metabolism (one locus), regulatory and cell signaling (two loci), secretion systems (one locus), transport (five loci), catalytic activity (one locus), DNA binding (two loci), and drug resistance (one locus) (Table 1). In addition, nine loci were predicted to encode proteins of unknown function (Table 1).

The texture and consistency of bacterial growth on modified Wilbrink's (MW) agar medium of all mutants and the wild-type strain of *X. albilineans* were similarly slightly viscous, with the exception of mutants affected in EPS and LPS biosynthesis. The texture of colonies of these latter mutants in loci XALc_2693 (*xanB*), XALc_2697 (*rmlB*), XALc_2701, XALc_2705, XALc_2706 (*gmd*), and XALc_2707 (*rmd*) was dry and easily identified when bacteria were subcultured with a transfer loop, functionally confirming the predicted involvement of these loci in production of surface polysaccharides.

Mutant M564 affected locus XALc_1762 (predicted to encode a membrane fusion protein) and failed to grow on MW agar medium supplemented with cephalixin at 25 mg/liter, novobiocin at 30 mg/liter, cycloheximide at 50 mg/liter, and benomyl at 12.5 mg/liter (WCNCB selective medium) used to isolate *X. albilineans* from plants. After further investigation, it was shown that this mutant was unable to grow on MW medium supplemented with novobiocin at 5 mg/liter whereas the wild-type strain was able to grow on MW medium supplemented with novobiocin at up to 60 mg/liter.

Pathogenicity-related loci encoding hypothetical proteins with unknown functions were scattered across the chromosome of *X. albilineans*. XALc_0007 is located near the start of chromosome replication and upstream of three biopolymer transport proteins encoded by *exbB*, *exbD1*, and *exbD2*. Orthologs of XALc_0007 exist in all bacterial species belonging to the *Xanthomonadaceae* family.

XALc_0639 is located downstream of *prpB*, encoding a methylisocitrate lyase, and upstream of *prpC*, encoding a 2-methylcitrate synthase, suggesting that XALc_0639 is involved in citrate metabolism. Interestingly, this locus has only been reported previously in the genome of *X. axonopodis* pv. *vasculorum* (the causal agent of gumming of sugarcane) and *X. vasicola* pv. *musacearum* (the causal agent of banana bacterial wilt), two xylem-invading pathogens.

XALc_0742 is located upstream of genes *waaG* and *waaL* and in the same gene cluster as *waaM* and *waaA*, which are all involved in LPS biosynthesis. Therefore, XALc_0742 may also be involved in LPS production. Orthologous genes of XALc_0742 exist in all bacterial species belonging to the *Xanthomonadaceae* family. Mutations in XALc_0742 are expected to have a polar effect on downstream genes because it is the first open reading frame (ORF) of the gene operon.

Genes surrounding XALc_0885, XALc_0886, XALc_2347, and XALc_2682 did not give any relevant clues regarding a possible function for these loci. However, no orthologs of XALc_0885 were found in any other bacterial species and only one was found for XALc_0886 in the genome of *X. axonopodis* pv. *vasculorum*, suggesting that these two contiguous loci are specific for interactions with sugarcane. Orthologs of XALc_2347 exist in all sequenced xanthomonads and in *Stenotrophomonas* spp. but not in *Xylella fastidiosa*, which is missing the entire XALc_2343 to XALc_2348 operon. Orthologs of XALc_2682 exist in all bacterial species belonging to the *Xanthomonadaceae* family.

XALc_2704 is located in a gene region involved in biosynthesis of cell surface polysaccharides, including XALc_2701, XALc_2702, XALc_2705, and XALc_2706. Orthologs of XALc_2705 and XALc_2706 in *X. campestris* pv. *campestris* (XC3630 and XC3627, respectively) were reported as pathogenicity-related genes (Qian et al. 2005) (Table 1). XALc_2704 might encode a protein with a similar function, although the mutant in this ORF (M694) did not show any obvious difference in texture and consistency compared with the wild-type strain. Interestingly, this locus was not found in any other bacterial species. XALc_2704 is the last ORF of the operon and

Table 1. Transposon mutants of *Xanthomonas albilineans* affected in pathogenicity

