Applied and Environmental Microbiology, Sept. 2007, p. 5523–5530 Vol. 73, No. 17
0099-2240/07/$08.00+0 doi:10.1128/AEM.00577-07

Substrate Specificity-Conferring Regions of the Nonribosomal Peptide Synthetase Adenylation Domains Involved in Albicidin Pathotoxin Biosynthesis Are Highly Conserved within the Species *Xanthomonas albilineans*▽

Adeline Renier,[1] Eric Vivien,[1] Stéphane Cociancich,[1] Philippe Letourmy,[2] Xavier Perrier,[3] Philippe C. Rott,[1] and Monique Royer[1]*

UMR CIRAD-INRA-Montpellier SupAgro Biologie et Génétique des Interactions Plante-Parasite, Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Campus International de Baillarguet,[1] and UPR Biostatistique[2] and UPR Multiplication Végétative,[3] Centre de Coopération Internationale en Recherche Agronomique pour le Développement, Avenue Agropolis, Montpellier, France

Received 13 March 2007/Accepted 3 July 2007

Albicidin is a pathotoxin produced by *Xanthomonas albilineans*, a xylem-invading pathogen that causes leaf scald disease of sugarcane. Albicidin is synthesized by a nonribosomal pathway via modular polyketide synthase and nonribosomal peptide synthetase (NRPS) megasynthases, and NRPS adenylation (A) domains are responsible for the recognition and activation of specific amino acid substrates. DNA fragments (0.5 kb) encoding the regions responsible for the substrate specificities of six albicidin NRPS A domains from 16 strains of *X. albilineans* representing the known diversity of this pathogen were amplified and sequenced. Polymorphism analysis of these DNA fragments at different levels (DNA, protein, and NRPS signature) showed that these pathogenicity loci were highly conserved. The conservation of these loci most likely reflects purifying selective pressure, as revealed by a comparison with the variability of nucleotide and amino acid sequences of two housekeeping genes (*atpD* and *efp*) of *X. albilineans*. Nevertheless, the 16 strains of *X. albilineans* were differentiated into several groups by a phylogenetic analysis of the nucleotide sequences corresponding to the NRPS A domains. One of these groups was representative of the genetic diversity previously found within the pathogen by random fragment length polymorphism and amplified fragment length polymorphism analyses. This group, which differed by three single synonymous nucleotide mutations, contained only four strains of *X. albilineans* that were all involved in outbreaks of sugarcane leaf scald. The amount of albicidin produced in vitro in agar and liquid media varied among the 16 strains of *X. albilineans*. However, no relationship among the amount of albicidin produced in vitro and the pathotypes and genetic diversity of the pathogen was found. The NRPS loci contributing to the synthesis of the primary structure of albicidin apparently are not involved in the observed pathogenicity differences among strains of *X. albilineans*.

Xanthomonas albilineans is a systemic, xylem-invading pathogen that causes leaf scald disease, one of the major diseases of sugarcane (interspecific hybrids of *Saccharum* spp.) (24, 25). Leaf scald symptoms include leaf chlorosis, necrosis, wilting, and plant death. Since the end of the 1980s, outbreaks of the disease in several sugarcane-producing locations, such as the Dominican Republic, France (Guadeloupe), Mauritius, Taiwan, and the United States (Florida, Louisiana, and Texas), have occurred. To understand the nature of these outbreaks, a first study of the worldwide genetic variation of the pathogen by pulsed-field gel electrophoresis (PFGE) was undertaken. A total of 218 strains from 31 geographic locations were distributed among eight groups, and the recent disease outbreaks were associated with strains of the pathogen belonging to a single group called PFGE group B (14).

X. albilineans produces a toxin called albicidin that has phytotoxic and antibiotic properties (1). Albicidin targets chloroplastic DNA gyrase (17) and plays a key role in pathogenesis because albicidin-defective mutants are no longer able to cause disease symptoms (2, 5, 6). This pathotoxin inhibits chloroplast DNA replication, blocks chloroplast differentiation, and is therefore responsible for the characteristic chlorotic leaf streaks and whitening of the foliage (1). Additionally, albicidin inhibits prokaryotic DNA replication and is bactericidal to a range of gram-positive and gram-negative bacteria (3, 4). Low yields of albicidin production by *X. albilineans* (0.2 mg of purified toxin per liter of culture) have slowed studies into the purification and characterization of the toxin, and the chemical structure of albicidin remains unknown (1).

Recently, the entire albicidin biosynthetic gene cluster from *X. albilineans* strain Xa23R1 from Florida was cloned and sequenced (27, 28). This cluster contains 20 open reading frames (*albI* to *albXX*), including three large polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) genes. Transposon mutagenesis and complementation studies demonstrated that these three PKS and NRPS genes are required for albicidin production (18, 27, 28). PKS and NRPS are megasynthases organized into repeated functional units known

* Corresponding author. Mailing address: UMR BGPI, CIRAD, TA A-54/K, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France. Phone: 33 (0)4 99 62 48 44. Fax: 33 (0)4 99 62 48 08. E-mail: monique.royer@cirad.fr.

▽ Published ahead of print on 13 July 2007.

TABLE 1. Modules, domains, NRPS signatures, and predicted NRPS substrates of megasynthases encoded by the three albicidin PKS and NRPS genes (*albI*, *albIV*, and *albIX*)

Megasynthase (size [aa])[a]	Module	Domain(s)[b]	Amino acid or aryl acid NRPS signature[c]	Predicted NRPS substrate
AlbI (6,879)	PKS-1	AL-ACP		
	PKS-2	KS-KR-ACP-ACP		
	PKS-3	KS-PCP		
	NRPS-1	C-A-PCP	AVKYVANDAK*	Unknown
			AFVYVANDAK**	Unknown
	NRPS-2	C-A?-PCP		
	NRPS-3	C-A-PCP	AVKYVANDAK*	Unknown
			AFVYVANDAK**	Unknown
	NRPS-4	C		
AlbIV (941)	NRPS-5	A-PCP	DLTKIGEVGK*	Asparagine
AlbIX (1,959)	NRPS-6	A-PCP	AIKYFSIDMK*	Unknown
			AFVYFSIDMK**	Unknown
	NRPS-7	C-A-PCP-TE	AIKYFSIDMK*	Unknown
			AFVYFSIDMK**	Unknown

[a] aa, amino acids.
[b] NRPS and PKS domains are abbreviated as follows: AL, acyl coenzyme A ligase; ACP, acyl carrier protein; KS, ketoacyl synthase; KR, ketoreductase; PCP, peptidyl carrier protein; C, condensation; and TE, thioesterase. A? indicates that the deleted A domain in NRPS-2 may be not functional.
[c] *, amino acid signature; **, aryl acid signature.

