

Ralstonia solanacearum Strains from Martinique (French West Indies) Exhibiting a New Pathogenic Potential^{∇†}

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We investigated a destructive pathogenic variant of the plant pathogen *Ralstonia solanacearum* that was consistently isolated in Martinique (French West Indies). Since the 1960s, bacterial wilt of solanaceous crops in Martinique has been caused primarily by strains of *R. solanacearum* that belong to either phylotype I or phylotype II. Since 1999, anthurium shade houses have been dramatically affected by uncharacterized phylotype II strains that also affected a wide range of species, such as *Heliconia caribea*, cucurbitaceous crops, and weeds. From 1989 to 2003, a total of 224 *R. solanacearum* isolates were collected and compared to 6 strains isolated in Martinique in the 1980s. The genetic diversity and phylogenetic position of selected strains from Martinique were assessed (multiplex PCRs, *mutS* and *egl* DNA sequence analysis) and compared to the genetic diversity and phylogenetic position of 32 reference strains covering the known diversity within the *R. solanacearum* species complex. Twenty-four representative isolates were tested for pathogenicity to *Musa* species (banana) and tomato, eggplant, and sweet pepper. Based upon both PCR and sequence analysis, 119 Martinique isolates from anthurium, members of the family *Cucurbitaceae*, *Heliconia*, and tomato, were determined to belong to a group termed phylotype II/sequovar 4 (II/4). While these strains cluster with the Moko disease-causing strains, they were not pathogenic to banana (NPB). The strains belonging to phylotype II/4NPB were highly pathogenic to tomato, eggplant, and pepper, were able to wilt the resistant tomato variety Hawaii7996, and may latently infect cooking banana. Phylotype II/4NPB constitutes a new pathogenic variant of *R. solanacearum* that has recently appeared in Martinique and may be latently prevalent throughout Caribbean and Central/South America.

The bacterial wilt pathogen, *Ralstonia solanacearum* (Smith) Yabuuchi et al., is widespread in tropical, subtropical, and warm temperate regions where it is a major constraint to the production of diverse crop species, including both monocots and dicots (41). *R. solanacearum* causes bacterial wilt on many host plants, and the disease is called by many different names, including Moko and Bugtok diseases of bananas and brown rot disease of potato. The host range of *R. solanacearum* is unusually wide for a plant pathogen, including over 450 host species in 54 botanical families (14). *R. solanacearum* is a highly heterogeneous bacterial species; based on phenotypic properties, the species has been divided into five races (related to the ability to wilt members of the family *Solanaceae* (race 1), banana (race 2), potato and tomato in temperate conditions (race 3), ginger (race 4), and mulberry (race 5) (2, 17, 30) and six biovars (metabolic profiles, related to the ability to metabolize three sugar alcohols and three disaccharides) (15–17). The assessment of the genetic diversity of *R. solanacearum* employing restriction fragment length polymorphism analysis resulted

in the identification of two clusters of strains denoted divisions 1 (“Asiaticum”) and 2 (“Americanum”) (3, 4). Recently, Fegan and Prior (9) proposed a new hierarchical classification scheme, based on sequence analysis of the internal transcribed spacer region, the endoglucanase (*egl*) gene, and the *hrpB* gene, that defines phylotypes as “a monophyletic cluster of strains revealed by phylogenetic analysis of sequence data” (8). Four phylotypes were distinguished. Phylotype I corresponds to the “Asiaticum” division 1 of Cook et al. (3) and contains strains belonging to biovars 3, 4, and 5. Phylotype II corresponds to the “Americanum” division 2 of Cook and Sequeira (4) and contains strains belonging to biovar 1/race 1, biovar 1/race 2 (Moko disease-causing strains), biovar 2/race 3, and biovar 2T strains. Phylotype III contains strains from Africa and the Indian Ocean, which belong to biovars 1 and 2T. Phylotype IV contains strains from Indonesia, some strains from Japan, and a single strain from Australia, belonging to biovars 1, 2, and 2T. Phylotype IV also contains the closely related species *Ralstonia syzygii* and the blood disease bacterium. Each phylotype can be further subdivided into sequovars based on differences in the sequence of a portion of the endoglucanase (*egl*) gene. The phylotyping scheme proposed by Fegan and Prior (9) is broadly consistent with the former phenotypic and molecular typing schemes and adds valuable information about the geographical origin and in some cases the pathogenicity of strains. In a recent study (13) assessing

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hybridization of genomic DNA from 18 strains, representative of the known diversity within the *R. solanacearum* species complex, to a microarray representative of 99% of the genes from reference strain GMI1000, the hierarchical clustering of strains perfectly matched the previous classification in four phylotypes (13).