Mutant	Locus ^w	PR gene ^x	Functional category ^y	Predicted product ^z	Gene	Pathogenicity ^y	
						DS	ESC
M655	XALc_0007	...	Function unknown	Conserved hypothetical protein	...	++	++
M1133	XALc_0007	...	Function unknown	Conserved hypothetical protein	...	–	+
M516	XALc_0028	...	Transport	ABC transporter permease	...	–	–
M791	XALc_0297 (1)	...	Regulatory and cell signaling	Two component system sensor response regulator hybrid protein	...	+++	++
M227	XALc_0557	...	Function unknown	OmpA-family protein	...	–	+
M468	XALc_0557	...	Function unknown	OmpA-family protein	...	–	–
M573	XALc_0557	...	Function unknown	OmpA-family protein	...	–	–
M768	XALc_0557	...	Function unknown	OmpA-family protein	...	–	+++
M1152	XALc_0557	...	Function unknown	OmpA-family protein	...	–	–
M352	XALc_0567	...	Purine biosynthesis	Amidophosphoribosyltransferase	<i>purF</i>	–	–
M510	Intergenic region upstream of XALc_0590	...	DNA binding	DNA-binding protein HU (histone-like protein HU)	<i>hupB</i>	+	++
M73	XALc_0624	XOO0878 (Xoo), XOCORF_1172 (Xoc)	Fatty acid biosynthesis	3-oxoacyl-(acyl-carrier-protein) synthase 3	<i>fabH</i>	–	+++
M550	XALc_0624	XOO0878 (Xoo), XOCORF_1172 (Xoc)	Fatty acid biosynthesis	3-oxoacyl-(acyl-carrier-protein) synthase 3	<i>fabH</i>	+	++
M770	XALc_0624	XOO0878 (Xoo), XOCORF_1172 (Xoc)	Fatty acid biosynthesis	3-oxoacyl-(acyl-carrier-protein) synthase 3	<i>fabH</i>	+	+++
M74	XALc_0639 (2)	...	Function unknown	Hypothetical protein	...	++	+++
M75	XALc_0643 (1)	...	Transport	TonB-dependent outer-membrane transporter	...	+	+++
M697	XALc_0723 (1)	...	Transport	TonB-dependent outer-membrane transporter	...	+	+
M1019	XALc_0739	...	Carbohydrate metabolism	NADP dependent malic enzyme	<i>maeB</i>	+	+
M1127	XALc_0742	...	Function unknown	Hypothetical protein	...	+	+
M676	XALc_0885 (4)	...	Function unknown	Hypothetical protein	...	++	+++
M79	XALc_0886 (3)	...	Function unknown	Transmembrane hypothetical protein	...	+	++
M1162	XALc_0911	...	Fatty acid biosynthesis	Acyl-CoA thioesterase II	<i>tesB</i>	+++	+++
M375	XALc_1042	...	DNA binding	Single-stranded DNA-binding protein	<i>ssb</i>	–	+
M39	XALc_1068	...	Catalytic activity	Dipeptidyl peptidase precursor	...	++	++
M1009	XALc_1434 (1)	...	Regulatory and cell signaling	Two component system sensor response regulator hybrid protein	...	+++	++
M564	XALc_1762	...	Drug resistance	Membrane fusion protein (confers resistance to novobiocin)	...	++	+++
M388	XALc_2347 (1)	...	Function unknown	Hypothetical protein	...	+	+
M139	XALc_2426	...	Transport	Biopolymer transport TolQ protein	<i>tolQ</i>	–	++++
M326	XALc_2656	XC3565 (Xcc)	Secretion systems	General secretion pathway protein M	<i>xpsM</i>	–	+
M624	XALc_2682	...	Function unknown	Conserved hypothetical protein with conserved region DUF1212 (putative membrane protein)	...	–	+++
M1190	XALc_2682	...	Function unknown	Conserved hypothetical protein with conserved region DUF1212 (putative membrane protein)	...	++++	+++
M427	XALc_2693	XOO0796 (Xoo)	EPS and LPS biosynthesis	Mannose-6-phosphate isomerase	<i>xanB</i>	–	–
M51	XALc_2697	...	EPS and LPS biosynthesis	dTDP-glucose 4,6-dehydratase	<i>rmlB</i>	+	+++
M820	XALc_2701 (4)	...	EPS and LPS biosynthesis	Glycosyl transferase	...	+++	++
M694	XALc_2704 (4)	...	Function unknown	Conserved hypothetical protein	...	++	++
M374	XALc_2705 (5)	XC3630 (Xcc)	EPS and LPS biosynthesis	Glycosyl transferase	...	–	–
M416	XALc_2705 (5)	XC3630 (Xcc)	EPS and LPS biosynthesis	Glycosyl transferase	...	+	+
M631	XALc_2705 (5)	XC3630 (Xcc)	EPS and LPS biosynthesis	Glycosyl transferase	...	+	+
M903	XALc_2705 (5)	XC3630 (Xcc)	EPS and LPS biosynthesis	Glycosyl transferase	...	+	+
M967	XALc_2706 (5)	XC3627 (Xcc)	EPS and LPS biosynthesis	GDP-mannose 4,6-dehydratase	<i>gmd</i>	++	+++
M1116	XALc_2707 (5)	...	EPS and LPS biosynthesis	NDP-hexose oxidoreductase	<i>rmd</i>	–	–
M67	XALc_2843	XOO3860 (Xoo)	Amino acid biosynthesis	Glycine/serine hydroxymethyltransferase	<i>glyA</i>	+	++++
M61	XALc_2974 (1,5)	...	Transport	Magnesium and cobalt transport protein	<i>corA</i>	++	++
M9	XALc_3092	...	Nitrogen metabolism	Nitrogen regulatory protein P-II	<i>glnB1</i>	+++	+++

^y Pathogenicity in sugarcane is rated as disease severity (DS) and extent of stalk colonization (ESC) compared with wild-type disease severity and stalk colonization, respectively. Symbols: – = 0 to 10%, + = 11 to 30%; ++ = 31 to 50%, +++ = 51 to 70%, and ++++ = 71 to 95%. DS and ESC were calculated with data obtained for 9 to 15 leaves and 3 to 5 stalks, respectively.

^w Based on the genome sequence of *X. albilineans* GPE PC73 (Pieretti et al. 2009). Orthologous genes of these loci exist in all sequenced species of the *Xanthomonadaceae* family (*Stenotrophomonas*, *Xanthomonas*, and *Xylella*) with the exception of (1) locus not present in *Xylella fastidiosa*, (2) = locus present only in *Xanthomonas albilineans*, *X. axonopodis* pv. *vasculorum*, and *X. vasicola* pv. *musacearum*, (3) = locus present only in *X. albilineans* and *X. axonopodis* pv. *vasculorum*, (4) locus present only in *X. albilineans*, and (5) = locus not present in *Stenotrophomonas maltophilia*.

^x Pathogenicity-related (PR) orthologous genes: Xcc = *X. campestris* pv. *campestris* 8004 (Qian et al. 2005), Xoo = *X. oryzae* pv. *oryzae* KACC10331 (Wang et al. 2008), Xoc = *X. oryzae* pv. *oryzicola* BLS303 (Wang et al. 2007).

^y EPS = exopolysaccharide and LPS = lipopolysaccharide.

^z OmpA = outer-membrane protein A.

mutations in this locus should not result in polar effects on downstream genes.

An outer-membrane protein is required for both disease-symptom expression and sugarcane stalk colonization by *X. albilineans*.