as modules, each of which is responsible for a discrete stage of polyketide or polypeptide chain elongation (7). Moreover, each module comprises a series of catalytic domains which act sequentially to assemble and modify the molecule. Among these domains, the acyltransferase domains and the adenylation domains (A domains) in PKS and NRPS, respectively, play an important role in the recognition and activation of a specific substrate because of their specificity for their cognate substrates (7, 15, 31). The substrate binding pocket of the phenylalanine NRPS A domain of the gramicidin S synthetase (GrsA) from *Brevibacillus brevis* was identified previously by crystal structure analysis as a stretch of about 100 amino acid residues between highly conserved motifs A4 and A5 (13). Based on sequence analyses of known A domains in relation to the crystal structure of the GrsA (Phe) substrate binding pocket, two similar models have been published to predict the amino acid substrate recognized by an unknown NRPS A domain (8, 32). These predictive models postulate specificity-conferring signatures for NRPS A domains consisting of critical amino acid residues putatively involved in amino acid substrate specificity. In addition, based on the crystal structure of an aryl acid-activating domain, an aryl acid specificity NRPS signature within the A4-A5 region of aryl acid-specifying NRPS modules was also identified previously (21).

The three albicidin PKS and NRPS genes (*albI*, *albIV*, and *albIX*) of strain Xa23R1 encode six albicidin NRPS A domains (Table 1) (28): (i and ii) AlbI module NRPS-1 and AlbI module NRPS-3 A domains that are 96.4% identical in the A4-A5 region (these two domains carry the same NRPS signatures, which did not match any defined amino acid or aryl acid code); (iii) an AlbI module NRPS-2 A domain that contains an incomplete and nonfunctional A4-A5 region; (iv) an AlbIV module NRPS-5 A domain that carries an asparagine signature; and (v and vi) AlbIX module NRPS-6 and AlbIX module NRPS-7 A domains that are 100% identical in the A4-A5 region (these two domains carry the same NRPS signatures, which also did not match any defined amino acid or aryl acid code). Detailed analyses of AlbI and AlbIX NRPS-1, NRPS-3, NRPS-6, and NRPS-7 signatures suggested that the four corresponding modules specify unknown nonproteinogenic aromatic substrates (28).

The genetic variation among albicidin biosynthetic genes was first characterized by restriction fragment length polymorphism (RFLP) analysis. Total genomic DNA from 137 strains of *X. albilineans* from worldwide locations was hybridized with two DNA probes harboring the entire albicidin biosynthetic gene cluster (9). Fourteen haplotypes and two major genetic groups (albicidin gene RFLP [ALB-RFLP] group A and ALB-RFLP group B) were identified, and strains that were isolated after recent outbreaks of leaf scald disease belong to group ALB-RFLP group B. Albicidin genetic diversity is very similar to the previously described genetic diversity of the pathogen based on the whole genome (14). Although no relationship between variation in albicidin biosynthetic genes and variation in the pathogenicity of *X. albilineans* strains was found (9, 10), these results showed that outbreaks of sugarcane leaf scald were linked to variation in the gene sequences coding for albicidin biosynthesis.

Because albicidin is a major factor involved in the pathogenicity of *X. albilineans* and because the albicidin primary structure is dictated partly by the substrate specificity-conferring regions of the six albicidin NRPS A domains, we investigated the polymorphism of these regions in 16 strains of the pathogen. Variation in the corresponding loci was then compared to variation in two housekeeping genes (*atpD* and *efp*) of *X. albilineans* and to variation in the amounts of albicidin produced in vitro by strains of the pathogen.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The sources of bacterial strains and their relevant characteristics are given in Table 2. *X. albilineans* strains were cultured routinely on modified Wilbrink's medium at 28°C without benomyl (26). *Escherichia coli* strains were grown on Luria-Bertani agar or in Luria-Bertani broth at 37°C and were maintained and stored according to standard protocols (30).

Design of PCR primers. Primer pairs were designed to amplify 0.5-kb DNA fragments encoding the substrate specificity-conferring regions of the six albicidin NRPS A domains based on the *albI*, *albIV*, and *albIX* sequences of *X. albilineans* strain Xa23R1 (GenBank accession number AJ586576). The NRPS gene primers corresponded to each side of the A4-A5 regions, which include all key residues of the NRPS signatures (28, 32). The nucleotide sequences encoding AlbI NRPS-1 and AlbI NRPS-3 A domains are 96.4% identical, and those encoding AlbIX NRPS-6 and AlbIX NRPS-7 A domains are 100% identical. Therefore, single amplification products corresponding to both AlbI NRPS-1 and AlbI NRPS-3 (referred to hereinafter as NRPS-1/3) and to both AlbIX NRPS-6 and AlbIX NRPS-7 (referred to hereinafter as NRPS-6/7) were expected. Primer pairs to amplify housekeeping genes *atpD* and *efp* were designed by S. Boudon (personal communication) from the genome sequence of *X. axonopodis* pv. *citri* (GenBank accession number NC_003919). Primers were purchased from Eurogentec (Seraing, Belgium) and are listed in Table 3.

PCR conditions. DNA templates were prepared by suspending a freshly grown colony in 100 μl of sterilized water. PCR amplifications were performed with the automated thermal cycler PTC-100 (MJ Research, Inc., Watertown, MA). The 25-μl PCR mix consisted of 5 μl of the bacterial suspension, 2.5 μl of 10× PCR buffer without $MgCl_2$ (Eurobio, Les Ulis, France), 120 μM deoxynucleoside triphosphate mix, 2.0 mM $MgCl_2$ (Eurobio), 0.5 μM (each) primers, 1 U of EurobioTaqII (Eurobio), and the amount of sterilized distilled water required to obtain the final volume. The PCR program was 95°C for 2 min; 25 cycles at 95°C for 1 min, the melting temperature (T_m) for 1 min, and 72°C for 1 min; and a final 72°C extension for 5 min. T_ms were calculated with the OligoAnalyser 3.0 program (Integrated DNA Technologies, Inc., Coralville, IA) and were tested and optimized for PCR amplification. For each pair of primers, the optimized T_m