From this hierarchical scheme, a phylotype-specific multiplex PCR (Pmx-PCR) was derived (9) and a *Musa*-specific multiplex PCR (Mmx-PCR) which distinguishes three subgroups within *R. solanacearum* Moko disease-causing strains, sequevar 3 (MLG 24 sensu (3), sequevar 4 (MLG 25), and sequevar 6 (MLG 28). Within sequevar 4, Moko disease-causing strains could be distinguished from strains not pathogenic on banana (34).

In the French West Indies, bacterial wilt has been the most damaging disease of solanaceous crops for decades (33). *R. solanacearum* was first found on tomato plants in Martinique in 1965 (6) and was responsible for great yield losses on eggplant crops during the early 1980s (24). However, although bananas are an extensive agroindustry crop, *R. solanacearum* has never been isolated from banana, and Moko disease has never been reported in Martinique. Prior and Steva reported in 1990 (36) that the *R. solanacearum* populations consisted of a majority of biovar 3 strains (phylotype I) and a minority of biovar 1 strains (phylotype II), all being previously typed as race 1. In April 1999, unusual strains of *R. solanacearum* were isolated from diseased anthurium (*Anthurium andreanum*) plants developing leaf yellowing and necrosis, as well as rotting of the rhizome with abundant bacterial ooze. This bacterial disease has caused severe damage in the main anthurium production areas in the central humid highland regions of the island of Martinique (25). In December 2001, *R. solanacearum* was identified as the causal agent of the first outbreaks of wilting cantaloupe (*Cucumis melo* L.) in the southeastern part of the island of Martinique. Other cases of bacterial wilt were then reported in 2002 on cucumber (*Cucumis sativus* L.), pumpkin (*Cucurbita moschata* L.), zucchini (*Cucurbita pepo* L.), and in 2003 on watermelon (*Citrullus lunatus*) (45). Two *R. solanacearum* strains (ANT307 and ANT1121) isolated from the first outbreaks on anthurium have been previously found to cluster into phylotype IIB sequevar 4 (IIB/4) (34), which contains Moko disease-causing strains (8). Employing the phylotype classification scheme and DNA-based molecular tools (9, 34), we examined the genetic diversity and phylogeny of these strains. We have also examined the pathogenicity of these strains of *R. solanacearum* causing bacterial wilt to anthurium and cucurbits in Martinique.

(A portion of these results has previously been presented elsewhere [44].)

MATERIALS AND METHODS

Bacterial strains. Strains ($n = 262$) used in this study are listed in Table 1 and in Table S1 in the supplemental material. Strains were provided by authors and obtained from collections (Collection Française de Bactéries Phytopathogènes [CFBP], Angers, France; Coleção de Culturas de Fitobactérias do Instituto Biológico [IBSBF], São Paulo, Brazil). Among the non-Martinique strains (called foreign strains in Table 1), one strain from Trinidad was included, as it was isolated in 2006 from a diseased anthurium in the northern range of the island (source, W. Elibox, University of West Indies, St. Augustine, Trinidad). Strains from Brazil that were also isolated from cucurbits and *Musa* were included as well. The Martinique strains ($n = 232$) consisted of six strains described