Five independent Tn5 insertions that produced few or no leaf disease symptoms were located in XALc_0557 (Fig. 1), which

is predicted to encode an OmpA family outer-membrane protein that was named XaOmpA1. A putative Shine-Dalgarno sequence was identified upstream of the start codon and, at least in the genome of sequenced strain *X. albilineans* GPE PC73, XALc_0557 is preceded by XALc_0556 in the opposite orientation on the complementary DNA strand (Pieretti et al. 2009). Additionally, XALc_0557 is followed by a predicted Rho-independent terminator, indicating that this locus is not

Fig. 1. Distribution of single-transposon insertion mutants showing reduced pathogenicity phenotype in locus XALc_0557 (1,101 bp) of *Xanthomonas albilineans* XaFL07-1. The striped area at the 3' end of the coding sequence of XALc_0557 (= 557) encodes the OmpA domain. Position of the insertion site in each mutant is indicated by a flag. Mutant identification numbers are indicated on top of each flag. The loop downstream of the locus represents a predicted Rho-independent terminator. Locus XALc_0556 (= 556) located upstream of XALc_0557 is predicted to encode an oxidoreductase. Locus XALc_0558 (= 558) located downstream of XALc_0557 is predicted to encode a conserved hypothetical protein.

Fig. 2. Leaf scald symptoms caused by *Xanthomonas albilineans* after inoculation of sugarcane 'CP80-1743'. **A**, Pencil-line stripes (pl) and necrosis (n) produced by wild-type XaFL07-1 1 month after plant inoculation. **B**, Symptomless leaf inoculated with XaOmpA1-mutant M227 1 month after inoculation. **C**, Effect of *X. albilineans* XaFL07-1 and XaOmpA1-mutant M227 on growth of stalks of sugarcane 'CP80-1743' 2 months after plant inoculation. Stalks labeled a, b, and c were inoculated with XaFL07-1, distilled water, and mutant M227, respectively. White vertical bars represent growth size of seven internodes after inoculation and removal of leaves.

part of an operon and that transposon insertions in XALc_0557 should not result in polar effects on downstream genes.

All XaOmpA1-mutants of *X. albilineans* produced albicidin in amounts equivalent to the wild type in vitro but produced no or very few pencil line stripes on sugarcane leaves (Fig. 2A and B). Additionally, although leaves inoculated with XaOmpA1-mutant M227 were symptomless, the pathogen was present in these leaves, from which it was easily reisolated on MW medium (data not shown). In separate experiments, mean disease severity (DS) of the five mutants varied between 0 and 0.4 (on a 0-to-6 scale), whereas mean DS of the wild-type strain of *X. albilineans* was between 4.0 and 5.6. Mean extent of stalk colonization (ESC) varied between 0 and 14 (on a 0-to-100 scale) for mutants M227, M468, M573, and M1152, whereas mean ESC of the wild-type strain of *X. albilineans* was between 76 and 98. In one experiment, mutant M768 colonized a greater length of the sugarcane stalk than the other mutants (mean ESC = 60) but population densities of this mutant were lower than those of the wild-type strain (mean ESC = 98). When growth of sugarcane stalks was measured 2 months after inoculation with i) wild-type XaFL07-1, ii) distilled water (control), and iii) mutant M227, only the wild-type strain affected stalk size (Fig. 2C). The growth rate of all XaOmpA1-mutants in liquid MW medium and in liquid minimal medium was lower than the growth rate of wild-type *X. albilineans* (Fig. 3; data not shown). Although XaOmpA1-mutants grew more slowly and reached lower population sizes than the wild-type strain in both media (Fig. 3), XaOmpA1-mutants were able to reach high population densities (optical density at 600 nm [OD₆₀₀] of 0.25 to 0.30 = 10⁹ CFU/ml). All mutants except mutant M768 were also less motile than the wild-type strain in MW 0.25% agar medium (Fig. 4).

XALc_0557 showed 81 to 83% amino acid identity to orthologous loci in other *Xanthomonas* spp. (Table 2). This identity was also very high in other members of the *Xanthomonadaceae* family: 78% with two strains of *Stenotrophomonas maltophilia* and 68% with two strains of *Xylella fastidiosa*. Surprisingly, locus XALc_0557 showed high amino acid identity (68%) to an *ompA* locus of *Clostridium* sp. strain M62/1. With the exception of the *Clostridium* sp., these identity values are similar to those observed with housekeeping genes such as *gyrB* (Table 2), suggesting that locus XALc_0557 is highly conserved among species of the *Xanthomonadaceae* family and under purifying selection. Only the *ompA* domain of the protein showed some identity (37 to 40%) with proteins encoded by the xylem-invading plant pathogen *Ralstonia solanacearum* (strain GMI1000) and the human pathogen *Escherichia coli* (strain K-12 substrain MG1655), confirming that locus XALc_0557 is specific to members of *Xanthomonadaceae*.

Pathogenicity of albicidin-deficient mutant isolates.

Albicidin is a small molecule synthesized by a unique, large, and mixed polyketide synthase–nonribosomal peptide synthetase (NRPS) gene cluster (Royer et al. 2004). Surprisingly, no Tn5 insertions were found in any of the albicidin biosynthesis genes, despite the fact that they cover approximately 1.4% of the *X. albilineans* genome. Therefore, we produced five site-specific deletion mutant isolates of *albXXI* (= *xabA*), a gene which encodes a phosphopantetheinyl transferase, an enzyme involved in post-translational activation of NRPS and required for albicidin biosynthesis (Huang et al. 2000; Royer et al. 2004). Polymerase chain reaction (PCR) and albicidin assays confirmed that *albXXI* was deleted in *ΔalbXXI* mutant isolates of XaFL07-1 and that these isolates do not produce any detectable albicidin in vitro (data not shown).

