Variation in Albicidin Biosynthesis Genes and in Pathogenicity of *Xanthomonas albilineans*, the Sugarcane Leaf Scald Pathogen

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ABSTRACT

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Total genomic DNA from 137 strains of *Xanthomonas albilineans* from worldwide locations was hybridized with two DNA probes that together harbor the entire 49-kb albidin biosynthesis gene cluster and two additional 3-kb genomic regions required for albidin production. Fourteen haplotypes and two major genetic groups (albidin [ALB]-restriction fragment length polymorphism [RFLP] A and ALB-RFLP B) were identified, and strains that were isolated after recent outbreaks of leaf scald disease belonged to group ALB-RFLP B. Albidin genetic diversity was very similar to the previously described genetic diversity of the

pathogen based on the whole genome. No relationship was found between variability of albidin biosynthesis genes and the amount of albidin produced in vitro by *X. albilineans*. Leaf scald-susceptible sugarcane cv. H70-144 was inoculated with 20 strains of the pathogen belonging to different ALB-RFLP haplotypes. Among them, 10 strains from Guadeloupe belonged to the same ALB-RFLP group but differed in the amount of albidin produced in vitro. Strains were distributed in at least three different pathogenicity groups based on symptom severity and pathogen population density in the stalk. These two pathogenicity factors varied concurrently; however, no relationship between variation in albidin biosynthesis genes, variation in the amount of albidin produced in vitro, and variation in pathogenicity of *X. albilineans* was found. Further investigation is necessary to identify other genes involved in pathogenicity of *X. albilineans*.

Xanthomonas albilineans is a systemic, xylem-invading pathogen that causes leaf scald of sugarcane (*Saccharum* interspecific hybrids). This disease occurs in at least 66 countries in the world and can cause severe yield losses (48). Leaf symptoms vary from a single, white, narrow, sharply defined stripe to complete wilting and necrosis of infected leaves, leading to plant death in the most susceptible cultivars. A common symptom in mature cane is the abnormal development of side shoots on stalks (48). Observation of symptoms on leaves and of stalk side shoots generally is used to assess disease severity and resistance after artificial inoculation of sugarcane (17,19,33,50).

A major toxic compound called albidin, specifically produced by *X. albilineans*, plays a key role in pathogenicity (4,6,7,58,59). Albidin-deficient mutants no longer incite disease symptoms and genetically modified sugarcane plants expressing an albidin-detoxifying gene are resistant to leaf scald (59). In plants, albidin inhibits DNA replication of proplastids, thus blocking plastid development (7). Albidin also has antibiotic activity and inhibits replication of several gram-positive and gram-negative bacteria, including *Escherichia coli* (5).

Recently, the entire 49-kb albidin biosynthesis gene cluster was cloned and sequenced from *X. albilineans* strain Xa23R1 from Florida (47,51). This cluster, included in a genomic region named XALB1, contains three large polyketide synthetase (PKS) and nonribosomal peptide synthase (NRPS) genes as well as several putative modifying, regulatory, and resistance genes. Two additional 3-kb regions of the genome of *X. albilineans* strain Xa23R1, namely XALB2 and XALB3, also were found to be in-

involved in albidin production. XALB2 contains a single gene coding for a phosphopantetheinyl transferase which is required for posttranslational activation of PKS or NRPS enzymes (29,51). XALB3 contains a single gene coding for HtpG protein for which the role in albidin production has not been elucidated (57).

The sugarcane leaf scald pathogen is spread mainly in infected sugarcane cuttings (48). However, aerial transmission of *X. albilineans* also has been shown to occur in Florida (11), Guadeloupe (17,32), and Mauritius (2). In Guadeloupe, two strains of *X. albilineans* differed in their capacity to colonize the sugarcane leaf surface and subsequently to cause disease (17). In addition to colonization of the leaf canopy, colonization of the sugarcane stalk is another critical step in disease progress. Leaf scald symptoms are associated with high population densities of *X. albilineans* in the stalk, and disease severity is correlated to population densities in the apex of the stalk (45,50). The capacity of *X. albilineans* to colonize sugarcane stalks or to cause symptoms varies according to the strain of the pathogen, indicating the existence of different pathotypes within the species (17,38). Interactions between sugarcane cultivars and strains of the pathogen suggests the occurrence of races within *X. albilineans* (1,38).

For the last two decades, leaf scald outbreaks have occurred in several geographical locations, such as Cuba (21), Florida (12), Guadeloupe (49), Guatemala (41), Louisiana (26), Mauritius (2), Taiwan (10), and Texas (30). Most, if not all, of these outbreaks were attributed to genetic variants of *X. albilineans* identified by restriction fragment length polymorphism with pulse-field gel electrophoresis (RFLP-PFGE) (19). Eight PFGE groups (A to H) were described. Most strains associated with new outbreaks belonged to PFGE group B. Strains isolated in Florida after the outbreak of leaf scald, belonging to PFGE group B, had greater propensity for aerial transmission than strains belonging to PFGE group A, isolated prior to disease reemergence (19). More re-

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cently, strains belonging to PFGE group B also were classified in a single genetic group, AFLP III, by amplified fragment length polymorphism (AFLP) with a worldwide collection of 162 strains of *X. albilineans* (42). However, no relationship has yet been identified between genetic variation of the pathogen and variation in pathogenicity.

The objective of our study was to characterize the genetic variation of *X. albilineans* with respect to genes involved in albicidin production, and to identify any relationship between this variation and variation in pathogenicity of *X. albilineans*.

MATERIALS AND METHODS

Bacterial strains. The 137 strains (74 from worldwide locations and 63 from Guadeloupe) of *X. albilineans* used in this study are listed in Tables 1 and 2. Bacterial strains stored at -20°C as turbid water suspensions were retrieved by plating on modified Wilbrink's (MW) agar medium for 3 days at 28°C (46). Serological characteristics of strains from Guadeloupe were determined as described by Rott et al. (49). For short-term storage between experiments, bacterial strains routinely were plated on MW agar medium and stored at 5°C .

E. coli DH5 α strain (Life Technologies Inc., Gaithersburg, MD) was stored at -20°C as a turbid water suspension supplemented with 15% glycerol (vol/vol) and grown on Luria-Bertani (LB) agar medium for 24 h at 37°C before use (52). Between short-term experiments, *E. coli* cultures were stored on LB agar at 5°C .

RFLP analysis of albicidin biosynthesis genes. The restriction enzymes *HincII* and *PstI* (Promega Corp., Madison, WI) were used for RFLP analysis because they generated reliable and easily identifiable DNA fingerprints in preliminary experiments (data not shown).

Two DNA plasmids covering the entire 49-kb albicidin biosynthesis gene cluster and the genomic regions XALB2 and XALB3 were used as probes for RFLP analysis. Plasmid pALB571 harbors a 37-kb part of the albicidin biosynthesis gene cluster, including the three large PKS and NRPS genes (47,51). Plasmid pBKS/K7 harbors the other 12-kb part of the albicidin biosynthesis gene cluster and two 3-kb DNA fragments corresponding to XALB2 and XALB3 genomic regions, respectively (E. Vivien, personal communication).