by Prior and Steva (36) in 1990, and 225 strains collected during (i) routine analyses (1989 to 2002) of the SPV (Plant Protection Service), and (ii) surveys carried out from 1999 to 2003 by FREDON (Regional Pest Management Federation), and CIRAD (International Center of Agronomic Research for Development). Strains were collected from the highland central humid areas of the island of Martinique (FREDON-SPV surveys 1999, 2000, 2001, and early 2002), from the main production zone for anthurium, from contaminated anthurium shade houses, from weeds (to search for alternative hosts), and from vegetable crops throughout the island (CIRAD survey 2002 to 2003). Diseased plants (leaf, stem, and rhizome) were sampled and cultured to isolate *R. solanacearum*. Samples were taken from the oozing stems of rotted rhizomes, surface-disinfected leaves of anthurium, stems of wilted cucurbits and other solanaceous plants, and roots and stems of symptomless weeds. The following culture media were used: K+ medium, modified from Kelman's triphenyltetrazolium chloride (TZC) medium (21) (Bacto Peptone, 10 g · liter⁻¹; glucose, 5 g · liter⁻¹; Casamino Acids, 1 g · liter⁻¹; yeast extract, 1 g · liter⁻¹; agar, 15 g · liter⁻¹; TZC, 50 mg · liter⁻¹; pH 7.4), and modified Granada and Sequeira (MGS) medium (12), as modified by Poussier et al. (32) (agar, 18 g · liter⁻¹; tryptone, 1 g · liter⁻¹; Bacto Peptone, 10 g · liter⁻¹; glycerine, 6.3 ml · liter⁻¹; pH 7.2; crystal violet, 3 mg · liter⁻¹; polymyxin B sulfate, 10 mg · liter⁻¹; tyrothricine, 20 mg · liter⁻¹; chloramphenicol, 5 mg · liter⁻¹; TZC, 25 mg · liter⁻¹; propiconazole, 0.04 ml · liter⁻¹; and penicillin G, 20 U · liter⁻¹). Strains were purified by streaking single colonies on K+ medium which was incubated at 28°C for 48 h. Three loopfuls of bacterial culture were then transferred in 3 ml of double-distilled sterile water, and the cultures were stored at room temperature. Strains from the CIRAD collection (CIR strains) were cryopreserved in glycerol-CPG broth as described by Denny and Hayward (5) and stored in a -70°C freezer. A set of 31 strains were deposited at the CFBP under the accession numbers 6776 to 7017 (Table 1 and Table S1 in the supplemental material). For PCR diagnosis, strains were incubated for 1 or 2 days on CPG medium (Casamino Acids, 1 g · liter⁻¹; Bacto Peptone, 10 g · liter⁻¹; glucose, 5 g · liter⁻¹; agar, 15 g · liter⁻¹; pH 7.2) at 28°C, one loopful of bacterial culture was added per ml of double-distilled sterile water, and the suspension was homogenized by vortexing and used directly as the template for PCR.

DNA typing. Two mx-PCR procedures were applied to characterize strains based on the hierarchical classification scheme described previously (9, 34). Pmx-PCR (11) was performed to identify which phylotype a strain belonged to. When the strain belonged to phylotype II, Mmx-PCR (35) was performed to identify whether the strain belonged to sequevar 3, 4, or 6 (Moko disease-inducing strains). Briefly, Pmx-PCR includes the primers 759/760 (29) as an internal marker providing the 280-bp reference PCR product which is specific for the *R. solanacearum* species and a set of four phylotype-specific forward primers (Nmult:21:1F [5'-CGTTGATGAGGCGCGCAATTT-3'], Nmult:21:2F [5'-AAGTTATGGACGGTGGAAAGTC-3'], Nmult:22:InF [5'-ATTGCCAAGACGAGAGAAGTA-3'], and Nmult:23:AF [5'-ATTACGAGAGCAATCGAAAGATT-3']), with a unique and conserved reverse primer (Nmult:22:RR [5'-TCGCTTGACCCTATAACGAGTA-3']), targeted in the 16S-23S intergenic spacer region (internal transcribed spacer). This Pmx-PCR produces the following phylotype-specific PCR products: a 144-bp amplicon from phylotype I strains, a 372-bp amplicon from phylotype II strains, a 91-bp amplicon from phylotype III strains, and a 213-bp amplicon from phylotype IV strains. *Taq* polymerase and deoxynucleoside triphosphates (dNTPs) were from Promega France (Charbonnières-les-Bains, France), and primers (11) were obtained from Eurogentec (Seraing, Belgium). PCRs were performed in a PCR Express thermocycler (Hybaid, Teddington, United Kingdom). PCR products were resolved through 2% agarose gels in Tris-acetate-EDTA (TAE) buffer and revealed by staining in 0.5 µg · ml⁻¹ ethidium bromide. Amplicon sizes were estimated by comparison to a 100-bp DNA ladder (Invitrogen, Life Technologies, Cergy-Pontoise, France). Mmx-PCR includes four sets of forward and reverse primers amplifying genomic DNA fragments by a subtractive hybridization approach (35): Mus35-F (5'-GCAGTAAAGAAACCCGGTGT-3') and Mus35-R (5'-TCTGGCGAAA GACGGGATGG-3'), Mus20-F (5'-CGGGTGGCTGAGACGAATATC-3') and Mus20-R (5'-GCCTTGTCAGAAATCCGAATG-3'), Mus06-F (5'-GCTGGCA TTGCTCCCGTCCAC-3') and Mus06-R (5'-TCGCTTCCGCCAAGACGC-3'), and Si28-F (5'-CGTTCCTTGTGTCAGCGATGG-3') and Si28-R (5'-CCCGT GTCACCCCGATAGC-3'). This Mmx-PCR provides sequevar-specific PCR products: a 400-bp product indicates the strain belongs to sequevar 3 (equivalent to MLG 24 of Cook and Sequeira [3]); a 220-bp product indicates the strain belongs to sequevar 6 (equivalent to MLG 28 of Cook and Sequeira [3]); a 351-bp product indicates the strain belongs to sequevar 4 (equivalent to MLG 25 of Cook and Sequeira [3]). Within sequevar 4, strains of the SFR ecotype (insect-transmitted strains whose colony morphology is small, fluidal, and round sensu French and Sequeira [11]) produce an additional amplicon of 167 bp as do