TABLE 2. Origins and characteristics of *X. albilineans* strains

Strain	Origin	Year of isolation	Author who first characterized strain	Pathogenicity in sugarcane cv. H70–144[a]	PFGE group (haplotype)[b]	Serovar[c]	ALB-RFLP group (haplotype)[a]	NRPS phylogenetic group[d]	NRPS-2 phylogenetic group[d]	*atpD* phylogenetic group[d]	Albicidin production[e] in:	
											Agar medium	Liquid medium
LS156R1[f]	Australia		R. Birch					I	I	I	N	N
BRA115	Brazil	1993	C. O. N. Cardoso	Low	B (B-11)	3	B (HB1)	III	III	II	L	L-N
HVO005	Burkina Faso	1980	M. Chatenet		F (F-01)	2	A (HA7)	II	II	II	H	L
HVO082	Burkina Faso	1989	M. Granier	High	C (C-04)	2	A (HA2)	II	II	II	H	N
DOM097	Dominican Republic	1991	M. J. Davis		A (A-01)	1		II	II	II	H-M	M
Xa23R1[g]	Florida	1993	M. J. Davis	Low	A (A-03)	1	A (HA1)	I	I	I	H	H-M
FIJ080	Fiji	1961	D. W. Dye	Low	E (E-02)	3	A (HA8)	I	I	I	H-M	M
GLP056	Guadeloupe	1988	P. Rott		B (B-01)	1	B (HB2)	III	III	II	M	H
MTQ058	Martinique	1989	P. Rott		I (I-01)	3	A (HA9)	I	I	I	H-M	N
MTQ078	Martinique	1957	J. A. Spence	Low	B (B-01)	1	B (HB2)	III	III	II	M	H-M
PNG130	Papua New Guinea	1993	M. Chatenet	Low	H (H-01)	2	A (HA10)	I	I	I	M	L-N
REU173	Réunion Island	1995	J.-C. Girard	Nil	D (D-17)	1	A (HA6)	II	II	II	N	N
REU174	Réunion Island	1995	J.-C. Girard		D (D-15)	1		II	II	II	M	L-N
REU209	Réunion Island	1995	J.-C. Girard		J (J-01)	1	A (HA2)	II	II	II	H-M	L
LKA070	Sri Lanka	1962	A. C. Hayward		G (G-01)	3	A (HA4)	II	II	II	M	L
TWN052	Taiwan	1988	C. T. Chen	High	B (B-01)	1	B (HB3)	III	III	II	H	H-M

[a] Data are from Champoiseau et al. (reference 9 and unpublished data). No data for strain DOM097 are given because the information in reference 9 corresponds to a mislabeled strain.
[b] Data are from Davis et al. (14) and P. Rott et al. (unpublished data).
[c] Data are from Yang et al. (34) and Davis et al. (14).
[d] Data are from this study.
[e] Data are from this study. H, high; M, medium; L, low; N, none. The data on the production of albicidin are based on the *E. coli* GIR diameters on agar medium and on the MIDs for *E. coli* growth in liquid medium (Tables 5 and 6).
[f] Spontaneously Rif^r derivate of LS156 (LS155-*xabA*::Tn*5* Km^r St^r Tox$^-$ [18]). Km^r, Rif^r, and St^r indicate resistance to kanamycin, rifampin, and streptomycin, respectively. Tox$^-$, deficient in albicidin production.
[g] Spontaneously Rif^r derivate of wild-type Xa23 from sugarcane (Florida) (27).

varied between 52 and 54°C. A 5-μl aliquot of each amplified product was analyzed by electrophoresis through a 1.5% agarose gel.

DNA sequencing. The automated sequencing of PCR amplicons was carried out with double-stranded DNA by dideoxynucleotide chain termination using an ABI BigDye terminator cycle sequencing kit and an automated sequencer according to the procedure specified by the manufacturer (Perkin-Elmer). Both strands were sequenced with the primers used to amplify the PCR products. This service was provided by Genome Express (Grenoble, France).

Sequence analysis. DNA sequences corresponding to the four A domains and those of the two housekeeping genes were edited with BioEdit software (16) and aligned with DNAMAN software (version 5.2.2; Lynnon Biosoft, Vaudreuil, Quebec, Canada). Because the DNA fragments corresponding to the substrate specificity-conferring regions of NRPS-1 and NRPS-3 were amplified with the same set of primers, their respective sequences could not be differentiated. Therefore, in all NRPS-1/3 sequences of the 16 strains of *X. albilineans*, the 14 nucleotides known to vary between the NRPS-1 and NRPS-3 sequences were replaced with the corresponding letter of the degenerate DNA alphabet. Percentages of identity and homology, mean numbers of differences between sequences per synonymous nucleotide position (Ks) and per nonsynonymous position (Ka), and the Ka/Ks ratios were calculated with Swaap 1.0 software as described by Pride and Blaser (23).

Phylogenetic analyses. Aligned sequences were analyzed with DARwin 4.0 software (22). The Jukes-Cantor model (20) was used to correct distances for multiple substitutions, and phylogenetic trees were constructed by the neighbor-joining method (29). The robustness of nodes of the phylogenetic trees was assessed based on 1,000 bootstrap resamplings, and bootstrap values were used as labels for internal nodes of the complete trees. Bootstrap values and positions in the phylogenetic trees were used to assign the bacterial strains to different clusters.

Albicidin production assays and experimental designs. Albicidin production by strains of *X. albilineans* was tested with two microbiological assays based on the activity of albicidin against *E. coli*. The first assay was performed with agar plates as previously described (27), and the second assay was performed with

TABLE 3. Primers used for PCR and sequencing and characteristics of sequenced fragments

Gene, module, region	Source strain (accession no.)	Primer name	Primer sequence (5′–3′)	Exptl T_m (°C)	Length of sequenced fragment (bp)
albI, NRPS-1/3, A4-A5	*X. albilineans* Xa23R1 (AJ586576)	NRPS1-For	TATGCGCAGCTGTACAACTG	54	455
		NRPS1-Rev	AGCACGTATACCTCGGTGTT		
albI, NRPS-2, A4-A5	*X. albilineans* Xa23R1 (AJ586576)	NRPS2-For	TTCTGCCGAACTGGCGAATA	54	474
		NRPS2-Rev	GGCTGTGTGATCCACAATTC		
albIV, NRPS-5, A4-A5	*X. albilineans* Xa23R1 (AJ586576)	NRPS5-For	ATTGCATCTATACCTCCGGC	54	592
		NRPS5-Rev	GGCTTGTTCACATAGCCGAT		
albIX, NRPS-6/7, A4-A5	*X. albilineans* Xa23R1 (AJ586576)	NRPS6-For	CCTACGTGATGTATACCTCC	54	524
		NRPS6-Rev	TTGTTCGTCGAGCACATGGA		
atpD	*X. axonopodis* pv. *citri* (NP_643956)	atpD-For	GGGCAAGATCGTTCAGAT	52	833
		atpD-Rev	GCTCTTGGTCGAGGTGAT		
efp	*X. axonopodis* pv. *citri* (NP_642696)	efp-For	GTCAAGAACGGCATGAAGA	54	455
		efp-Rev	TCGTCCTGGTTGACGAAC		