Total genomic DNA was extracted from 3-day-old cultures of *X. albilineans* grown from single colonies in 5 ml of MW broth for 48 h at 28°C using a Qiagen extraction DNeasy Tissue kit (Qiagen SA, Courtaboeuf, France) according to the manufacturer's recommendations. DNA concentrations were adjusted to 60 to 80 ng/ μl before enzymatic restriction. DNA was restricted for 3 h at 37°C in a 20- μl reaction mixture (1 $\times$ buffer, RNase at 10 $\mu\text{g}/\text{ml}$, 4 mM spermidine, and DNA at 60 to 80 ng/ μl) containing 10 units of enzyme. Restriction products were separated by gel electrophoresis in 0.5 $\times$ Tris-borate-EDTA buffer at 5.1 V cm^{-1} for 5 h, stained with ethidium bromide, and visualized by UV transillumination. Southern transfer of DNA fragments onto nitrocellulose membranes was performed as recommended by

TABLE 1. Characteristics of *Xanthomonas albilineans* strains from worldwide locations used in this study

Strain	Origin	Year	Author	Haplotype PFGE ^w	Albicidin production group ^{x,y}	Haplotype RFLP ^{571y}	Haplotype RFLP ^{K7y}	Combined haplotype ^y
BRB101	Barbados	1992	P. Rott	B-01	H	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
BLZ102	Belize	1992	P. Rott	B-10	H	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
BLZ103	Belize	1992	P. Rott	B-15	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
BLZ105	Belize	1992	P. Rott	B-19	H	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
BRA050	Brazil	1988	R. Neto	B-11	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
BRA051	Brazil	1988	R. Neto	B-09	M	H ⁵⁷¹ -B1	HK ⁷ -B1	HB1
BRA115	Brazil	1993	C. O. Cordaso	B-11	M	H ⁵⁷¹ -B1	HK ⁷ -B1	HB1
BRA118	Brazil	1993	C. O. Cordaso	B-10	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
BRA120	Brazil	1993	C. O. Cordaso	B-21	H	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
HVO005	Burkina Faso	1980	M. Chatenet	F-01	M	H ⁵⁷¹ -A5	HK ⁷ -A4	HA7
HVO031	Burkina Faso	1984	M. Chatenet	C-02	M	H ⁵⁷¹ -A1	HK ⁷ -A2	HA2
HVO082	Burkina Faso	1989	M. Granier	C-04	H	H ⁵⁷¹ -A1	HK ⁷ -A2	HA2
CMR029	Cameroon	1984	P. Rott	C-02	M	H ⁵⁷¹ -A1	HK ⁷ -A2	HA2
CMR041	Cameroon	1986	M. Granier	C-02	M	H ⁵⁷¹ -A1	HK ⁷ -A2	HA2
TCD228	Chad	1997	M. Chatenet	...	M	H ⁵⁷¹ -A1	HK ⁷ -A3	HA5
COL217	Colombia	1996	J. I. Victoria	...	H	H ⁵⁷¹ -A1	HK ⁷ -A3	HA5
DOM096	Dominican Republic	1991	M. J. Davis	B-01	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
DOM097	Dominican Republic	1991	M. J. Davis	A-01	...	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
FJI079	Fiji	1961	D. W. Dye	E-01	M	H ⁵⁷¹ -A2	HK ⁷ -A5	HA8
FJI080	Fiji	1961	D. W. Dye	E-02	M	H ⁵⁷¹ -A2	HK ⁷ -A5	HA8
FJI106	Fiji	1961	D. W. Dye	E-02	M	H ⁵⁷¹ -A2	HK ⁷ -A2	HA3
GUF215	French Guyana	1996	J.-H. Daugrois	...	M	H ⁵⁷¹ -A2	HK ⁷ -A5	HA8
GLP007	Guadeloupe	...	M. Chatenet	E-01	M	H ⁵⁷¹ -A2	HK ⁷ -A5	HA8
GLP012	Guadeloupe	...	L. Féreol	B-01	H	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
GLP043	Guadeloupe	1987	J. Montillet	B-01	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
GLP056	Guadeloupe	1988	P. Rott	B-01	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
GLP057	Guadeloupe	1988	P. Rott	B-01	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
GLP059	Guadeloupe	1989	M. Granier	B-01	H	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
GLP094	Guadeloupe	1991	P. Rott	B-06	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
GLP229	Guadeloupe	1998	J.-H. Daugrois	...	M	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
GLP230	Guadeloupe	1998	J.-H. Daugrois	...	H	H ⁵⁷¹ -B2	HK ⁷ -B1	HB2
CIV039	Ivory Coast	1986	P. Rott	C-02	H	H ⁵⁷¹ -A1	HK ⁷ -A2	HA2
CIV040	Ivory Coast	1986	M. Granier	C-02	M	H ⁵⁷¹ -A1	HK ⁷ -A2	HA2

(continued on next page)

^w Data from Davis et al. (19); PFGE = pulse-field gel electrophoresis.

^x Grouping is based on growth inhibition ring (GIR) and on Student-Newman-Keuls test of least square means comparison with six GIR values. Abbreviations: H = highly producing group (GIR > 15.0 mm), M = medium-producing group (GIR = 10.1 to 15.0 mm), L = low-producing group (GIR = 0.1 to 10.0 mm), and N = no albicidin production (GIR = 0 mm). Strains of L and H albicidin-producing groups are significantly different at $P = 0.05$. With a few exceptions, strains of albicidin-producing group M are not significantly different at $P = 0.05$ from strains of groups H and L.

^y This study; RFLP = restriction fragment length polymorphism.

^z P. Rott (unpublished data).

the manufacturer (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, England). Plasmids pALB571 and pBKS/K7 were labeled and used as probes using the random priming technique of the Megaprime hybridization kit and P³²dCTP (Amersham Pharmacia Biotech UK Ltd.). DNA fragments that hybridized with radiolabeled probes were visualized by exposure of radiographic films for 1 to 3 days, depending on radioactive intensity.

Reliability of the technique was controlled by comparing the experimental RFLP patterns of strain Xa23R1 with the theoretical RFLP patterns of the same strain based on the nucleotide sequences of XALB1, XALB2, and XALB3 DNA regions (accession nos. AJ586576, AJ586577, and AM039379, respectively). The theoretical RFLP patterns were determined with DNA Strider software (version 1.0; Institut de Recherche Fondamentale, Commissariat à l'Energie Atomique, Paris). DNA banding patterns obtained with the four enzyme/plasmid combinations (*HincII*/pALB571, *HincII*/pBKS/K7, *PstI*/pALB571, and *PstI*/pBKS/K7) were determined separately by visual analysis. Binary scores were attributed based on the presence or absence (1 or 0, respectively) of DNA bands. Binary matrices were used to calculate similarity between paired haplotypes with Dice's similarity index (22). Cluster analysis of the distance matrix was conducted by the unweighted pair group method with arithmetic means (UPGMA) procedure (54). Similarity calculations and cluster analyses were performed with the DARwin program (version 4.0; CIRAD-FLHOR, Montpellier, France).

Albicidin production assay. Albicidin production was quantified in vitro by a microbiological assay as described by Birch and Patil (5). Briefly, 10 µl of a 10⁹ CFU/ml suspension of *X. albilineans* was spotted in the center of a petri dish containing 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) and incubated at 28°C. After 5 days of growth, the *X. albilineans* culture and the agar medium were overlaid with 3 ml of 1.5% agar medium containing 10 µl of an *E. coli* overnight culture (optical density at 540 nm = 0.56). The plates then were incubated at 37°C for 24 h. Albicidin production was quantified by calculating the width of the *E. coli* growth inhibition ring (GIR) as $(D - d)/2$, where D = diameter of the *E. coli* growth inhibition ring and d = diameter of the *X. albilineans* culture. Three plates were inoculated per strain and experiment. Two separate experiments, assay 1 and assay 2, were conducted with strains from worldwide locations and strains from Guadeloupe, respectively. Both assays were replicated (trials 1 and 2 of each assay) and a third trial (trial 3) was performed with 10 strains from Guadeloupe, to confirm albicidin production results obtained in assay 2 and to identify different albicidin production groups.