TABLE 1. *Ralstonia solanacearum* strains used in this study

Strain ^a	Alternate name	Yr of isolation	Host	Origin ^b	Biovar ^c	PhyloTYPE/sequence determined by ^d :		Plant pathogenicity tested on ^e :	Reference(s) ^f	GenBank accession no. for:	
						PCR	Trees			egl	musS
Strains from Martinique											
CFBP2955	MT3	1984	Tomato	Le Lamentin	3	I	I		36		
CFBP2957	RUN27	1987	Tomato	Case Pilote	1	II	IIA/5		34, 36	AF295265	EF371845
CFBP2964	MA4	1983	Eggplant	Schoelcher	3	I			36		
CFBP2970	MPI	1985	<i>Capsicum</i> sp.	Fort de France	3	I			36		
CFBP2972	RUN30	1986	Potato	Le Lamentin	1	II	IIA/5		34, 36	AF295264	AY756807
CFBP2976	Mb1	1987	<i>Ensete ventricosum</i>	Le Lamentin	3	I			36		
RS25		1997	Tomato	Le Lamentin		II		Tomato	TS		
CFBP6786	SPV98-1537	1998	Tomato	Fort de France		II/4NPB	IIB/4		TS	EF371823	EF371858
CFBP6784	ANT307	1999	<i>Anthurium</i> sp.	Saint Joseph	1	II/4NPB	IIB/4	AAA banana	35; TS	EF371813	AY756742
SPV99-1119	RUN283	1999	<i>Anthurium</i> sp.	Morne Rouge	1	II/4NPB	IIB/4		TS	EF371829	EF371863
SPV99-1541	RUN280	1999	<i>Anthurium</i> sp.	Gros Morne	1	II/4NPB	IIB/4		TS	EF371822	EF371857
CFBP6801	RUN279	2001	<i>Heliconia caribea</i>	Gros Morne		II/4NPB	IIB/4		TS	EF371836	EF371873
CFBP6800	RUN281	2001	<i>Anthurium</i> sp.	Gros Morne		II/4NPB	IIB/4		TS	EF371825	EF371865
SPV02-60144	RUN284	2002	Cucumber	Fond Saint Denis		II/4NPB	IIB/4		TS	EF371832	EF371867
CFBP6795	SPV02-60152	2002	Pumpkin	Saint Esprit	1	II/4NPB	IIB/4		TS	EF371837	EF371874
SPV02-60037	RUN285	2002	Cantaloupe	Vauclin		II/4NPB	IIB/4		TS	EF371815	EF371850
CFBP6797	CFBP6797	2002	<i>Solanum americanum</i>	Saint-Joseph		II/4NPB	IIB/4		TS	EF371820	EF371855
CIR02-005(1)		2002	<i>Anthurium ferriense</i>	Saint-Joseph		II/4NPB		AAAB and AAA banana, <i>Solanaceae</i>	TS		
CIR02-035		2002	Sweet pepper	Marigot		I		<i>Solanaceae</i>	TS		
CIR02-045		2002	Eggplant	Morne des Esses		I		<i>Solanaceae</i>	TS		
CFBP6783	RUN17	2002	<i>Heliconia caribea</i>	Morne Rouge		II/4NPB	IIB/4	AAAB and AAA banana, <i>Solanaceae</i>	TS	EF371817	EF371852
CFBP6782	RUN18	2002	<i>A. andreaeanum</i>	Morne Rouge		II/4NPB	IIB/4	AAAB and AAA banana, <i>Solanaceae</i>	TS	EF371819	EF371854
CIR02-080	CIR02-083	2002	<i>Pandanus</i> sp.	Le Vauclin		II/4NPB	IIB/4	AAAB and AAA banana, <i>Solanaceae</i>	TS	EF371827	EF371861
CFBP6782		2002	<i>A. ferriense</i>	Le Vauclin		II		AAA banana	TS		
CIR02-136(4)		2002	Tomato	Ste Anne		II		AAA banana	TS		
CIR02-141		2002	Tomato	Le Lamentin		II		AAA banana	TS		