In the first greenhouse experiment, sugarcane ‘CP80-1743’ was inoculated with XaFL07-1 and the five *ΔalbXXI* mutant

isolates of XaFL07-1 (eight plants were inoculated with the wild-type strain at 10⁹ CFU/ml and four plants were inoculated per mutant isolate at 10⁹ CFU/ml). Within 10 days to 3 weeks, the wild-type strain and also all five mutant isolates produced leaf scald symptoms on inoculated leaves, including pencil line stripes, yellow chlorosis, and necrosis (Fig. 5). However, pencil line stripes produced by the albicidin-deficient mutant isolates were not as wide as those usually produced by XaFL07-1 (0.1 versus 0.2 to 0.3 mm). These pencil lines were also more diffuse and yellowish or red in color whereas those usually produced by XaFL07-1 were well defined and generally white in color. Pathogen population densities in leaves were similar for the wild type (mean of 1.8 × 10⁹ CFU/g of fresh matter determined with 5 leaf samples) and for albicidin-defective mutant isolates (mean of 1.0 × 10⁹ CFU/g of fresh matter determined with 10 leaf samples). More than 1,500 CFU of *ΔalbXXI* mutant isolates were tested for albicidin production after isolation from symptomatic leaves, and all tested negative. In contrast, the 1,000 CFU of XaFL07-1 isolated from symptomatic leaves all produced albicidin in vitro. The pathogen was also successfully reisolated from internodes of the 8 stalks inoculated with XaFL07-1 and from the 20 stalks inoculated with the mutant isolates.

In a second greenhouse experiment, sugarcane ‘CP80-1743’ was inoculated with XaFL07-1 and mutant *ΔalbXXI* isolate M8 at three different inoculum concentrations (six plants were inoculated per strain and per inoculum concentration: 10⁹, 10⁸, and 10⁷ CFU/ml). As in the first experiment, XaFL07-1 and the albicidin-deficient mutant induced leaf scald symptoms as described in the first experiment, within 10 days and 3 weeks. DS observed on leaves that developed after inoculation and extent of stalk colonization by the pathogen were not different between the wild-type strain and the albicidin-deficient mutant at all inoculum concentration used (Table 3). Additionally, mutant *ΔalbXXI* isolate M8 affected sugarcane growth (reduced size of internodes) similarly to XaFL07-1 (Fig. 5). However, the population size of the albicidin-deficient mutant at stalk internode +6 was significantly lower than the population size of the wild-type strain at the same location: 2.0 × 10⁶ CFU per gram of fresh matter versus 1.0 × 10⁷ CFU per gram of fresh matter. More than 1,000 CFU of *ΔalbXXI* isolate M8 were tested for albicidin production after isolation from inoculated stalks, and all tested negative.

DISCUSSION

A simple mutagenesis system based on a transposon-transposase synaptic complex (transposome) proved to be very efficient in producing Tn5 transposon mutants of *X. albilineans*. As previously shown in other bacterial species (Guihabert et al. 2001; Wang et al. 2008), transpositions occurred at generally random sites on the *X. albilineans* chromosome. However, identification of several transposon insertions in a few loci also suggests that the transposon insertion sites are not absolutely random. The genome size of *X. albilineans* is approximately 3.8 Mb (Pieretti et al. 2009) and a screen of only 1,216 mutants does not begin to approach a saturation scale (1 insertion per average-size gene of 1,000 bp). Nevertheless, the approach was useful for the identification of new candidate pathogenicity factors in *X. albilineans*, which is a unique species of *Xanthomonas*. Surprisingly, a high frequency (2.7%) of nonredundant pathogenicity-affected mutants was obtained (ribosomal mutants excluded), whereas only 0.45, 0.3, and 0.2% mutants were impaired in virulence in a large-scale screening of *X. campestris* pv. *campestris* (Qian et al. 2005), *X. oryzae* pv. *oryzae* (Wang et al. 2008), and *X. oryzae* pv. *oryzicola* (Wang et al. 2007), respectively. It is possible that pathogenicity-related

genes are present as a higher percentage of the total in the reduced genome of *X. albilineans*.

The *X. albilineans* genome encodes 12 large nonhomologous NRPS in six regions, and these NRPS are activated by a specific phosphopantetheinyl transferase encoded by *albXXI*. In all, 3 of the 12 NRPS of *X. albilineans*, encoded in the same gene cluster called XALB1, were previously described as involved in albicidin biosynthesis (Royer et al. 2004). The products encoded by the nine other NRPS are currently unknown. Surprisingly, we did not identify any Tn5 mutants affected in albicidin biosynthesis. Furthermore, and in contrast to expected results (Birch 2001), albicidin-deficient mutant isolates produced by deletion mutagenesis were still able to produce disease symptoms such as necrosis, resulting in partial or total leaf death. Use of different strains of the pathogen, different sugarcane cultivars, or different environmental conditions affecting symptom expression may explain this conflicting result. Regardless, one implication of these results is that the antipathogenesis approach to control leaf scald disease, based on specific degradation of albicidin (Birch 2001), may not be effective with all strains of *X. albilineans* or under all environmental conditions.

Although albicidin-deficient mutant M8 of strain XaFL07-1 was able to spread efficiently in the leaf and the stalk xylem, its population densities in the upper part of the stalk (internode +6) were slightly lower than those of wild-type strain XaFL07-1. Therefore, albicidin may only contribute to full pathogenicity of at least some strains of *X. albilineans*. Other molecules encoded by NRPS may also contribute to full pathogenicity of *X. albilineans*. Mutagenesis and complementation studies of multiple NRPS genes will be necessary to investigate this hypothesis.

Xanthan gum produced by *Xanthomonas* spp. is an EPS widely used in the food, cosmetic, and oil industries as a thick-

ening agent. It contributes to *Xanthomonas* spp. epiphytic survival (Dunger et al. 2007) and also to pathogenicity of *X. axonopodis* pv. *citri*, the causal agent of citrus canker (Rigano et al. 2007). Although *X. albilineans* does not produce xanthan gum and is not a slime-former on sucrose-rich media, polysaccharides are present on its cell surface, as evidenced by slightly viscous cultures that become dry when genes involved in EPS or LPS production are mutated. Surface polysaccharides, including EPS and LPS, represent the predominant structures on all bacterial surfaces, and they are often important players in plant-pathogen interactions. LPS is increasingly recognized as a major pathogen-associated molecular pattern factor (Jackson 2009; Patil et al. 2007). Mutants of *X. albilineans* affected in production of surface polysaccharides were not only affected in symptom production but also in capacity to spread in the sugarcane stalk xylem. The transposon insertion site of mutant M51 was found to be located in gene *rmlB* that is predicted to encode a dTDP-glucose 4,6-dehydratase. This enzyme is known to be involved in LPS biosynthesis (Patil et al. 2007). The LPS provides a critical protection function against plant defenses (Kingsley et al. 1993); therefore, this result may be expected. To our knowledge, this is the first report of a critical role of *rmlB* in plant pathology.