Pathogenicity assay. A replicated greenhouse experiment was set up in Montpellier (France) to assess pathogenicity of 20 strains of *X. albilineans* by measuring disease severity on leaves and stalk colonization by the pathogen. Of these 20 strains, 10 originated from worldwide locations and were representative of the genetic diversity of albicidin biosynthesis genes identified in

TABLE 1. (continued from preceding page)

Strain	Origin	Year	Author	Haplotype PFGE ^w	Albicidin production group ^{x,y}	Haplotype RFLP ^{571y}	Haplotype RFLP ^{K7y}	Combined haplotype ^y
KEN006	Kenya	...	M. Chatenet	D-11	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
MTQ032	Martinique	1932	J. A. Spence	B-01	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
MTQ058	Martinique	1989	P. Rott	I-01 ^z	M	H ⁵⁷¹ -A2	H ^{K7} -A6	HA9
MTQ078	Martinique	1957	J. A. Spence	B-01	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
MUS062	Mauritius	1956	G. Orian	B-13	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
PNG130	Papua New Guinea	1993	T. Shigaki	H-01 ^z	L	H ⁵⁷¹ -A4	H ^{K7} -A7	HA10
REU008	Reunion	1983	F. Léoville	D-01	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU009	Reunion	1983	J.-C. Girard	D-07	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU100	Reunion	1992	J.-H. Daugrois	C-01	M	H ⁵⁷¹ -A1	H ^{K7} -A2	HA2
REU148	Reunion	1992	J.-H. Daugrois	D-03	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU172	Reunion	1995	J.-C. Girard	D-15 ^z	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU173	Reunion	1995	J.-C. Girard	D-17 ^z	N	H ⁵⁷¹ -A3	H ^{K7} -A3	HA6
REU175	Reunion	1995	J.-C. Girard	D-16 ^z	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU176	Reunion	1995	J.-C. Girard	D-18 ^z	H	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU185	Reunion	1995	J.-C. Girard	D-15 ^z	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU189	Reunion	1995	J.-C. Girard	...	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU200	Reunion	1995	J.-C. Girard	D-01 ^z	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU207	Reunion	1995	J.-C. Girard	D-15 ^z	M	H ⁵⁷¹ -A1	H ^{K7} -A3	HA5
REU209	Reunion	1995	J.-C. Girard	J-01 ^z	M	H ⁵⁷¹ -A1	H ^{K7} -A2	HA2
LKA070	Sri Lanka	1962	A. C. Hayward	G-01	M	H ⁵⁷¹ -A2	H ^{K7} -A1	HA4
TWN052	Taiwan	1988	C. T. Chen	B-01	M	H ⁵⁷¹ -B3	H ^{K7} -B1	HB3
TWN107	Taiwan	1993	C. T. Chen	B-01	M	H ⁵⁷¹ -B3	H ^{K7} -B1	HB3
TWN109	Taiwan	1993	C. T. Chen	B-01	H	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
TWN110	Taiwan	1993	C. T. Chen	B-01	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
TWN111	Taiwan	1993	C. T. Chen	B-01	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
INC002	Unknown	...	D. C. Harris	B-01	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
USA081	United States	...	H. Koike	...	M	H ⁵⁷¹ -A1	H ^{K7} -A2	HA2
USA083	Florida, United States	1990	M. Davis	B-01	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
USA129	Florida, United States	1993	J. Comstock	B-01	H	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
USA135	Florida, United States	1993	J. Comstock	B-01	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
Xa23R1	Florida, United States	1996	P. Rott	...	H	H ⁵⁷¹ -A1	H ^{K7} -A1	HA1
HAW073	Hawaii, United States	1965	H. Koike	A-01	M	H ⁵⁷¹ -A1	H ^{K7} -A1	HA1
HAW112	Hawaii, United States	1993	S. Schenck	A-05	H	H ⁵⁷¹ -A1	H ^{K7} -A1	HA1
USA156	Texas, United States	1993	T. Isakeit	B-01	H	H ⁵⁷¹ -B4	H ^{K7} -B1	HB4
USA165	Texas, United States	1994	T. Isakeit	B-08	H	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
USA167	Texas, United States	1993	T. Isakeit	B-01	H	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
USA169	Texas, United States	1993	T. Isakeit	B-01	H	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
VNM235	Vietnam	1998	P. Rott	...	M	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
VNM236	Vietnam	1998	P. Rott	...	H	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
VNM237	Vietnam	1998	P. Rott	...	H	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
ZIM071	Zimbabwe	1965	R. A. Lelliot	C-04	H	H ⁵⁷¹ -A1	H ^{K7} -A2	HA2

this study: BLZ103, BRA115, HVO082, DOM097, FJI080, MTQ078, PNG130, TWN052, TWN107, and Xa23R1 (Table 1). Ten strains from Guadeloupe were chosen based on their variable albicidin production measured in this study: GPEDC23, GPEDC38, GPEF4, GPEDC61, GPEG5, GPET16, GPE48, GPESJ2, GPEP14B, and GPESJ47 (Table 3). The experiment was conducted with disease-free tissue-cultured plants of sugarcane cv. H70-144 which is susceptible to leaf scald (50). Plants were propagated in vitro and transferred to the greenhouse as described by Feldmann et al. (23). Four-week-old greenhouse plantlets were planted individually in 6-liter pots containing a mixture of peat, compost, and volcanic rock (2:1:2, vol/vol/vol). During plant growth, secondary shoots were removed systematically to keep a single stalk per plant.

Inoculum of the pathogen was prepared from 3-day-old cultures on MW agar at 28°C. Four-month-old plants were inoculated with 10⁸ CFU/ml bacterial suspensions by the decapitation technique as described by Rott et al. (50). Control plants inoculated with sterile distilled water were included in the experiment to evaluate any naturally occurring cross contamination (19). After inoculation, plants were distributed in the greenhouse using a randomized complete block design with nine replications of one plant each. Trial A and trial B were duplications of the experiment in two separate greenhouses.

Disease severity was assessed as described by Rott et al. (45), with a few modifications. Briefly, disease symptoms were recorded on noninoculated leaves 4, 6, 8, 10, and 15 weeks after inoculation using a symptom severity scale ranging from 0 to 5,

TABLE 2. Characteristics of *Xanthomonas albilineans* strains from Guadeloupe used in this study

Strain ^x	Origin	Sampling date	Sugarcane cultivar	Isolated from ^y	Haplotype RFLP ^{571z}	Haplotype RFLP ^{K7z}	Combined haplotype ^z
GPEDC2, GPEDC23, GPEDC27	Duclos	November 1999	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEDC35, GPEDC38	Duclos	January 2000	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEF1, GPEF4, GPEF5, GPEF6	Duclos	December 2000	B69566	Leaf symptom	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEDC61, GPEDC62, GPEDC63, GPE DC64	Duclos	December 2000	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEDC80, GPEDC81	Duclos	July 2001	B69566	Stalk	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEDC76, GPEDC77, GPEDC79	Duclos	October 2001	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEDC82, GPEDC84, GPEDC88	Duclos	January 2002	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEG7, GPEG8	Godet	December 1999	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEG5, GPEI1, GPEGI5, GPEGK9	Godet	December 1999	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPET16	Godet	May 2000	B69566	Stalk	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEG39, GPEG40, GPEG43, GPEG44	Godet	July 2001	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEG37	Godet	September 2001	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEDC2b	Roujol	February 2002	B69566	Leaf symptom	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPE53, GPE55, GPE57, GPE60, GPE61	Roujol	February 2002	B69566	Leaf symptom	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPE47, GPE48	Saint-Jean	October 1999	B69566	Leaf symptom	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPE49	Saint-Jean	October 1999	B8008	Leaf symptom	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPESJ2, GPESJ3	Saint-Jean	November 1999	B8008	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPESJ11	Saint-Jean	January 2000	B8008	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPESJ16	Saint-Jean	January 2000	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPEO8B, GPEP14B, GPEQ13B	Saint-Jean	April 2001	B69566	Stalk	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPESJ28, GPESJ31	Saint-Jean	September 2001	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPESJ34, GPESJ36	Saint-Jean	September 2001	B8008	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPESJ37, GPESJ41, GPESJ42, GPESJ43, GPESJ45	Saint-Jean	September 2001	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPESJ46, GPESJ47	Saint-Jean	October 2001	B8008	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2
GPESJ49, GPESJ51, GPESJ53	Saint-Jean	October 2001	B69566	Leaf surface	H ⁵⁷¹ -B2	H ^{K7} -B1	HB2

^x Single colonies were isolated on selective agar medium (XAS) after 5 days of growth at 28°C (18). All strains belong to serovar I.