CIR02-204		2002	Cucumber	Ste Anne		II/4NPB	IIB/4	AAAB and AAA banana, <i>Solanaceae</i>	TS		
CFBP6781	CIR02-142(1)	2002	Tomato	Ste Anne		II/4NPB	IIB/4	<i>Solanaceae</i>	TS	EF371830	EF371864
CFBP6780	RUN287	2002	Tomato	Ste Anne		II/4NPB	IIB/4	AAAB and AAA banana	TS	EF371835	EF371872
CFBP6779	RUN288	2002	<i>Ginnia indica</i>	Le Lamentin		II	IIA	AAAB and AAA banana	TS	EF371816	EF371851
CFBP6778	RUN289	2002	Tomato	Le Lorrain		II/4NPB	IIB/4	<i>Solanaceae</i>	TS	EF371818	EF371853
CFBP7012	CIR02-229(1)	2002	Pumpkin	Le Marin		II/4NPB	IIB/4	AAAB and AAA banana, <i>Solanaceae</i>	TS	EF371821	EF371856
CFBP6777	RUN291	2002	Watermelon	Basse-Pointe		II/4NPB	IIB/4	<i>Solanaceae</i>	TS	EF371824	EF371859
CFBP7013	RUN292	2003	Tomato	Saint-Esprit		I		<i>Solanaceae</i>	TS		
CIR03-076		2003	Tomato	Macouba		II		Tomato	TS		
CIR03-077		2003	Tomato	Macouba		II/4NPB	IIB/4	AAAB and AAA banana, <i>Solanaceae</i>	TS	EF371826	EF371860
CFBP7017	RUN293	2003	Watermelon	Macouba		II/4NPB	IIB/4	<i>Solanaceae</i>	TS	EF371828	EF371870
CFBP7016	RUN294	2003	<i>A. andreaeanum</i>	Gros Morne		II	IIA/5		TS	EF371842	EF371866
CFBP7015	RUN295	2004	Plantain	Trinité		II/4NPB	IIB/4		TS	EF371834	EF371862
CIR04-006/16F	RUN296	2004	Plantain	Trinité		II/4NPB	IIB/4		TS		
Foreign strains											
CFBP7014	CIR06-024	2006	<i>A. andreaeanum</i>	Trinidad		II/4NPB	IIB		TS	AF371831	EF371875
IBSBF1503	RUN302	1999	<i>Cucumis sativus</i>	Brazil		II/4NPB	IIB/4		TS	EF371840	EF371868
IBSBF1546	RUN298	2001	<i>Fuchsia</i> sp.	Brazil		II	IIA		TS	EF371838	EF371877
IBSBF1454	RUN303	1999	<i>Cucurbita pepo</i>	Brazil		II/4NPB	IIB/4		TS	EF371844	EF371876
IBSBF1712	RUN299	2002	<i>Pelargonium</i> × <i>hortorum</i>	Brazil		II	IIB		TS	EF371833	EF371869
IBSBF1900	RUN301	2000	<i>Musa</i> sp.	Brazil		II/6	IIA/6		TS	EF371839	EF371871
Reference strains											
GMI1000	RUN54		Tomato	French Guyana		I	I/13 to 18		9	AF295251	AY756804
CFBP2968	RUN58		Eggplant	Guadeloupe		I	I/13 to 18		34	AF295252	AY756800
JT1523	RUN333		Potato	Reunion Island		I	I/13 to 18		34	AF295253	AY756803
CFBP765	RUN34		Tobacco	Japan		I	I/13 to 18		34	AF295253	AY756738
NCPPB3190	RUN78		Tomato	Malaysia		I	I/13 to 18		34	AF295254	AY756764
ACH92	RUN158		Ginger	Australia		I	I/13 to 18		34	AF295255	AY756801
R292	RUN91		<i>Morus alba</i>	China		I	I/2		34	AF295263	AY766776
ICMP7963	RUN55		Potato	Kenya		II	IIA/7		34	AF295262	AY756799
K60	CFBP2047		Tomato	United States		II	IIA/7		34		
A3909	RUN9		Heliconia	Hawaii		II/6	IIA/6		34	EF371812	AY756753