TABLE 4. Sequence polymorphism of gene fragments corresponding to four NRPS A domains and two housekeeping genes of 16 strains of *X. albilineans*[a]

Gene, module, region	No. of allelic variants	% Identity	% Homology	Ks	Ka	Ka/Ks ratio
albI, NRPS-1/3, A4-A5	4	99.75 ± 0.24	99.83 ± 0.30	0.45 ± 0.45	0.07 ± 0.13	0.162 ± 0.162
albI, NRPS-2, A4-A5	5	99.43 ± 0.39	99.73 ± 0.29	2.14 ± 1.71	0.12 ± 0.13	0.055 ± 0.086
albIV, NRPS-5, A4-A5	4	99.69 ± 0.33	99.83 ± 0.25	1.03 ± 1.09	0.08 ± 0.11	0.074 ± 0.064
albIX, NRPS-6/7, A4-A5	8	99.33 ± 0.47	99.93 ± 0.18	2.75 ± 2.02	0.03 ± 0.08	0.011 ± 0.056
atpD	4	98.46 ± 1.63	99.79 ± 0.23	6.76 ± 7.16	0.09 ± 0.1	0.014 ± 0.01
efp	0	100	100	0	0	0

[a] Values given with "±" are means ± standard deviations.

liquid medium. The same inoculum was used to start both assays as follows. Ten microliters of 110-μl precultures of *X. albilineans* in saccharose-peptone (SP) medium (2% sucrose, 0.5% peptone) at an optical density at 600 nm of 0.1 (approximately 10^9 CFU/ml) was spotted onto each plate of saccharose-peptone-agar medium (2% sucrose, 0.5% peptone, and 1.5% agar). The remaining 100 μl of each preculture was deposited into 25 ml of SP medium.

Agar plates were distributed in a growth chamber at 28°C by using a randomized complete block design with four replications and were incubated for 5 days. Plates were then overlaid with a mixture of *E. coli* DH5α (10^7 cells in 2 ml of distilled water) and 2 ml of molten 1.5% (wt/vol) Noble agar (Difco) at approximately 65°C and examined for growth inhibition zones after 24 h at 28°C. The bioassay was repeated independently three times.

Tubes inoculated with SP medium were incubated under agitation at 120 rpm at 28°C for 5 days. Two-milliliter aliquots of bacterial cultures were then centrifuged for 3 min at 11,000 × *g*, and 1.5-ml aliquots of the supernatant were diluted 2-, 4-, and 10-fold. Fifty microliters of undiluted supernatant and 50 μl of each dilution were deposited into wells of a 96-well microtitration plate (but only the 60 inner wells of each plate were used). Fifty microliters of a 200-times-diluted culture of *E. coli* DH5α at an optical density at 600 nm of 0.4 was then added to each well. The supernatant dilution series corresponding to each strain of *X. albilineans* were distributed among the wells of the microtitration plates by using a randomized incomplete block design with four replications. Each microtitration plate represented one block, and two plates constituted one replication of 16 strains of *X. albilineans*. Microtitration plates under agitation at 120 rpm were incubated at 28°C for 24 h and then examined for the maximum inhibitory dilution (MID). The optical density of the contents of each well was determined using an MRX microplate reader (Dynatech Laboratories), and the MID was defined as the last dilution at which no growth of *E. coli* DH5α was detected. The bioassay was repeated independently three times.

Statistical analysis. All bioassay data were subjected to analysis of variance using Tukey's range test with version 13 software (Minitab Inc., Paris, France).

RESULTS

Variability of NRPS A domains. Four to eight allelic variants (sequences differing by at least one nucleotide) corresponding to each NRPS module were identified among the 16 strains of *X. albilineans* (Table 4). No deletion or insertion in any of the gene fragments was found when sequences were compared to the sequence of reference strain Xa23R1, and substitutions were distributed all across the DNA sequences. Percentages of identity and homology of the NRPS A domains (A4-A5 regions) of the 16 strains of *X. albilineans* varied from 99.33% (module NRPS-6/7) to 99.75% (module NRPS-1/3) and from 99.73% (module NRPS-2) to 99.93% (module NRPS-6/7), respectively (Table 4). Mean numbers of differences between sequences per synonymous nucleotide position (Ks) and per nonsynonymous nucleotide position (Ka) varied from 0.45 to 2.75 and from 0.03 to 0.12, respectively. The Ka/Ks ratio was clearly below 1 whatever the locus, and it varied between 0.01 and 0.16.

Variability of housekeeping genes. Four allelic variants of the *atpD* gene (encoding the B subunit of the F_oF_1 ATPase complex) were found, but no variants of the *efp* gene (encoding elongation factor P) were discovered (Table 4). The percentages of identity of the *atpD* and *efp* genes were 98.46 and 100%, respectively. The percentages of homology of the *atpD* and *efp* genes were 99.79 and 100%, respectively. The Ks and Ka were 6.76 and 0.09 for the *atpD* gene, respectively, and nil for the *efp* gene. The Ka/Ks ratio for the *atpD* gene was 0.014.

Diversity of sequences and clustering of strains of *X. albilineans*. Although the degree of genetic variation was low, the 16 strains of *X. albilineans* were distributed among several groups and subgroups by phylogenetic analysis with the sequence of each NRPS module or the concatenated sequence of the four modules. The topologies of the phylogenetic trees varied slightly according to each module (data not shown). Strain grouping was, however, very similar for NRPS-2 sequences and the concatenated sequence of the four NRPS modules, and the 16 strains of the pathogen were distributed among three major groups (Fig. 1): group I contained five strains (MTQ058, FIJ080, Xa23R1, LS156R1, and PNG130), group II comprised seven strains (HVO005, LKA070, HVO082, DOM097, REU173, REU174, and REU209), and group III contained four strains (TWN052, GLP056, MTQ078, and BRA115). All strains of group III belonged to PFGE group B and to ALB-RFLP group B (Table 2). The 16 strains of *X. albilineans* were distributed between two groups by phylogenetic analysis with the sequence of the *atpD* housekeeping gene (Fig. 2): group I contained five strains (MTQ058, FIJ080, Xa23R1, LS156R1, and PNG130), and group II included the remaining 11 strains. Additionally, all sequences of strains LS156R1 (from Australia) and Xa23R1 (from Florida) were identical, whatever the locus. The same result for strains FIJ080 (from Fiji) and MTQ058 (from Martinique) was observed.