^y Leaf surface: isolated from water droplets sampled from the leaf surface of sugarcane plants.

^z This study; RFLP = restriction fragment length polymorphism.

TABLE 3. In vitro albicidin production by *Xanthomonas albilineans* as determined with a microbiological assay^x

	GIR (in mm)						Variance analysis ^y			
	Assay 1			Assay 2						
	Trial 1	Trial 2	Mean	Trial 1	Trial 2	Mean	DF	Mean square	F value	P > F
Mean (N) ^z	13.9 (63)	13.8 (71)	13.8	13.2 (56)	14.2 (61)	13.7	...	...	...	...
Variance	9.3	9.4	...	30.2	3.5	...	...	...	...	...
Maximum	21	19.5	...	27	19.5	...	...	...	...	...
Minimum	0	0	...	0.5	8	...	...	...	...	...
t test	P = 0.83	...	...	P = 0.04	...	...	...	...	...	...
Assay 1 source										
Strain (error)	...	...	...	...	...	...	72 (58.9)	45.3 (4)	11.3	0.0001
Trial (error)	...	...	...	...	...	...	1 (59.3)	0.00016 (3.9)	0	0.995
Strain × trial	...	...	...	...	...	...	59	4	7.9	0.0001
Error	...	...	...	...	...	...	260	0.5	...	...
Assay 2 source										
Strain (error)	...	...	...	...	...	...	60 (53)	50.1 (40.3)	1.2	0.211
Trial (error)	...	...	...	...	...	...	1 (53)	92.7 (40.1)	2.3	0.134
Strain × trial	...	...	...	...	...	...	53	40.4	107.9	0.0001
Error	...	...	...	...	...	...	219	0.4	...	...

^x Growth inhibition ring (GIR) of 73 strains from worldwide locations (Assay 1) and 61 strains from Guadeloupe (Assay 2) was measured in two separate and duplicated experiments. Trials 1 and 2 are independent replications of each experiment.

^y Statistical analysis was performed with a mixed model of variance analysis considering the strain-trial interaction as random source of variation.

^z Calculated for N strains with mean values of three plates for each strain.

where 0 = no symptoms, 1 = one or two pencil-line streaks, 2 = more than two pencil-line streaks, 3 = leaf chlorosis or bleaching, 4 = leaf necrosis, and 5 = death of the plant. Inoculated plants were rated individually based on the score of the leaf showing the most severe symptom. Disease severity was expressed as $DS = 100 \times (1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4 + 5 \times N_5) / 5N_T$, where N_i = number of plants with score i and N_T = total number of plants. It was used to calculate the area under disease progress curve (AUDPC) for statistical analysis (8).

Pathogen populations were determined 15 weeks after plant inoculation in three different locations on the stalk: SL_0 , SL_{-4} , and SL_{+10} . SL_0 corresponded to the location of two to three shortened 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 the entry point of the pathogen into the sugarcane stalk. SL_{-4} corresponded to the fourth internode below SL_0 and SL_{+10} to the tenth internode above SL_0 . Stalks were sampled individually and cut at the soil level with pruning shears. The rind of stalk fragments comprising SL_0 , SL_{-4} , or SL_{+10} was cleaned with absorbent paper and flamed with alcohol to ensure external sterilization. Each stalk location then was cut transversally, in the middle, with pruning shears. Cut sections were pressed firmly on *X. albilineans* selective growth medium (18) for several seconds. The stalk fragment then was re-cut 0.5 cm below the first section and the new section was processed in the same manner as the first one. Pruning shears were systematically flamed with alcohol between each sample. Growth of *X. albilineans* was recorded after 5 days of incubation of agar plates at 28°C. Pathogen population densities were assessed 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. Visual identification of *X. albilineans* colonies or confluent growth was verified randomly by serology (49). The average score of the two sections per stalk location was used for statistical analysis of data.

Statistical analysis. All statistical analyses were performed with SAS (version 8.2; SAS Institute Inc., Cary, NC). The GIR values were analyzed by ANOVA to compare albicidin production by the different strains of *X. albilineans*. When the null hypothesis in ANOVA was rejected, groupings were determined based on pairwise multiple comparison procedure using the test of Student-Newman-Keuls (SNK). Disease severities and stalk colonization intensities each were compared with the General Linear Models procedure after data rank transformation (13). Groupings were identified with the mean comparison test of SNK.

RESULTS

Variability in albicidin biosynthesis genes. The experimental Xa23R1 banding patterns were in agreement with the theoretical restriction profiles assuming (i) predicted DNA fragments differing in size by less than 28 bp were not distinguishable on experimental banding patterns and (ii) fragments below 0.4 or 0.7 kb were not observed with pBKS/K7 and pALB571 probes, respectively (Fig. 1).

Banding patterns were observed for all strains when the total genomic DNA of the 137 strains of *X. albilineans* was hybridized with probes pALB571 and pBKS/K7 after *HincII* and *PstI* restriction. Banding patterns varied with strains of *X. albilineans* and each banding pattern was assigned to a haplotype. Following restriction with *HincII*, nine haplotypes (H^{571} -A1 to A5 and H^{571} -B1 to B4) and eight haplotypes (H^{K7} -A1 to A7 and H^{K7} -B1) were obtained with probes pALB571 and pBKS/K7, respectively (Tables 1 and 2). The *HincII* banding patterns obtained with the two

probes were combined for each strain, and 14 combined haplotypes (HA1 to HA10 and HB1 to HB4) were generated. These combined haplotypes were distributed into two major genetic groups (ALB-RFLP A and ALB-RFLP B) by cluster analysis (Fig. 2). Similarity among the 74 strains from worldwide locations ranged from 20 to 100%. Group ALB-RFLP A (combined haplotypes HA1 to HA10) included 36 strains that showed strain similarity higher than 75%. Group ALB-RFLP B (combined haplotypes HB1 to HB4) included 38 strains that showed strain similarity higher than 85%. The 63 strains from Guadeloupe that were isolated from 1999 to 2002 had 100% similarity. They all showed the profile of combined haplotype HB2 and, therefore, were ranked in genetic group ALB-RFLP B.

After restriction of genomic DNA of the 137 strains of *X. albilineans* with *PstI*, 10 haplotypes (P^{571} -A1 to A4 and P^{571} -B1 to

Fig. 1. Experimental restriction fragment length polymorphism banding patterns and corresponding sizes (in bp) of theoretical DNA fragments of *Xanthomonas albilineans* strain Xa23R1 after hybridization with molecular probes pALB571 and pBKS/K7, following restriction of total genomic DNA with **A**, *HincII* and **B**, *PstI* enzymes. Molecular probes pALB571 and pBKS/K7 together cover the entire 49-kb albicidin biosynthesis gene cluster and two additional 3-kb genomic regions required for albicidin biosynthesis (all cloned from *X. albilineans* strain Xa23R1). DNA fragments below the size of the smallest fragments shown in the figure were not observed experimentally. FUS are DNA fragments of unpredictable size (unknown flanking sequences).

B6) and 6 haplotypes (P^{K7}-A1 to A6) were obtained with probes pALB571 and pBKS/K7, respectively. In all, 12 combined haplotypes were identified (data not shown). Cluster analysis based on *Pst*I-combined haplotypes resulted in the same genetic groups as those described above for *Hinc*II: ALB-RFLP A and ALB-RFLP B.