Similarly, several other loci that are pathogenicity related in *X. albilineans* have not been reported thus far as encoding pathogenicity factors in other *Xanthomonas* spp. These include proteins with unknown functions, a membrane-fusion protein conferring resistance to novobiocin, and transport proteins (*tolQ*, *corA*, and TonB-dependent outer-membrane transporters [TBDT]). TonB-dependent transport is a mechanism for active uptake across the outer membrane of gram-negative bacteria (Schauer et al. 2008). TBDT are outer-membrane proteins known for the active transport of iron complexes and vitamin

Fig. 3. Growth of *Xanthomonas albilineans* XaFL07-1 (wild type) and XaOmpA1-mutants M227 and M468 in modified Wilbrink's (MW) liquid medium and in liquid minimal medium (MM). Growth measured for 7 to 8 days after inoculation of each medium is expressed as optical density at 600 nm (OD_{600}), and each point represents the mean of four different tubes per strain. Bars represent standard deviation.

B₁₂, and also nickel and different carbohydrates. In *X. campestris* pv. *campestris*, one TBDT transports sucrose with a very high affinity, suggesting that it might be a sucrose scavenger, and mutants in the corresponding gene are affected in pathogenicity (Blanvillain et al. 2007). Therefore, TBDT also may play a role in bacterial adaptation to plants, involving scavenging plant carbohydrates (Blanvillain et al. 2007; Jackson 2009; Schauer et al. 2008). TBDT are overrepresented in *Xanthomonas* spp. (Blanvillain et al. 2007) and 35 TBDT loci were identified in the genome of *X. albilineans* (E. Lauber, unpublished data). Orthologous genes of the two pathogenicity-related TBDT of *X. albilineans* exist in other pathogenic xanthomonads but they have not been associated with pathogenicity thus far.

Most of the pathogenicity-related genes identified herein will require confirmation by genetic complementation because observed phenotypes may also be due to spontaneous ectopic mutations or to polar effects of insertions on downstream genes. However, isolation of three, four, and five independent mutations in loci *fabH* (3-oxoacyl-[acyl-carrier-protein] synthase 3), XALc_2705 (glycosyl transferase), and XALc_0557 (XaOmpA1), respectively, strongly support the conclusion that these loci are relevant pathogenicity factors in *X. albilineans*. Additionally, locus XALc_2705 is located next to two other genes (*gmd* and *rmd*) involved in LPS synthesis and for which each independent mutation resulted in loss of pathogenicity, strongly supporting their role in pathogenicity. Furthermore, several genes identified herein, such as *fabH*, *glyA*, or *xanB* are orthologues of genes involved in pathogenicity of other related bacterial pathogens.

The transposon insertion site of five independent mutants (M227, M468, M573, M768, and M1152) was found to be located in locus XALc_0557 which is predicted to encode an outer-membrane protein (XaOmpA1). Transposon insertions in this locus resulted in mutants of *X. albilineans* that were greatly affected in pathogenicity. These mutants could no longer produce leaf symptoms or efficiently spread in the stalk xylem. OmpA proteins play an essential role in virulence of several human pathogens (Ristow et al. 2007; Singamsetty et al. 2008; Smith et al. 2007; Wang and Kim 2002). Although orthologous genes showing high protein identity to *X. albilineans* XALc_0557 exist in other species of *Xanthomonadaceae*, and extensive transposon mutagenesis was performed in model species such as *X. campestris* pv. *campestris* (Qian et al. 2005) and *X. oryzae* pv. *oryzae* (Wang et al. 2008), this particular OmpA-family protein, thus far, has not been suspected of involvement in symptom development and spread in xylem vessels by a bacterial plant pathogen. A second OmpA-family protein (XC4223 or XCC4132) also exists in *X. albilineans* (XALc_0045) and was found to be essential for symptom expression in *X. campestris* pv. *campestris* but its function is unknown (Qian et al. 2005). Similarly, an OmpA mutant showing reduced symptom expression was reported in *X. oryzae* pv. *oryzae* (Wang et al. 2008) but this locus does not show homology to locus XALc_0557 of *X. albilineans*.

An XALc_0557 homolog, MopB, was suspected to be involved in xylem adhesion and pathogenicity of *Xylella fastidiosa* but this suspicion was not confirmed (Bruening et al. 2004). However, because *X. albilineans* and *Xylella fastidiosa*

Fig. 4. Motility of *Xanthomonas albilineans* **A**, XaFL07-1 and **B**, XaOmpA1-mutant M227 in modified Wilbrink's 0.25% agar medium. Bacteria in the white halo in **A** are *X. albilineans*. Picture was taken 11 days after stab inoculation of the medium.

Table 2. Amino acid identity of ompA locus (XALc_0557) and housekeeping gene *gyrB* (XALc_0004) of *Xanthomonas albilineans* to orthologous loci in other bacterial species^y

Taxon	XALc_0557 ompA (366 aa)			<i>gyrB</i> = XALc_0004 (820 aa)		
	Ortholog	Length (aa)	Identity ^z	Ortholog	Length (aa)	Identity ^z
<i>X. oryzae</i> pv. <i>oryzae</i> MAFF 311018	XOO_3490	365	306/366 (83%)	XOO_0004	814	701/821 (85%)
<i>X. axonopodis</i> pv. <i>citri</i> 306	XAC1012	365	302/366 (82%)	XAC0004	814	702/821 (85%)
<i>X. campestris</i> pv. <i>vesicatoria</i> 85-10	XCV1042	365	302/366 (82%)	XCV0004	832	702/821 (85%)
<i>X. campestris</i> pv. <i>campestris</i> ATCC 33913	XCC0935	365	299/366 (81%)	XCC0004	814	707/821 (86%)
<i>Stenotrophomonas maltophilia</i> K279a	Smlt0955	366	293/371 (78%)	Smlt0005	819	706/820 (86%)
<i>S. maltophilia</i> R551-3	Sml_0800	366	289/367 (78%)	Sml_0004	819	705/820 (85%)
<i>Xylella fastidiosa</i> 9a5c	XF0343	389	262/381 (68%)	XF0005	814	632/822 (76%)
<i>X. fastidiosa</i> Temecula1	PD1709	389	262/381 (68%)	PD0005	814	635/822 (77%)
<i>Clostridium</i> sp. strain M62/1	CLOM621_09126	294	202/297 (68%)	CLOM621_07162	636	298/562 (53%)
<i>Escherichia coli</i> K-12 substrain MG1655	b0957	346	48/120 (40%)	b3699	804	497/810 (61%)
<i>Ralstonia solanacearum</i> GM11000	RSc2380	209	41/108 (37%)	RSc3440	842	464/841 (55%)