When reference strain Xa23R1 was compared to the 15 other strains of *X. albilineans*, seven polymorphic nucleotides in the sequence corresponding to that flanked by motifs A4 and A5 of the NRPS A domain of module NRPS-2 were identified. Among these nucleotides, three specific synonymous substitutions were closely related to the distribution of strains TWN052, GLP056, MTQ078, and BRA115 into NRPS-2 phylogenetic group III, PFGE group B, and ALB-RFLP group B (Table 2). Concurrently, these substitutions were related to the distribution of the other 11 strains into NRPS-2 phylogenetic groups I and II, several PFGE groups, and ALB-RFLP group A. This result was confirmed by an additional study of the NRPS-2 sequences of 32 strains of *X. albilineans* from various geographical locations, including 12

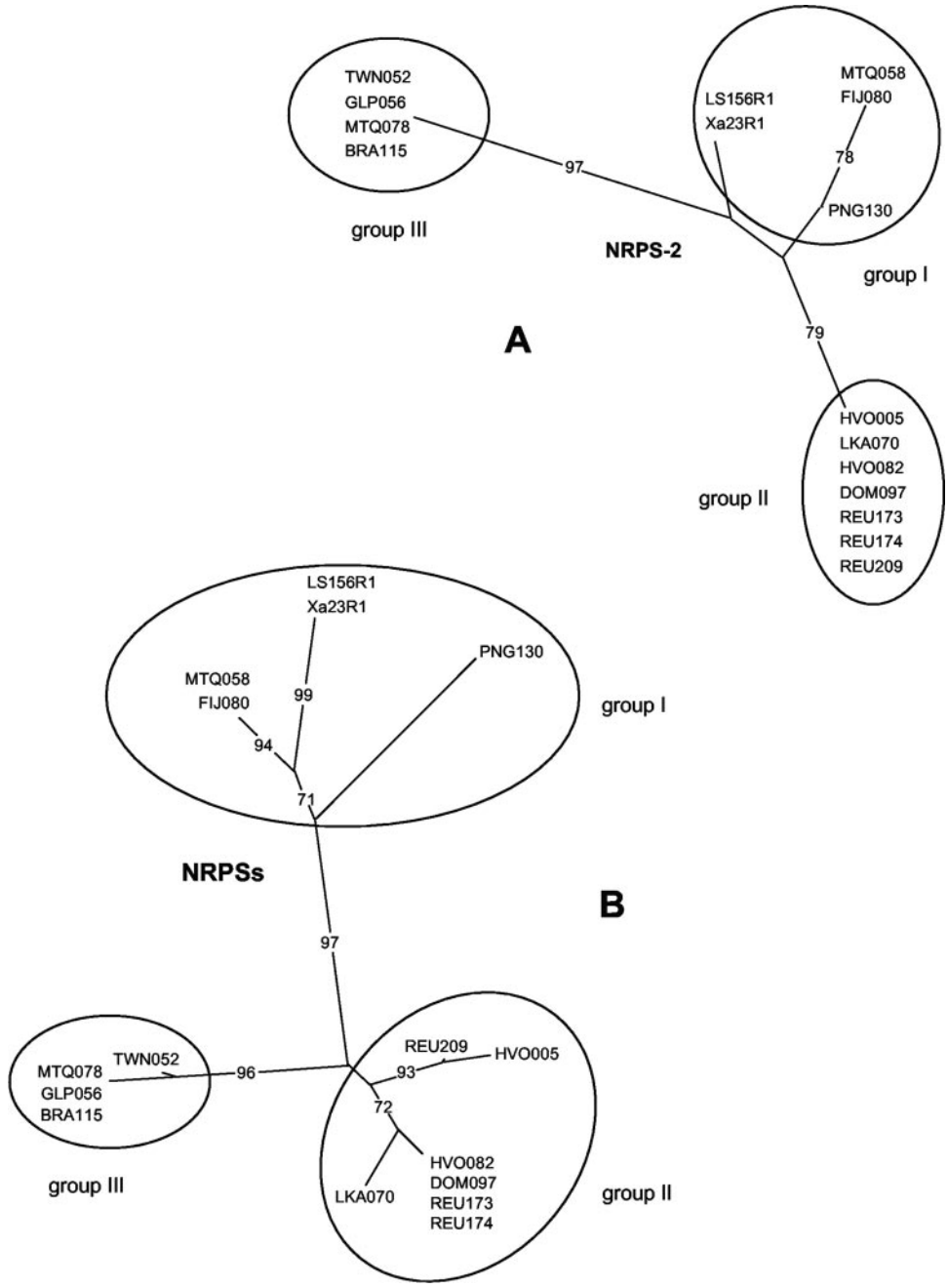

FIG. 1. Neighbor-joining trees for *Xanthomonas albilineans* constructed by using the A domains of the NRPS-2 modules (A) and the concatenated A domains of NRPS modules 1/3, 2, 5, and 6/7 (B) of 16 strains. The trees are based on distances corrected according to the Jukes-Cantor model, and only bootstrap values higher than 70% (from 1,000 bootstrap resamplings) are displayed at nodes.

strains belonging to several PFGE groups and ALB-RFLP group A and 20 strains belonging to PFGE group B and ALB-RFLP group B (data not shown).

Variability of the selectivity-conferring codes of albicidin NRPSs. The selectivity-conferring codes (the amino acid NRPS signatures or the aryl acid NRPS signatures) of albicidin synthases AlbI, AlbIV, and AlbIX in all 16 strains of *X. albilineans* were identical, with one exception. The NRPS signature of NRPS-1 in strain LKA070 from Sri Lanka differed from the signature of the other 15 strains by the replacement of a valine with an alanine at the ninth amino acid position.