Variability in albicidin production. The *E. coli* GIR for 73 strains of *X. albilineans* from worldwide locations and 61 strains from Guadeloupe varied between strains from 0 to 21 mm and from 0.5 to 27 mm, respectively (Table 3). GIR mean values were very similar between the two groups of strains and between the two duplications of each experiment (trials 1 and 2). No significant difference between GIR means and variances of trials 1 and 2 was found for worldwide strains ($P = 0.83$). In contrast, a signifi-

cant difference ($P = 0.04$) was found for the strains from Guadeloupe because of high differences in variances among trials (Table 3). For each group of strains, preliminary analysis revealed high significant differences in albicidin production between strains ($P < 0.0001$). Additionally, occurrence of significant strain-trial interaction ($P < 0.0001$) suggested random or uncontrolled variability of albicidin production by several strains between trials (data not shown). For these reasons, the strain effect in variation source of GIR was analyzed under GLM procedure by a mixed model ANOVA, considering strain-trial interaction as a random source of variation (Table 3). No strain effect ($P = 0.211$) was found for the strains from Guadeloupe, probably because of a high level of strain-trial variation.

Fig. 2. Genetic relatedness of 74 *Xanthomonas albilineans* strains from worldwide locations based on restriction fragment length polymorphism (RFLP). After restriction with enzyme *Hinc*II, total genomic DNA was hybridized with DNA probes pALB571 and pBKS/K7. These two probes together cover the entire 49-kb albicidin biosynthesis gene cluster and two additional 3-kb genomic regions required for albicidin biosynthesis (all cloned from *X. albilineans* strain Xa23R1). Banding patterns obtained with the two probes were combined in 14 linearly combined haplotypes (HA1 to HA10 and HB1 to HB4). Combined haplotypes were used for hierarchical cluster analysis by the unweighted pair group method with arithmetic means. Genetic groups ALB-RFLP A and B were resolved at the 0.75 similarity level (see scale bar at the top left of the figure).

In contrast, the strain effect of strains from worldwide locations was highly significant ($P = 0.0001$) and three albicidin production groups were identified (Table 1): a high production group of 23 strains (group H) with GIR mean values ranging from 15.2 to 19.9 mm, an intermediate production group of 48 strains (group M) with GIR mean values ranging from 10.4 to 15 mm, and a low or weak production group formed by only one strain (group L) with a GIR mean value of 9.5 mm. No albicidin production was measured for strain REU173 (group N) in any of the trials.

A third albicidin production trial was performed with 10 strains from Guadeloupe representative of the variability of albicidin

amount produced in trials 1 and 2 of assay 2. Mean GIR values obtained in the three trials were analyzed, and high significant trial effect ($P < 0.0001$) and strain effect ($P = 0.0072$) were found. The 10 strains were distributed in three different groups of albicidin production based on mean GIR. However, additional differences existed within groups, as shown by statistical data (Table 4).

Variability in pathogenicity based on disease severity. The first leaf scald symptoms were observed on inoculated sugarcane leaves 2 to 4 weeks after inoculation of cv. H70-144 in the greenhouse experiment (trials A and B). However, only symptoms that

TABLE 4. In vitro albicidin production by 10 strains of *Xanthomonas albilineans* from Guadeloupe as determined with a microbiological assay^v

Strains	Albicidin production (GIR)				Group ^z	Variance analysis ^x			
	Mean (square mean) ^w			Exp. mean ^y		DF	Mean square	<i>F</i> value	<i>P</i> > <i>F</i>
	Trial 1	Trial 2	Trial 3						
GPEF4	26.3 (0.4)	15.4 (0.9)	25.7 (1.8)	22.5 a	H	...	...	...	...
GPEDC38	16.0 (0)	16.5 (0.3)	29.7 (0.9)	20.7 b	H	...	...	...	...
GPEG5	11.5 (0)	14.9 (0.1)	25.5 (0.7)	17.3 c	H	...	...	...	...
GPESJ47	13.7 (0.2)	11.1 (0.3)	26.7 (0.6)	17.1 c	H	...	...	...	...
GPET16	9.0 (1.3)	13.3 (0.3)	25.5 (0.3)	15.9 d	H	...	...	...	...
GPEDC61	11.2 (0.2)	14.5 (0)	16.7 (0.2)	14.1 e	M	...	...	...	...
GPE48	5.7 (0.4)	13.1 (0.1)	22.8 (1.2)	13.9 e	M	...	...	...	...
GPEDC23	8.5 (0.7)	13.5 (0)	18.2 (0.2)	13.4 e	M	...	...	...	...
GPEP14B	5.7 (0.8)	8.5 (0.5)	12.2 (0.9)	8.8 f	L	...	...	...	...
GPESJ2	1.0 (0.3)	12.2 (0.3)	12.8 (0.6)	8.7 f	L	...	...	...	...
Source									
Strain (error)	...	...	...	...	...	9 (18)	175.9 (45.7)	3.8	0.0072
Trial (error)	...	...	...	...	...	2 (18)	940.4 (45.6)	20.6	<0.0001
Strain × trial	...	...	...	...	...	18	45.9	59.6	<0.0001
Error	...	...	...	...	...	58	0.8	...	...

^v Growth inhibition ring (GIR) was measured in an experiment with three independent replications (trials 1, 2, and 3).
^w Values are the means of three plates for each strain.
^x Statistical analysis was performed with a mixed model of variance analysis considering the strain-trial interaction as a source of variation.
^y Experiment mean of the three trials. Values followed by the same letter are not significantly different at $P = 0.05$ according to the test of Student-Newman-Keuls.
^z Albicidin production group. Grouping is based on experiment mean GIR value, where H = highly producing group (GIR > 15.0 mm), M = medium-producing group (GIR = 10.1 to 15.0 mm), and L = low-producing group (GIR = 0.1 to 10.0 mm).

Fig. 3. Disease progress curves in sugarcane cv. H70-144 as determined by disease severity on leaves after inoculation with 20 strains of *Xanthomonas albilineans*: **A**, 10 strains from worldwide locations and **B**, 10 strains from Guadeloupe. The experiment was set up in Montpellier (France) in a greenhouse using a randomized complete block design with nine replications of one plant each. Foliar symptoms were scored 4, 6, 8, 10, and 15 weeks after inoculation on noninoculated leaves with a 0-to-5 scale. Each point of disease severity corresponds to the mean score of nine plants. Strains that did not incite any symptom in inoculated plants were not represented on the figure: strains Xa23R1, FJ1080, MTQ078, DOM097, and PNG130 (worldwide origin) and strain GPESJ47 (Guadeloupe). The experiment was duplicated once but only results of trial A are shown in the figure.

subsequently appeared on noninoculated leaves were recorded to differentiate strains of the pathogen. The first leaf scald symptoms on noninoculated leaves were recorded 4 weeks after inoculation. At the end of trial A, 15 weeks after stalk inoculation, 5 of 10 strains from worldwide locations and 9 of 10 strains from Guadeloupe had incited disease symptoms on the leaves (Fig. 3). Great differences in disease severity also were observed 15 weeks after inoculation and disease severity ranged from 0 to 84 and 0 to 78 for strains from worldwide locations and strains from Guadeloupe, respectively (Fig. 3). Disease progress patterns were different among strains, and three disease severity groups were identified based on AUDPC values (Table 5). The first group contained the most aggressive strains for which disease severity increased rapidly up to 10 weeks after inoculation, and then only slightly changed up to 15 weeks after inoculation. At the end of the experiment, 15 weeks after inoculation, disease severity of the most aggressive strains varied from 56 to 84. The second group, the intermediate group, consisted of less aggressive strains. At the end of the experiment, disease severity of these strains ranged from 9 to 20. The third group contained nonaggressive or weakly aggressive strains for which symptoms were never or only occasionally recorded during the experiment. Disease severity of strains from this group was mostly nil and never exceeded a value of 7.