^y Amino acid (aa) sequences of XALc_0557 and XALc_0004 of *Xanthomonas albilineans* were used to search for similar sequences of other bacterial species in the National Center for Biotechnology Information databases using the BLAST algorithm. Orthologs that showed highest similarities to those of *X. albilineans* are cited herein, with the exception of the orthologs in *R. solanacearum* and *E. coli* that are used for comparison.

^z Amino acids identity = number of identical amino acids/total number of compared amino acids.

are phylogenetically relatively close (Pieretti et al. 2009), and because both species are xylem-limited pathogens lacking Hrp T3SS, it seems likely that the XALc_0557 homolog in *Xylella fastidiosa* is at least essential for xylem colonization in this

plant pathogen. Very recently, Chen and associates (2010) reported that a mutation in *mopB* (equivalent to XALc_0557 in *X. albilineans*) in *X. campestris* pv. *campestris* Xc17 from Taiwan causes loss of pathogenicity (i.e., capacity to cause symp-

Fig. 5. Leaf scald symptoms caused by *Xanthomonas albilineans* after inoculation of sugarcane ‘CP80-1743’. **A**, Symptomless leaf inoculated with water. Pencil-line stripes (pl) produced by **B**, wild-type XaFL07-1 and **C**, mutant $\Delta albXXI$ M8 defective in albicidin production. Pencil-line stripes (pl) and necrosis (n) produced by **D**, wild-type XaFL07-1 and **E**, mutant $\Delta albXXI$ M8. Severe leaf necrosis (n) produced by **F**, wild-type XaFL07-1 and **G**, mutant $\Delta albXXI$ M8. All leaves were photographed 1 month after plant inoculation. **H**, Effect of *X. albilineans* on sugarcane growth: reduced internode size of stalks caused by **a**, wild-type XaFL07-1 and **c**, mutant $\Delta albXXI$ M8 compared with **b**, distilled water. White vertical bars represent growth size of seven internodes 2 months after inoculation and removal of leaves.

toms of cabbage black rot). In *Pseudomonas reinekei*, a species isolated from an enrichment derived from a polluted stream, outer-membrane protein OprF (an OmpA-family protein) plays an important role under severe nutrient-limiting conditions, during which it acts as a sensing or response protective barrier (Bobadilla Fazzini et al. 2009). XaOmpA1 of *X. albilineans* is a previously unrecognized and essential pathogenicity factor in *X. albilineans* that could have a similar protective barrier function in the nutrient-poor environment of the xylem. XaOmpA1-mutants of *X. albilineans* were affected in vitro in growth rate and in motility and in planta in invasion of the sugarcane stalk xylem. Therefore, XaOmpA1 probably contributes to bacterial fitness. The precise role of this protein in *X. albilineans* fitness, as well as the possible roles of OmpA proteins in other xanthomonads, is currently under investigation.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Strain XaFL07-1 of *X. albilineans* was isolated in 2007 from a diseased sugarcane leaf that was sampled at the United States Department of Agriculture Sugarcane Field Station at Canal Point, FL. It was cultured routinely at 30°C on MW agar medium containing sucrose (10 g), peptone (5 g), K₂HPO₄·3H₂O (0.50 g), MgSO₄·7H₂O (0.25 g), Na₂SO₃ (0.05 g), agar (15 g), and distilled water (1 liter), pH 6.8 to 7.0. Transposon mutants of XaFL07-1 were cultured on the same medium supplemented with kanamycin at 20 mg/liter. All strains of *X. albilineans* were stored at -80°C as turbid water suspensions and retrieved before transposon mutagenesis or plant inoculation by plating on MW agar medium. *E. 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).

X. albilineans electroporation with a Tn5 transposase-transposon complex (transposome).

Two-day-old liquid cultures of *X. albilineans* XaFL07-1 in MW medium were grown at 30°C to mid-exponential growth phase (OD₆₀₀ = 0.4 to 0.5). Cells were incubated on ice for 30

min and were collected by centrifugation at 4,000 × g at 4°C for 15 min. Cell pellets were washed and resuspended two times, first in 50 ml and then in 25 ml of chilled distilled water. Cell pellets were then resuspended in 1 ml of sterile, ice-cold distilled water, and cells (50 µl) were aliquoted and stored at -80°C. The EZ-Tn5 <KAN-2>Tnp Transposome complex (Epicentre Biotechnologies, Madison, WI, U.S.A.) was used for transposon mutagenesis of *X. albilineans*. Transposome DNA (20 ng) was electroporated into 40 to 50 µl of electrocompetent cells of *X. albilineans* at 18 kV/cm, using an Eppendorf 2510 electroporator (Westbury, NY, U.S.A.). Following electroporation, 1 ml of liquid MW was added to the mix and the culture was grown at 30°C for 2 to 3 h at 120 rpm. Then, 50-µl aliquots of undiluted and 10× and 100× diluted cultures in liquid MW were spread on MW agar plates containing kanamycin at 20 mg/liter for selection of transposon mutants.

Cloning, sequencing, and analysis of Tn5-tagged regions of *X. albilineans*.