Albicidin production. In the agar bioassay, the diameters of the *E. coli* growth inhibition rings (GIRs) varied from 0 to 18.7 mm among the 16 strains of *X. albilineans*, and four albicidin production groups were identified (Table 5): a high-level-production group of eight strains corresponding to mean GIR diameters ranging from 15.3 to 18.7 mm, an intermediate-level-production group of five strains corresponding to mean

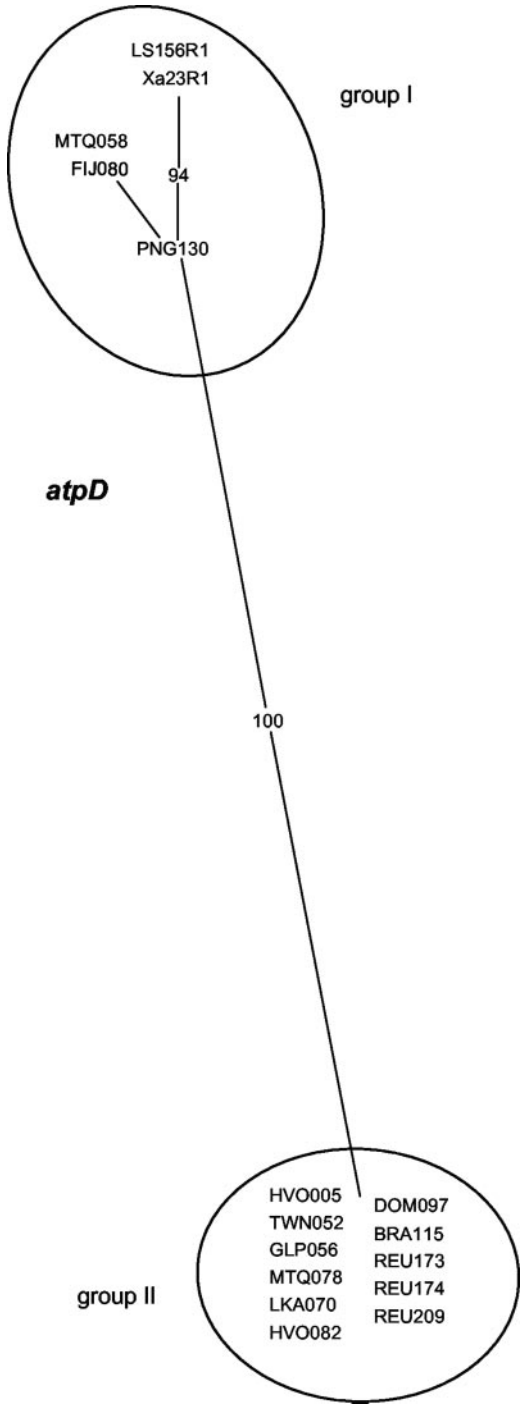

FIG. 2. Neighbor-joining tree of *Xanthomonas albilineans* constructed by using the *atpD* genes of 16 strains. The tree is based on distances corrected according to the Jukes-Cantor model, and only bootstrap values higher than 70% (from 1,000 bootstrap resamplings) are displayed at nodes.

GIR diameters ranging from 12.8 to 14.4 mm, and a low-level-production group comprising only one strain corresponding to a mean GIR diameter of 9.3 mm. No albicidin production by two strains, LS156R1 and REU173, was detected in any of the agar assays. Among strains of the high-level-production group, reference strain Xa23R1 produced the highest level of albicidin.

In the liquid medium assay, the mean MIDs for the 16 strains of *X. albilineans* varied from 0- to 5.3-fold, and four groups were identified (Table 6): a high-level-production group of four strains for which mean MIDs ranged from 3.4- to 5.3-fold; an intermediate-level-production group of two strains for which mean MIDs were 2.0- and 2.1-fold, and a low-level-production group comprising six strains for which mean MIDs ranged from 0.1- to 0.7-fold. No albicidin production by four strains, MTQ058, HVO082, LS156R1, and REU173, was detectable.

Relationship between genetic variation and variation in pathogenicity and metabolic characteristics of 16 strains of *X. albilineans*. The degrees of genetic variability of key sequences of albicidin biosynthesis genes and the amounts of albicidin produced in vitro from 16 strains from worldwide locations were compared (Tables 2 and 6). High degrees of variability in the amounts of albicidin produced in vitro (high-level production to no production) by strains that were ranked in the same PFGE group, serovar, NRPS phylogenetic group, or *atpD* phylogenetic group were shown. For example, strains BRA115 (from Brazil), GLP056 (from Guadeloupe), MTQ058 (from Martinique), and TWN052 (from Taiwan) all belonged to PFGE group B, ALB-RFLP group B, and NRPS-2 phylogenetic group III, but their levels of albicidin production varied from low to high. Similarly, strains belonging to the same PFGE group, serovar, NRPS phylogenetic group, or *atpD* phylogenetic group showed variation in pathogenicity. For example, strains BRA115, MTQ078, and TWN052, which belonged to the same genetic groups, differed in levels of pathogenicity from low to high (Table 2).

TABLE 5. Albicidin production in agar medium by 16 strains of *X. albilineans*

Strain	GIR diam[a] (mm)	Production group[b]
Xa23R1	18.7 a	H
HVO005	17.2 b	H
HVO082	17.1 b	H
TWN052	16.9 b	H
DOM097	16.6 bc	H-M
MTQ058	16.1 bc	H-M
FIJ080	15.9 bc	H-M
REU209	15.3 bcd	H-M
REU174	14.4 cd	M
GLP056	14.4 cd	M
LKA070	14.1 d	M
MTQ078	13.3 d	M
PNG130	12.8 d	M
BRA115	9.3 e	L
LS156R1	0 f	N
REU173	0 f	N

[a] Values are the means of results for 12 plates from three independent assays of four plates each. Values followed by the same letter(s) are not significantly different at P of 0.05 according to Tukey's test.

[b] Grouping is based on mean GIR diameters and statistical results. Abbreviations: H, high (GIR diameter, >15.0 mm); M, medium (GIR diameter, 10.1 to 15.0 mm); L, low (GIR diameter, 0.1 to 10.0 mm); N, no albicidin production (GIR diameter, 0 mm). Strains attributed to two groups (H-M) are strains that belong to the high-level-production group based on the numerical measurement of albicidin production but for which this numerical value was not significantly different from values for strains of the medium-level-production group after statistical analysis of data.

TABLE 6. Albicidin production in liquid medium by 16 strains of *X. albilineans*

Strain	MID (*n*-fold) (rank)[a]	Production group[b]
GLP056	5.3 (15.1) a	H
MTQ078	3.8 (14.1) ab	H-M
TWN052	4.5 (13.7) ab	H-M
Xa23R1	3.4 (13.2) ab	H-M
FIJ080	2.0 (12.1) b	M
DOM097	2.1 (11.0) b	M
HVO005	0.7 (8.0) c	L
LKA070	0.7 (7.0) cd	L
REU209	0.4 (6.5) cd	L
BRA115	0.5 (6.2) cde	L-N
PNG130	0.5 (6.2) cde	L-N
REU174	0.1 (4.8) de	L-N
MTQ058	0 (4.5) e	N
HVO082	0 (4.5) e	N
LS156R1	0 (4.5) e	N
REU173	0 (4.5) e	N

[a] Each MID is the mean of results for 12 tubes from three independent assays of four tubes each. Statistical analysis was performed after rank transformation of MIDs (12), and each rank value is the mean of results for 12 tubes. Values followed by the same letter are not significantly different at P of 0.05 according to Tukey's test.