In greenhouse trial B, 7 of 10 strains from worldwide origins and 8 of 10 strains from Guadeloupe incited symptoms on non-

inoculated leaves of cv. H70-144. At the end of the experiment, 15 weeks after inoculation, disease severity differed among strains and ranged from 0 to 60 and 0 to 24 for the strains from worldwide locations and strains from Guadeloupe, respectively. Although disease severity values of trial B were lower than those of trial A, similar disease progress patterns and groupings were obtained, with a few differences (Table 5). Strain GPEG5 from Guadeloupe belonged to the intermediate aggressive group in trial B and to the most pathogenic group in trial A. Strains GPEDC38 from Guadeloupe and BLZ103 from Belize were distributed in the intermediate aggressive group in trial A but in the most aggressive and nonaggressive groups, respectively, in trial B. Strain GPET16 from Guadeloupe was ranked in the nonaggressive group in trial A and in the intermediate group in trial B. Additionally, a few symptoms of leaf scald were recorded for strains DOM097 from Dominican Republic and PNG130 from Papua New Guinea in trial B, but none in trial A. In contrast, strain GPEP14B from Guadeloupe incited a few symptoms in plants of trial A but none in plants of trial B. However, the correlation of data obtained for all strains in trial A and trial B was high ($R = 0.88$), indicating reliability of these results.

Variability in pathogenicity based on stalk colonization. Fifteen weeks after plant inoculation, population densities of the pathogen were determined at three different locations of the sugarcane stalk (SL_0 , SL_4 , and SL_{+10}). The first location (SL_0) was the entry point of the pathogen into the stalk. The second

TABLE 5. Disease severity on leaves and intensity of stalk colonization in sugarcane cv. H70-144 after inoculation with 10 strains of *Xanthomonas albilineans* from worldwide locations (I) and 10 strains of *X. albilineans* from Guadeloupe (II)^a

Strain	Disease severity: AUDPC (rank) ^w		Group	Intensity of stalk colonization: bacterial population density (rank) ^x		Colonization group
	Trial A	Trial B		Trial A	Trial B	
I. Worldwide ^y						
TWN052	465.6 (85.4) a	222.2 (78.1) a	H	3.2 (74.8) a	3.9 (78.4) a	H
HVO082	278.9 (77.6) a	135.0 (61.6) b	H-I	2.6 (65.2) a	3.9 (79.1) a	H
TWN107	30.0 (50.8) b	42.8 (49.2) bc	I-L	2.2 (61.2) ab	2.3 (57.9) bc	I-L
BLZ103	32.8 (54.1) b	15.0 (44.1) c	I-L	0.3 (37.7) c	1.4 (47.8) c	I
BRA115	6.7 (34.6) c	0 (35.0) c	L	0 (33.5) c	0.1 (34.4) d	L
PNG130	0 (30.5) c	3.3 (39.1) c	L	0 (33.5) c	0 (31.0) d	L
DOM097	0 (30.5) c	2.8 (39.0) c	L	1.2 (48.7) bc	0 (31.0) d	I-L
MTQ078	0 (30.5) c	2.2 (38.9) c	L	0 (33.5) c	0 (31.0) d	L
FJI080	0 (30.5) c	0 (35.0) c	L	0 (33.5) c	0 (31.0) d	L
Xa23R1	0 (30.5) c	0 (35.0) c	L	0 (33.5) c	0 (31.0) d	L
II. Guadeloupe ^z						
GPEDC61	372.2 (82.7) a	54.4 (63.9) ab	H	2.8 (65.5) a	3.9 (70.6) a	H
GPEF4	281.1 (74.5) b	107.2 (75.4) a	H	2.6 (63.7) a	3.2 (56.9) ab	H
GPEG5	192.2 (68.5) b	39.4 (51.3) bc	H-I	2.8 (65.2) a	3.6 (61.2) ab	H
GPEDC38	108.8 (56.8) c	54.4 (54.0) abc	I	1.0 (45.9) ab	3.6 (64.2) ab	H-I
GPEDC23	59.4 (49.6) c	11.1 (39.8) bc	I	2.0 (56.3) a	2.7 (50.1) b	H-I
GPET16	8.3 (28.5) d	27.2 (47.3) bc	I-L	0.4 (36.5) b	2.2 (48.0) b	I-L
GPEP14B	6.7 (23.9) d	0 (27.0) c	L	0 (30.5) b	0.4 (26.5) c	L
GPE48	3.3 (23.6) d	6.7 (33.3) c	L	0 (30.5) b	0 (20.5) c	L
GPESJ2	2.2 (22.8) d	11.1 (35.9) c	L	0 (30.5) b	0 (20.5) c	L
GPESJ47	0 (20.5) d	0 (27.0) c	L	0 (30.5) b	0 (20.5) c	L

^a The experiment was set up in a greenhouse in Montpellier (France), using a randomized complete block design with nine replications of one plant each. Trial A and trial B are independent duplications of the experiment. Correlation (R^2) between AUDPC and bacterial population density values was 0.83 (trial A) and 0.91 (trial B) for strains from worldwide locations and 0.88 (trial A) and 0.74 (trial B) for strains from Guadeloupe. Disease severity and colonization groups are based on AUDPC and bacterial population density values, respectively, obtained in trial A and trial B. Abbreviations: H = highly aggressive group, I = intermediate aggressive group, and L = low or nonaggressive group.

^w Area under disease progress curve (AUDPC) was calculated with the scores (0-to-5 scale) of foliar symptoms on noninoculated leaves determined at 4, 6, 8, 10, and 15 weeks after inoculation. Each value of AUDPC corresponds to the mean value of nine plants. Statistical analysis was performed after rank transformation of AUDPC values (13), and each rank value corresponds to the mean value of nine plants. 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 (SNK).

^x Bacterial population densities were determined at three different locations of the sugarcane stalk: SL_0 (considered as the entry point of the pathogen into the sugarcane stalk), SL_4 (fourth internode below SL_0), and SL_{+10} (tenth internode above SL_0) following a 0-to-4 scale. Only results at SL_{+10} are presented in the table. In each column, values followed by the same letter are not significantly different at $P = 0.05$ according to the test of SNK.

^y Variance analysis was performed after rank transformation of AUDPC. Disease severity: strain, $DF = 9$, $P > F = <0.0001$ for trials A and B; bloc, $DF = 8$, $P > F = 0.741$ and 0.299 for trials A and B, respectively; error, $DF = 72$. Intensity: strain, $DF = 9$, $P > F = <0.0001$ for trials A and B; bloc, $DF = 8$, $P > F = 0.109$ and 0.979 for trials A and B, respectively; error, $DF = 72$.

^z Variance analysis was performed after rank transformation of bacterial population density data. Disease severity: strain, $DF = 9$, $P > F = <0.0001$ for trials A and B; bloc, $DF = 8$, $P > F = 0.057$ and 0.974 for trials A and B, respectively; error, $DF = 72$. Intensity: strain, $DF = 9$, $P > F = <0.0001$ for trials A and B; bloc, $DF = 8$, $P > F = 0.369$ and 0.023 for trials A and B, respectively; error, $DF = 72$.

location (SL_{-4}) was situated below SL_0 , at the lower part of the stalk. The third location (SL_{+10}) was situated above SL_0 , at the top of the stalk. SL_0 populations of *X. albilineans* were determined to control efficiency of plant inoculation and colonization of stalks by the pathogen after colonization of inoculated leaves. In trials A and B, SL_0 was colonized by all strains of the pathogen. Control plants did not show any symptoms and were free of *X. albilineans*, as determined by the measurement of pathogen population densities at different stalk locations.