MW liquid medium supplemented with kanamycin (20 mg/liter) was inoculated with cells from Tn5 transformants and incubated with shaking (120 rpm) at 30°C for 36 to 48 h. Cultures were centrifuged and total genomic DNA was extracted using the illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Piscataway, NJ, U.S.A.), according to the manufacturer's protocol. Plasmid pUC19 (Invitrogen Corporation, Carlsbad, CA, U.S.A.) was used to clone *Eco*R1-digested genomic DNA fragments. Transformed *E. coli* cells were spread on LB plates containing kanamycin at 20 to 50 mg/liter for screening of cloned DNA inserts containing Tn5-tagged regions. Plasmid DNA from kanamycin-resistant clones was sequenced with KAN-2 FP-1 forward primer and KAN-2 RP-1 reverse primer of the transposome kit. All sequencing was performed by the Interdisciplinary Center for Biotechnology Research DNA Sequencing Core Facility at the University of Florida. Sequenced fragments were blasted against the entire genome sequence of *X. albilineans* GPE PC73 from Guadeloupe (Pieretti et al. 2009), using the integrated annotation tool platform at INRA Toulouse (France).

Production of albicidin-defective mutants.

In order to make a marker-free deletion mutation in *albXXI*, a gene critical for albicidin biosynthesis (Royer et al. 2004), plasmid pALBXXI-AD, supplied by M. Marguerettaz (CIRAD Montpellier, France), was electroporated into electrocompetent cells of *X. albilineans* as described above. This plasmid contained a splice-overlap PCR product that carried both ends (each approximately 1 kb long) of the target ORF but not the target region (= *albXXI*). This PCR product was cloned into suicide vector pUFR080 (Castaneda et al. 2005), containing two selectable markers and the *sacB* gene. Following electroporation, 1 ml of liquid MW medium without sucrose but supplemented with glucose at 10 g/liter (GW medium) was added to the mix and the culture was grown at 30°C for 2.5 h at 120 rpm. Culture aliquots (50 µl) were then spread on GW agar plates containing kanamycin at 20 mg/liter. Six-day-old colonies were subcultured on kanamycin-supplemented GW medium, and the resulting culture was streaked on regular MW medium containing sucrose to select for double recombination and deletion of the target gene as described by Castaneda and associates (2005). Colonies that grew on sucrose were analyzed by PCR to verify deletion of *albXXI*. Absence of albicidin production by selected mutants was verified as described below.

Inoculation of sugarcane with *X. albilineans*.

All pathogenicity experiments were conducted at the University of Florida under greenhouse conditions using healthy

Table 3. Pathogenicity of *Xanthomonas albilineans* XaFL07-1 and albicidin-defective mutant M8 in sugarcane 'CP80-1743' 2 months after inoculation^w

Strain	Pathogenicity parameter ^x		
	DS	ESC	Population ^y
XaFL07-1	5.15 (1.200) a	92 (1.307) a	7.0 a
XaFL07-1 Δ <i>albXXI</i> M8	5.15 (1.197) a	91 (1.286) a	6.3 b
Variance analysis ^z			
Strain <i>F</i>	0.01	0.21	4.55
Strain <i>P</i> > <i>F</i>	0.9396	0.6503	0.0402

^w The experiment was set up in a greenhouse in Gainesville (Florida) using a randomized complete block design with six replications of one plant each. Sugarcane 'CP80-1743' was inoculated with each strain at 10⁹, 10⁸, and 10⁷ CFU/ml. No effect of inoculum concentration was found by preliminary variance analysis; therefore, data of each strain were combined for analysis, and values are the mean of 18 plants. Control plants inoculated with distilled water were symptomless and free of the pathogen, and data of these plants were not included in variance analysis.

^x Disease severity on leaves (DS) and extent of stalk colonization (ESC) values are on a 0-to-6 and 0-to-100 scale, respectively. Means in parentheses are arcsin square root transformed data expressed as proportions (DS/6 and ESC/100). Bacterial population densities are expressed as Log[(CFU/g of fresh matter + 1)].

^y Bacterial population density at internode I₄₋₆.

^z Variance analysis of DS and ESC data was performed after arcsin square root transformation of data expressed as proportions (DS/6 and ESC/100). In each column, values followed by the same letter are not significantly different at *P* = 0.05 according to the test of Student-Newman-Keuls.

plants of sugarcane 'CP80-1743', moderately susceptible to leaf scald. Inoculum of *X. albilineans* was prepared from 2-day-old agar cultures. Except where otherwise stated, sugarcane stalks with at least four to five internodes were inoculated with bacterial suspensions at 10^8 CFU/ml in sterile distilled water by a modified decapitation method. In this method, the spindle leaves on a stalk were cut off just below the third visible dewlap with pruning shears and 0.2 to 0.5 ml of inoculum was then deposited onto the cut surface.

Initial screening for reduced pathogenicity of transposon mutant strains.

In each screening experiment, approximately 200 sugarcane stalks were inoculated, each with a different transposon mutant strain. Leaf scald symptoms (pencil line stripes, chlorosis, and necrosis) were recorded on three to four emerging leaves 1 month after inoculation. Populations of *X. albilineans* were determined in three different stalk locations (I_{-1} , I_{+2} , and I_{+5}) by the stalk blot inoculation technique 2 months after inoculation. Internodes I_0 to I_{+4} corresponded to the location of the five stalk internodes that showed reduced growth after mechanical plant inoculation (cut leaves above the growing point). These internodes were attached to the inoculated leaves and, therefore, were considered to be entry points of the pathogen into the sugarcane stalk.

Stalks were sampled individually and cut at the soil level with pruning shears. The rind of stalk fragments comprising I_{-1} , I_{+2} , and I_{+5} was cleaned with absorbent paper and flamed with alcohol to ensure external sterilization. Each stalk location was then cut transversally, in the middle, with sterile pruning shears. Cut sections were firmly pressed onto two plates, one plate of WCNCB medium and one plate of MW agar medium supplemented with kanamycin at 20 mg/liter, novobiocin at 30 mg/liter, cycloheximide at 50 mg/liter, and benomyl at 12.5 mg/liter (WKNCB medium). Pruning shears were flamed with alcohol after each cut. Growth of *X. albilineans* was recorded after 5 days of incubation of agar plates at 30°C. Pathogen population densities were assessed in each internode using a 0-to-4 scale, where 0 = no bacterial colony in the stalk imprint, 1 = 1 to 10 colonies in the stalk imprint, 2 = more than 10 colonies or confluent growth of bacteria in less than 25% of the stalk imprint, 3 = confluent growth of bacteria in 25 to 75% of the stalk imprint, and 4 = confluent growth of bacteria in more than 75% of the stalk imprint. Only mutant strains that showed scores of 0 to 2 in all three stalk locations were considered for further pathogenicity assays.