[b] Grouping is based on mean MIDs and statistical results. Abbreviations: H, high (MID, >3-fold); M, medium (MID, 1.1- to 3-fold); L, low (MID, 0.1- to 1-fold); N, no albicidin production (MID, 0-fold). Strains attributed to two groups (H-M or L-N) are strains that belong to the first group based on the numerical measurement of albicidin production but for which this numerical value was not significantly different from values for strains of the second group after statistical analysis of data.

DISCUSSION

We investigated herein variations in the signature sequences of NRPS A domains which could result in the biosynthesis of different molecules of the pathotoxin albicidin. However, the signature sequences of the four studied A domains did not vary among 16 strains of *X. albilineans*, with one exception: the signature of module NRPS-1/3 of strain LKA070 varied at the ninth amino acid position. This residue involved in substrate specificity was a valine in strain LKA070, whereas it was an alanine in the remaining 15 strains of *X. albilineans*. However, it can be hypothesized that this small variation does not modify substrate specificity for the following reasons. (i) The valine-for-alanine substitution replaces a simple aliphatic amino acid with a branched aliphatic amino acid exhibiting the same physical and chemical properties; this substitution, therefore, does not result in significant stereochemical modifications which may modify the tertiary structure of the substrate binding pocket. (ii) Variations in amino acid residues within signature sequences of NRPS A domains exist in prediction models; for example, the ninth residue in the prediction model of Stachelhaus et al. is highly variable (32). Additionally, the prediction model of Challis et al. (8) considers only 8 of 10 residues from the previous model and excludes residues 9 and 10, which are considered less informative. (iii) Single valine-for-alanine substitutions in the signature sequences of the A domains of the fengycin synthase from *Bacillus subtilis* and the tyrocidine synthetase II from *Brevibacillus brevis* have been described previously, and these two domains both specify the same amino acid (32). The high degree of conservation among the signature sequences of NRPS A domains in 16 different strains of *X. albilineans* strongly supports the hypothesis that the albicidin NRPS substrate specificities are not involved in the observed pathogenic variation among strains of *X. albilineans*. There is still some uncertainty regarding the signature sequences of NRPS-1, NRPS-3, NRPS-6, and NRPS-7 modules because their related signatures do not exist in databases and the critical residues involved in specificity in these modules may be different from amino acid or aryl acid signatures. However, phylogenetic analyses of the amino acid sequences of the A domains of NRPS-1, NRPS-3, NRPS-6, and NRPS-7 modules did not reveal any nonsynonymous mutation putatively linked to variation in pathogenicity.

The complete nucleotide sequences corresponding to the NRPS A domains (A4-A5 regions) also showed very low levels of polymorphism, and the percentages of identity of the sequences encoding the six A domains of the 16 strains of *X. albilineans* varied only between 99.33 and 99.75%. Because genes may evolve at different speeds according to their category (19), the level of polymorphism in the A domain-encoding sequences of *X. albilineans* was compared to the level of polymorphism in two housekeeping genes of this pathogen. Functional constraints play an essential role in evolution speed, and functional genes are more conserved among strains. This trend toward conservation is especially evident for the housekeeping genes found within a species, a genus, or even different kingdoms (11, 19). Because they are highly conserved, housekeeping genes can be used to trace phylogenies, and they are a typical example of genes under purifying selective pressure. The mean percentages of identity of the sequences of the albicidin biosynthesis genes and those of the sequences of the housekeeping genes *atpD* and *efp* of the 16 strains of *X. albilineans* were similar. Additionally, the Ka/Ks ratios for the albicidin biosynthesis genes were not significantly different from those for the housekeeping gene *atpD*, suggesting that albicidin biosynthesis genes are subjected to the same natural selective pressure as genes that are highly conserved. Moreover, most of the nucleotide replacements were at the third codon position (data not shown), confirming the absence of evolution of these loci involved in toxin biosynthesis. These data also reflect the importance of functional albicidin biosynthesis genes in *X. albilineans*.

Several genetic groups of *X. albilineans* strains with neutral markers (14) and pathogenicity markers (9, 10) have been identified, and specific genotypes (PFGE group B and ALB-RFLP group B) of the pathogen have been associated with the leaf scald outbreaks that occurred during the last two decades. Although the degree of diversity of the NRPS A domains was low, three nucleotides corresponding to the NRPS-2 module also allowed us to identify the genotypes involved in leaf scald outbreaks, and therefore, an analysis of the entire genome of *X. albilineans* will no longer be necessary to differentiate these genotypes of the pathogen. However, these nucleotides are not informative regarding the genetic origin of the disease outbreak strains.

Levels of albicidin production varied among strains of the pathogen but also according to the bioassay. Several strains (HVO005, HVO082, MTQ058, and REU209) showed medium to high levels of albicidin production in the agar medium bioassay but low levels of production or no production in the liquid medium bioassay. Albicidin production, therefore, appears to depend very strongly on culture conditions, and levels

of in planta production of albicidin may also differ according to the sugarcane cultivar and plant growth conditions. No relationship between albicidin production and variation in pathogenicity or variation in the genetic characteristics of the 16 strains of *X. albilineans* was found. Therefore, differences in pathogenicity among strains of the pathogen are apparently not linked to the production of different albicidin molecules or to a higher level of production of albicidin by strains involved in the recent outbreaks of sugarcane leaf scald.

The results reported herein show that NRPS A domains in *X. albilineans* are highly conserved and that variation in the substrate specificities of these domains is therefore not involved in the observed pathogenicity differences among strains of this pathogen. Mutations in other sequences involved in albicidin biosynthesis (sequences encoding PKS modules or modification enzymes) may play a role in the variation in albicidin structure and pathogenicity. Structural analyses of albicidin molecules produced by the different strains of *X. albilineans* will be necessary to strictly validate this hypothesis. The recently described strategy to overproduce albicidin (33) is a promising approach to characterizing the structure of albicidin and to studying its variation. Additionally, genes other than those involved in albicidin biosynthesis must play a critical role in the variation in pathogenicity, and these genes remain to be identified. The annotation of the entire genome of *X. albilineans* that is in progress should result in their identification and study.

ACKNOWLEDGMENT

We thank Sylvain Boudon for kindly supplying sequence data on PCR primers used to amplify housekeeping genes.