In trial A, populations of *X. albilineans* at SL_{-4} differed between strains from worldwide locations ($P = 0.029$) and between strains from Guadeloupe ($P < 0.0001$). In trial B, these populations differed again between strains from Guadeloupe ($P < 0.0001$) but not between strains from worldwide locations ($P = 0.072$). Correlation of data obtained in the two trials was low, 0.51 and 0.23 for strains from Guadeloupe and strains from worldwide locations, respectively.

In trial A and in trial B, populations of *X. albilineans* at SL_{+10} differed between strains from worldwide locations ($P < 0.0001$) and between strains from Guadeloupe ($P < 0.0001$) (Table 5). Correlation of data obtained in the two trials was 0.91 and 0.85 for strains from worldwide locations and strains from Guadeloupe, respectively. At the end of trial A, 15 weeks after stalk inoculation, 5 of 10 strains from worldwide locations and 6 of 10 strains from Guadeloupe had colonized SL_{+10} (Fig. 4). The 10 strains from worldwide locations and the 10 strains from Guadeloupe were distributed in three and two different groups, respectively, based on pathogen population densities (Table 5). At the end of trial B, 5 of 10 strains from worldwide locations and 7 of 10 strains from Guadeloupe had colonized SL_{+10} (Fig. 4). The 10 strains from worldwide locations and the 10

strains from Guadeloupe were distributed in four and three different groups, respectively, based on pathogen population densities (Table 5).

Genetic and pathogenic characterization of 20 strains of *X. albilineans*. Genetic variability of albicidin biosynthesis genes, amount of albicidin produced in vitro, disease severity, and intensity of stalk colonization were compared for 10 strains from worldwide locations and 10 strains from Guadeloupe (Table 6). High variability in the amount of albicidin produced in vitro (albicidin groups L to H) was shown for strains that shared the same haplotype, particularly for strains from Guadeloupe (combined haplotype HB2). Additionally, strains from worldwide locations (combined haplotypes HA1, HA2, HA8, HA10, HB1, HB2, and HB3) appeared randomly distributed in different albicidin production groups (albicidin groups L to H). The different disease severity and colonization groups (L to H) correlated very well for either strains from worldwide locations or strains from Guadeloupe. Additionally, strains from Guadeloupe (combined haplotype HB2) and strains from worldwide locations (combined haplotypes HA1, HA2, HA8, HA10, HB1, HB2, and HB3) were distributed in at least three disease severity or colonization groups, regardless of haplotype and albicidin group.

DISCUSSION

The recent description and sequencing of genes involved in albicidin production provided the first tools to study variation of genes involved in pathogenicity of *X. albilineans* and variation in disease caused by this sugarcane pathogen. In this study, we explored genetic diversity of genes involved in albicidin production by *X. albilineans* in relation to variation of pathogenicity that was

Fig. 4. Population densities of *Xanthomonas albilineans* in sugarcane stalks of cv. H70-144 determined 15 weeks after inoculation with 10 strains from worldwide locations and 10 strains from Guadeloupe. The experiment was set up in a greenhouse in Montpellier (France) using a randomized complete block design with nine replications of one plant each. Population densities of the pathogen were determined with a 0-to-4 scale at three different locations of the sugarcane stalk: SL_0 (considered as the entry point of the pathogen into sugarcane stalk), SL_{-4} (fourth internode below SL_0), and SL_{+10} (tenth internode above SL_0). Only results at SL_{+10} are shown in the figure. Each bar corresponds to the mean value of nine stalks, and error bars refer to mean square. Strains: 1 = TWN052, 2 = HVO082, 3 = BLZ103, 4 = TWN107, 5 = BRA115, 6 = PNG130, 7 = DOM097, 8 = MTQ078, 9 = FJI080, 10 = Xa23R1 (worldwide origins); strains: 11 = GPEDC61, 12 = GPEF4, 13 = GPEG5, 14 = GPEDC38, 15 = GPEDC23, 16 = GPET16, 17 = GPEP14B, 18 = GPE48, 19 = GPESJ2, and 20 = GPESJ47 (Guadeloupe). Trials A and B are two independent duplications of the experiment.

assessed by albicidin production, disease severity on leaves, and pathogen population densities in the stalk.

Variability in albicidin biosynthesis genes was explored by RFLP and reliability of the technique was shown after comparison of strain Xa23R1 experimental banding patterns with theoretical banding patterns obtained from XALB1, XALB2, and XALB3 nucleotide sequences of this strain. The presence of fragments of unpredictable size (FUS) (Fig. 1) in experimental banding patterns resulted from unknown nucleotide sequences flanking regions XALB1, XALB2, and XALB3 in the genome of *X. albilineans*. RFLP characterization of the albicidin gene cluster of 137 strains of *X. albilineans* (representative of the genetic diversity already described within the species) with probes pALB571 and pBKS/K7 resulted in the identification of 14 combined haplotypes and two major genetic groups (ALB-RFLP A and ALB-RFLP B). The combined haplotypes correlated to the haplotypes of the genetic groups previously identified by Davis et al. (19) and Pilet et al. (42). In group ALB-RFLP A, combined haplotypes HA3, HA4, HA7, HA9, and HA10 are shared by single strains that belong to PFGE groups E, G, F, I, and H, respectively; combined haplotype HA1 is shared by 3 strains, 2 belonging to PFGE group A and 1 of undetermined PFGE group; combined haplotype HA2 is shared by 10 strains, 8 belonging to PFGE group C, 1 belonging to PFGE group J, and 1 of undetermined PFGE group; combined haplotype HA5 is shared by 13 strains, 10 belonging to PFGE group D and 3 of undetermined PFGE group; HA6 is shared by only 1 strain belonging to PFGE group D; and HA8 is shared by 4 strains, 3 belonging to PFGE group E and 1 of undetermined PFGE group. In genetic group ALB-RFLP B, combined haplotype HB1 is shared by 2 strains, HB2 by 96 strains (including 63 strains from Guadeloupe), HB3 by 2 strains, and HB4 belongs to a single strain. Ninety-five of these strains belong to PFGE group B, 5 are of undetermined PFGE group and 1 strain, DOM097 (haplotype HB2), belongs to PFGE group A. All strains that were isolated after the recent outbreaks of leaf scald disease in several locations in the world, such as Cuba (21), Florida (12),

Guadeloupe (49), Louisiana (26), Taiwan (10), and Texas (30), belong to ALB-RFLP and PFGE groups B. Therefore, our results, based on albicidin biosynthesis genes, confirm previous data which showed that recent outbreaks of the disease in several locations were associated with particular genetic variants of *X. albilineans* (19,21). Similar variation exists in genes involved in pathogenicity and other regions of the genome of *X. albilineans*, such as in repetitive DNA sequences (21). RFLP-based techniques have been used successfully to explore genetic variability specifically associated with pathogenicity, at species or pathovar level, of different phytopathogenic bacteria (14,20,24,34,40). However, polymerase chain reaction (PCR)-RFLP analysis of the *hrp* gene region of *Ralstonia solanacearum* showed phylogenetic relationships among strains of the pathogen that were similar to those identified by genomewide RFLP analysis (43). Similarly, pathovar-associated strains of *Pseudomonas syringae* could be identified by either specific probes carrying toxin-producing genes or a PCR-based technique, with primers amplifying enterobacterial repetitive intergenic consensus regions of the genome (36).

In Guadeloupe, the 63 strains of *X. albilineans* isolated between 1999 and 2002 in three sugarcane fields located in different climatic environments (Duclos, Godet, and Saint-Jean) all belonged to ALB-RFLP group B. Bacteria were sampled from symptomatic sugarcane stalks and leaves or from water droplets at the surface of both symptomatic and asymptomatic sugarcane leaves, as described by Daugrois et al. (17). In Guadeloupe, disease leaf symptoms and sugarcane stalk infections can result from epiphytic populations of *X. albilineans* (16,17), and most of the 63 strains of the pathogen were considered to originate from aerial contamination because they all were isolated from sugarcane fields established with disease-free tissue-cultured plants (17). Therefore, recent populations of *X. albilineans* in Guadeloupe appear genetically homogeneous, although several genetic variants, serovars, and pathotypes have been reported in the past on this island (17,19,49).