CFBP2958	Tomato	IIA/5	AF295266	AY756806
IPO1609	Potato	II	EF371814	EF371849
JT516	Potato	II	AF295258	AY756783
NCPBP987	Potato	II	AF295261	AY756785
UW477	Potato	II/ND	AF295260	AY756821
MOLK2	<i>Musa</i> sp.	IIA/3	EF371841	EF371848
UW9	Heliconia	II/3	AF295257	AY756744
CFBP1409	<i>Musa</i> sp.	II/3	EF371808	AY756751
CFBP1183	Heliconia	II/3	EF371805	AY756749
UW70	<i>Musa</i> sp. (plantain)	II/3	DO011550	AY756794
UW129	<i>Musa</i> sp. (plantain)	II/4	EF371811	AY756782
UW162	<i>Musa</i> sp. (plantain)	II/4A	AF295256	AY756795
ICMP6782	<i>Musa</i> sp.	II/6	DO011553	NA ^a
ICMP9600	<i>Musa</i> sp.	II/6	DO011554	NA
ANT307	<i>A. andreaeanum</i>	II/6	EF371813	AY756742
UW21	<i>Musa</i> sp.	II/6	DO011546	AY756758
CFBP3059	Eggplant	III	AF295270	AY756766
CFBP734	Potato	III/19 to 23	AF295274	AY756746
NCPBP332	Potato	III	AF295272	AY756766
JT525	<i>Pelargonium asperum</i>	III/19 to 23	AF295276	AY756786
RUN60	Potato	III	DO011558	AY756752
RUN71	Potato	IV	EF371804	AY756732
RUN83	Tomato	IV/10		

^a CFBP, Collection Françaises de Bactéries Phytopathogènes, Angers, France; IBSPF, Coleção de Culturas de Fito bacterias do Instituto Biológico, São Paulo, Brazil.

^b Strains were all isolated in Martinique (French West Indies) except for the foreign strains and reference strains.

^c The biovars were determined in microplates by the method of French et al. (10).

^d The phylotypes and sequevars determined by PCR were those determined by the Pmx-PCR and Mmx-PCR (9, 34). The strains marked II gave no signal when tested by the Mmx-PCR. The phylotypes and sequevars determined by trees were determined by *egl* and *mutS* sequence analyses.

^e Pathogenicity was tested as described in the text on plantain banana (AAB), Cavendish banana (AAA), members of the family *Solanaceae*, and/or tomato genotypes only.

^f Reference numbers are given with one exception. TS, this study.

^g NA, not available.

strains of the A ecotype (insect-transmitted strains from the Amazon basin, colonies are round and mucoid with no or very little formazan coloration; sensu French and Sequeira (11). Prior and Fegan (34) found that the sequevar 4 strains ANT307 and ANT1121 from Martinique (CFBP6784 and 6785 in our study), did not produce this additional amplicon. These strains have since been designated phylotype II/sequevar 4 NPB (II/4NPB), as they were also found to be nonpathogenic for banana (P. Prior and D. Dufael, personal communication in 1999). Strains that do not belong to the *Musa* group (sequevar 3, 4, or 6) do not produce any PCR product when subjected to Mmx-PCR.