REFERENCES

1. **Birch, R. G.** 2001. *Xanthomonas albilineans* and the antipathogenesis approach to disease control. Mol. Plant Pathol. **2:**1–11.
2. **Birch, R. G., and S. S. Patil.** 1983. The relation of blocked chloroplast differentiation to sugarcane leaf scald disease. Phytopathology **73:**1368–1374.
3. **Birch, R. G., and S. S. Patil.** June 1985. Antibiotic and process for the production thereof. U.S. patent 4,525,354.
4. **Birch, R. G., and S. S. Patil.** 1985. Preliminary characterization of an antibiotic produced by *Xanthomonas albilineans* which inhibits DNA synthesis in *Escherichia coli*. J. Gen. Microbiol. **131:**1069–1075.
5. **Birch, R. G., and S. S. Patil.** 1987. Correlation between albicidin production and chlorosis induction by *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Physiol. Mol. Plant Pathol. **30:**199–206.
6. **Birch, R. G., and S. S. Patil.** 1987. Evidence that an albicidin-like phytotoxin induces chlorosis in sugarcane leaf scald disease by blocking plastid DNA replication. Physiol. Mol. Plant Pathol. **30:**207–214.
7. **Cane, D. E., and C. T. Walsh.** 1999. The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases. Chem. Biol. **6:**319–325.
8. **Challis, G. L., J. Ravel, and C. A. Townsend.** 2000. Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chem. Biol. **7:**211–224.
9. **Champoiseau, P., J.-H. Daugrois, J.-C. Girard, M. Royer, and P. Rott.** 2006. Variation in albicidin biosynthesis genes and in pathogenicity of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Phytopathology **96:**33–45.
10. **Champoiseau, P., J.-H. Daugrois, I. Pieretti, S. Cociancich, M. Royer, and P. Rott.** 2006. High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe. Phytopathology **96:**1081–1091.
11. **Christensen, H., and J. E. Olsen.** 1998. Phylogenetic relationships of *Salmonella* based on DNA sequence comparison of *atpD* encoding the β subunit of ATP synthase. FEMS Microbiol. Lett. **161:**89–96.
12. **Conover, W. J., and R. L. Iman.** 1981. Rank transformations as a bridge between parametric and nonparametric statistics. Am. Stat. **35:**124–129.
13. **Conti, E., T. Stachelhaus, M. A. Marahiel, and P. Brick.** 1997. Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S. EMBO J. **16:**4174–4183.
14. **Davis, M. J., P. Rott, C. J. Warmuth, M. Chatenet, and P. Baudin.** 1997. Intraspecific genomic variation within *Xanthomonas albilineans*, the sugarcane leaf scald pathogen. Phytopathology **87:**316–324.
15. **Du, L., and B. Shen.** 2001. Biosynthesis of hybrid peptide-polyketide natural products. Curr. Opin. Drug Discov. Devel. **4:**215–228.
16. **Hall, T. A.** 1999. BioEdit: a user-friendly biological sequence alignments editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95–98.
17. **Hashimi, S. M., M. K. Wall, A. B. Smith, A. Maxwell, and R. G. Birch.** 2007. The phytotoxin albicidin is a novel inhibitor of DNA gyrase. Antimicrob. Agents Chemother. **51:**181–187.
18. **Huang, G., L. Zhang, and R. G. Birch.** 2001. A multifunctional polyketide-peptide synthetase essential for albicidin biosynthesis in *Xanthomonas albilineans*. Microbiology **147:**631–642.
19. **Jordan, K. I., I. B. Rogozin, Y. I. Wolf, and E. V. Koonin.** 2002. Microevolutionary genomics of bacteria. Theor. Popul. Biol. **61:**435–447.
20. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York, NY.
21. **May, J. J., N. Kessler, M. A. Marahiel, and M. T. Stubbs.** 2002. Crystal structure of DhbE, an archetype for aryl acid activating domains of modular nonribosomal peptide synthetases. Proc. Natl. Acad. Sci. USA **99:**12120–12125.
22. **Perrier, X., A. Flori, and F. Bonnot.** 2003. Data analysis methods, p. 43–76. *In* P. Hamon, M. Seguin, X. Perrier, and J. C. Glaszmann (ed.), Genetic diversity of cultivated tropical plants. Cirad, Montpellier, France.
23. **Pride, D. T., and M. J. Blaser.** 2002. Concerted evolution between duplicated genetic elements in *Helicobacter pylori*. J. Mol. Biol. **316:**629–642.
24. **Ricaud, C., and C. C. Ryan.** 1989. Leaf scald, p. 39–58. *In* C. Ricaud, B. T. Egan, A. G. Gillaspie, Jr., and C. G. Hughes (ed.), Diseases of sugarcane: major diseases. Elsevier, Amsterdam, The Netherlands.
25. **Rott, P., and M. J. Davis.** 2000. Leaf scald, p. 38–44. *In* P. Rott, R. A. Bailey, J. C. Comstock, B. J. Croft, and A. S. Saumtally (ed.), A guide to sugarcane diseases. La Librairie du Cirad, Montpellier, France.
26. **Rott, P., M. Abel, D. Soupa, P. Feldmann, and P. Letourmy.** 1994. Population dynamics of *Xanthomonas albilineans* in sugarcane plants as determined with an antibiotic-resistant mutant. Plant Dis. **78:**241–247.
27. **Rott, P. C., L. Costet, M. J. Davis, R. Frutos, and D. W. Gabriel.** 1996. At least two gene clusters are involved in albicidin production by *Xanthomonas albilineans*. J. Bacteriol. **178:**4590–4596.
28. **Royer, M., L. Costet, E. Vivien, M. Bes, A. Cousin, I. Pieretti, A. Savin, S. Megessier, M. Viard, R. Frutos, D. W. Gabriel, and P. C. Rott.** 2004. Albicidin pathotoxin produced by *Xanthomonas albilineans* is encoded by three large PKS and NRPS genes present in a gene cluster containing also several putative modifying, regulatory and resistance genes. Mol. Plant-Microbe Interact. **17:**414–427.
29. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
31. **Sieber, S. A., and M. A. Marahiel.** 2005. Molecular mechanisms underlying nonribosomal peptide synthesis: approaches to new antibiotics. Chem. Rev. **105:**715–738.
32. **Stachelhaus, T., H. D. Mootz, and M. A. Marahiel.** 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. **6:**493–505.
33. **Vivien, E., D. Pitorre, S. Cociancich, I. Pieretti, D. W. Gabriel, P. C. Rott, and M. Royer.** 2007. Heterologous production of albicidin: a promising approach to overproducing and characterizing this potent inhibitor of DNA gyrase. Antimicrob. Agents Chemother. **51:**1549–1552.
34. **Yang, P., P. Rott, L. Vauterin, B. Hoste, P. Baudin, K. Kersters, and J. Swings.** 1993. Intraspecific variability of *Xanthomonas albilineans*. Syst. Appl. Microbiol. **16:**420–426.