The origin of the genetic polymorphism revealed in this study could not be clearly identified due to complex banding patterns and the absence of several DNA fragments that could not be observed in our experimental conditions. However, it can be hypothesized that haplotype differentiation based on one or several polymorphic bands within and between ALB-RFLP groups was due to a single nucleotide mutation affecting the recognition site of the restriction enzymes. Different haplotypes of *X. albilineans* produced similar albicidin amounts in vitro. Additionally, RFLP in albicidin biosynthesis genes was not related to variation in albicidin production. The ubiquitous presence of syringomycin in genetically heterogeneous strains of *P. syringae* pv. *syringae* also was reported (36). These results strongly suggest that mutations in albicidin biosynthesis genes are silent mutations or mutations that do not affect the activity of biosynthesis enzymes or that occurred in noncoding regions, assuming that most of the toxin biosynthesis genes are highly conserved in phytopathogenic bacteria and that single nonsilent mutations in toxin-producing genes mostly resulted in nontoxigenic phenotypes (Tox-) (3,27). Because the two albicidin probes hybridized with the genomic DNA of all strains of *X. albilineans*, no large rearrangements, deletions, or insertions occurred in these strains of the pathogen. Additionally, the strains from Guadeloupe which belong to the same haplotype varied in albicidin production.

One strain of *X. albilineans* (REU173) did not produce any detectable albicidin. In this strain, a single nonsilent mutation in one (or more) albicidin gene(s) may be responsible for nontoxigenicity, as reported for toxins produced by other pathogenic bacteria (3) or fungi (55).

Variability in disease severity and intensity of stalk colonization was shown among the 10 strains from worldwide locations and the 10 strains from Guadeloupe, confirming the existence of

TABLE 6. Summary of genetic and pathogenic characteristics of 10 strains of *Xanthomonas albilineans* from worldwide locations and 10 strains of *X. albilineans* from Guadeloupe^w

Strain	Combined haplotype ^x	Albicidin production group ^y	Disease severity group ^z	Colonization group ^z
I. Worldwide				
TWN052	HB3	M	H	H
HVO082	HA2	H	H-I	H
BLZ103	HB2	M	I-L	I-L
TWN107	HB3	M	I-L	I
BRA115	HB1	M	L	L
PNG130	HA10	L	L	L
DOM097	HB2	...	L	I-L
MTQ078	HB2	M	L	L
FJI080	HA8	M	L	L
Xa23R1	HA1	H	L	L
II. Guadeloupe				
GPEDC61	HB2	M	H	H
GPEF4	HB2	H	H	H
GPEG5	HB2	H	H-I	H
GPEDC38	HB2	H	I	H-I
GPEDC23	HB2	M	I	H-I
GPET16	HB2	H	I-L	I-L
GPEP14B	HB2	L	L	L
GPE48	HB2	M	L	L
GPESJ2	HB2	L	L	L
GPESJ47	HB2	H	L	L

^w Abbreviations: H = high, M = medium, I = intermediate, and L = low.

^x Data from Figure 2.

^y Data from Tables 1 and 4 for locations worldwide and from Guadeloupe, respectively.

^z Data from Table 5I and II for locations worldwide and from Guadeloupe, respectively.

different pathotypes within *X. albilineans* (1,17,38). Interestingly, very similar levels of variation in symptom severity and pathogen population density in the sugarcane stalk were observed among strains of *X. albilineans* from different geographical origins and among strains that were isolated recently in a single geographic location (Guadeloupe). Additionally, for both sets of strains, a strong relationship was found between disease severity groups (based on symptom severity on leaves) and stalk colonization groups (based on pathogen population density in the upper part of the sugarcane stalk). This strong relationship was established with 20 different strains of *X. albilineans* and a single sugarcane cultivar. It confirmed the previous correlation between disease severity and stalk population in the upper part of the sugarcane stalk demonstrated with only a single strain of the pathogen and several cultivars differing in resistance to leaf scald (50).

Considerable diversity within the *syf* gene region and gene sub-clusters involved in syringomycin biosynthesis appeared to be involved in specific qualities relevant to plant-pathogen interactions (44). However, with most strains of *X. albilineans*, no obvious relationship was observed between variation in pathogenicity (disease severity or stalk colonization) and either albicidin genetic diversity or variability in albicidin production. Indeed, several weak pathogenic strains (disease severity and colonization groups L) produced low amounts of albicidin (albicidin production group L), but other weak pathogenic strains produced high amounts of the toxin in vitro and were expected to have better propensity to systemically colonize the sugarcane stalk (4). Additionally, strains belonging to either group ALB-RFLP A or B were found in each of the three disease severity groups, and high variability in disease severity was observed for genetically closely related strains from Guadeloupe. Similar variation in aggressiveness of genetically related strains of *R. solanacearum* isolated in a single geographic location was found by different DNA-based methods (28,31). Finally, strain REU173 of *X. albilineans*, that did not produce any detectable albicidin, was able to colonize the sugarcane stalk without inducing appearance of disease symptoms (data not shown).

The role of phytotoxins in the pathogenicity of *P. syringae* has been widely studied and led to contrasted observations. Syringomycin production did not stimulate bacterial growth in planta, whereas coronatine production resulted in significantly higher bacterial populations in mature lesions (27). Syringomycin hyper-producers were either indistinguishable from the wild type or markedly reduced in virulence (27). It is now largely assumed that variation in toxin production is regulated by sophisticated mechanisms in relation to the nutrient environment of phytopathogenic bacteria (9,27,44,56). It was shown recently that variation in syringomycin production by *P. syringae* pv. *syringae* was regulated by genes located outside the toxin biosynthesis gene cluster (37). Another possible feature of these complex mechanisms is the coordinated control of other virulence factors (extracellular polysaccharides and a variety of extracellular enzymes) by the toxin-associated genes (3,27). Similarly, variability in albicidin production and in pathogenicity of *X. albilineans* could not be related to variation in albicidin biosynthesis genes. This variability may be correlated to either albicidin regulation genes not identified so far or to other genes involved in pathogenicity, and particularly stalk colonization by the pathogen. Additionally, the possible existence of races of the pathogen that was reported by several authors (1,38) may affect strains gathering or classification in the different disease severity or stalk colonization groups identified herein. Therefore, inoculation of sugarcane cultivars differing in resistance to the disease would be of interest to look for relationships between variation in pathogenicity and variation in albicidin production or variability of albicidin biosynthesis genes.

The role of toxins to act as virulence factors to increase disease severity or exhibit a function necessary for plant pathogenicity

has been reported in different pathogenic bacteria (25,27). In *X. albilineans*, no *hrp* or *avr* genes have been found up to now (15,35). These genes, coding for essential pathogenicity factors of many plant-pathogenic bacteria, were not identified either in the recently sequenced genomes of two xylem-limited bacteria, *Xylella fastidiosa* and *Leifsonia xyli* subsp. *xyli*, pathogenic to citrus and sugarcane, respectively (39,53). In these pathogens, genes coding for plant cell-wall-degrading factors, exopolysaccharides, extracellular enzymes, adhesion, or other virulence factors probably play major roles in pathogenicity.

The role of albicidin in pathogenicity of *X. albilineans* was confirmed herein. However, variations in albicidin production and in pathogenicity of this pathogen were not related, exclusively, to the diversity of albicidin biosynthesis genes. Therefore, albicidin is necessary but not sufficient for leaf scald development, and it appears to be a virulence factor acting coordinately with other virulence factors. Identification of genes coding for these virulence factors and studying the contribution of these factors to pathogenicity, along with albicidin production, will be the next step in the comprehension of the molecular mechanisms involved in pathogenicity of *X. albilineans*.

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