Pathogenicity assays.

In each pathogenicity assay, sugarcane stalks were distributed in the greenhouse using a randomized complete block design with three to eight replications of one plant each. Leaf scald symptoms were recorded on emerging leaves 1 month after inoculation using a symptom severity scale ranging from 0 to 6, where 0 = no symptoms, 1 = 1 to 5 pencil-line stripes, 2 = 6 to 10 pencil-line stripes, 3 = more than 10 pencil-line stripes, 4 = leaf chlorosis or less than 10% leaf necrosis, 5 = 10 to 50% leaf necrosis, and 6 = 51 to 100% leaf necrosis. Inoculated stalks were rated individually based on the average score of the three inoculated leaves showing the most severe symptoms. Populations of *X. albilineans* were determined 2 months after inoculation in 10 (I_{-4} to I_{+5}) to 13 (I_{-4} to I_{+8}) stalk locations by the stalk blot inoculation technique, as described above. However, in experiments with albicidin-defective mutants, cut sections of stalk internodes were all pressed onto two plates each of WCNCB medium. Pathogen population densities in each internode were assessed using a 0-to-4 scale, as described above. ESC was expressed as $ESC = 100 [(1 \times N_1 + 2 \times N_2 + 3 \times N_3 +$

$4 \times N_4)/4 \times N_T]$, where N_i = number of internodes with score i and N_T = total number of internodes.

Determination of bacterial population densities in leaves and stalk internodes.

Leaf fragments (1 to 2 by 2 to 3 cm) or internode sections (3 to 5 by 4 to 6 mm) were weighed and then homogenized in 20-mM Tris-buffered saline buffer (2.42 g of Tris, 8 g of NaCl, and 1,000 ml of distilled water, pH 7.5). Homogenized tissues were kept at room temperature for 1 h, then serially diluted for quantification of bacteria. Serial dilutions were plated in triplicate on WCNCB or WKNCB according to the strain of *X. albilineans*. Dilutions were also plated on sucrose-peptone agar (SPA) medium (sucrose at 20 g/liter, peptone at 5 g/liter, and agar at 15 g/liter, pH 6.8 to 7.0) when bacteria were to be tested for albicidin production after plant isolation. Bacterial colonies were counted after 5 days of incubation at 30°C.

In vitro characteristics of *X. albilineans*.

Growth rate of *X. albilineans* mutants in liquid medium was initially assayed by inoculation of 50 ml of MW medium with 5 µl of a bacterial suspension at 10^9 CFU/ml of sterile distilled water. Glass flasks were incubated at 30°C on an orbital shaker at 100 rpm and OD_{600} of bacterial suspensions was measured every 8 h for 12 days using a spectrophotometer. The growth curves of *X. albilineans* and XaOmpA1 mutants M227 and M468 in MW medium and in minimal medium were determined with 50-ml Corning tubes containing 10 ml of medium. Minimal medium contained sucrose (10 g), L-methionine (0.1 g), $K_2HPO_4 \cdot 3H_2O$ (3 g), NaH_2PO_4 (1 g), NH_4Cl (1 g), $MgSO_4 \cdot 7H_2O$ (0.3 g), and distilled water (1 liter), pH 6.8 to 7.0 (Birch and Patil 1985b). Tubes were inoculated with 100 µl of a bacterial suspension at 10^9 CFU/ml of 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 7 to 8 days and OD_{600} of bacterial suspensions was measured using a biophotometer.

Swimming motility was assayed with MW medium and SPA medium, both containing only 0.25% (wt/vol) agar. Strains were stab inoculated with a sterile loop to the bottom of the petri dish, and all plates were inoculated at least in triplicate. Swim plates were incubated at 30°C for 5 to 7 days.

Albicidin production was quantified in vitro by a microbiological assay as described by Birch and Patil (1985b). Briefly, 6- to 8-day-old single colonies of *X. albilineans* on SPA medium were overlaid with 4 ml of a mix containing 2 ml of 1.5% noble agar (wt/vol) and 2 ml of an *E. coli* DH5α suspension (10^7 CFU/ml sterile distilled water). The plates were then incubated at 37°C for 24 to 48 h. Albicidin production was quantified by calculating the width of the *E. coli* growth inhibition ring as $(D - d)/2$, where D = diameter of the *E. coli* growth inhibition ring and d = diameter of the *X. albilineans* colony.

Statistical analysis.

Statistical analyses were performed with SAS (version 9.2; SAS Institute Inc., Cary, NC, U.S.A.).

ACKNOWLEDGMENTS

We thank J. Shine (Sugar Cane Growers Council, Belle Glade, FL, U.S.A.) and J. Comstock (United States Department of Agriculture, Canal Point, FL, U.S.A.) for supplying healthy sugarcane planting material and sugarcane infected by *Xanthomonas albilineans* XaFL07-1, M. Marguerettaz (CIRAD, Montpellier, France) for supplying the plasmid construct (pALBXXI-AD) that was used to produce albicidin-deficient mutant isolates of *X. albilineans*, J.-H. Daugrois (CIRAD, Guadeloupe, France)

for performing statistical analyses of data, and P. Rayside for excellent technical assistance. P. Rott was supported by a Marie Curie Outgoing International Fellowship from the European Commission.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

INRA iANT (integrated ANnotation Tool) complete annotation data for *Xanthomonas albilineans* GPE PC73 from Guadeloupe:
iant.toulouse.inra.fr/bacteria/prj/XANAL/DOC/cgi/index.cgi