PCR amplification and DNA sequencing of portions of the endoglucanase (*egl*) and DNA repair (*mutS*) genes. PCR amplification of a 750-bp region of the *egl* gene was performed by using the primer pair Endo-F (5'-ATGCATGCCGCTGGTCGCCG-3') and Endo-R (5'-GCGTTGCCCGGCACGAACACC-3'). The reaction mixture (total volume, 100 µl) contained PCR buffer (supplied by the manufacturer), 1.5 mM MgCl₂, 200 µM of each dNTP, 25 pmol of each primer, 2 µl of a turbid bacterial suspension as the template, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). PCR was performed using a MJ Research PTC200 thermocycler (Bio-Rad Laboratories, Inc., Hercules, CA) using the following protocol: (i) initial denaturation at 96°C for 9 min; (ii) 30 cycles, with 1 cycle consisting of 95°C for 1 min, 70°C for 1 min, and 72°C for 2 min; and (iii) a final extension step of 72°C for 10 min. PCR amplification of a 758-bp fragment of the terminal portion of the *mutS* gene was performed using a MJ Research PTC200 thermocycler. Reactions were carried out by the method of Prior and Fegan (34) in a total volume of 25 µl containing 1× PCR buffer (Promega), 1.5 mM MgCl₂, 200 µM of each dNTP, 0.8 µM dimethyl sulfoxide, 1.25 U of *Taq* polymerase (Promega), and 9 pmol each of the primers mutS-RsF.1570 (5'-ACAGCGCCTTGAGCCGGTACA-3') and mutS-RsR1926 (5'-GCTGATCACCGGCCGCAACAT-3'). Reaction mixtures were heated to 96°C for 5 min and then cycled through 35 cycles of 94°C for 60 s, 66°C for 60 s, and 72°C for 90 s, followed by a final extension period of 5 min at 72°C. Samples (5 µl) of reaction mixtures were examined by electrophoresis through 2% agarose gels in TAE buffer, and bands were revealed by staining in 0.5 µg ml⁻¹ ethidium bromide. PCR products were purified, and sequencing reaction was performed on both strands by Macrogen sequencing services (Kumchun-ku, Seoul, Korea) with the mutS and Endo primers mentioned above.

Sequence analysis. Portions of the sequences of the housekeeping DNA repair *mutS* gene located on the chromosome and of the endoglucanase (*egl*) virulence-related gene located on the megaplasmid were analyzed by using the ARB software environment (23). Sequences were manually aligned using the ARB sequence editor. Sequence data were analyzed using the three major phylogenetic approaches offered by the ARB software. Evolutionary relationships between sequences were computed by the following methods: (i) by using the algorithm of Jukes and Cantor (20), phylogenetic trees were constructed from the genetic distance data by using the neighbor-joining (NJ) method (37) with 5,000 bootstrap resamplings of the data to test the tree topologies; (ii) maximum likelihood methods using the FastDNAML program; and (iii) the maximum parsimony methods using the Phylip DNAPARS package implemented in the ARB software. From the pool of *mutS* and *egl* sequences included in this study (61 and 63 sequences, respectively), sequences from reference strains were retrieved, and sequences from newly described strains were deposited into the GenBank database (Table 1).

Pathogenicity tests. A subset of *R. solanacearum* strains was assembled to represent all phylotypes, with particular attention to phylotype II/4NPB strains. This subset of strains was tested for pathogenicity for different hosts: banana, tomato, sweet pepper, and eggplant (Table 1). Pathogenicity was assessed on Cavendish (AAA genome, cv. Grande Naine) and plantain (cooking banana) (AAB genome, cv. Dominico-Harton) banana, and different solanaceous hosts. Strains were transferred from glycerol-CPG broth onto K+ medium or Sequiera medium South Africa (SMSA) (5) and grown for 2 or 3 days at 28°C. Single mucoid colonies were then selected, streaked on CPG medium, and grown at 28°C for 48 h. Bacterial growth on a petri dish was harvested into 5 ml Tris buffer (0.01 M Tris, pH 7.2). Cell suspensions were spectrophotometrically adjusted to 10⁸ CFU · ml⁻¹ (optical density at 600 nm of 0.1) prior to use as a plant inoculum.

Cavendish and plantain bananas were tested in separate experiments. Banana and plantain plants were grown in a quarantine nursery in heat-sterilized potting mix. When they reached the four-leaf stage (approximately 40 cm high), they were infected by employing two techniques: injection of 0.1 ml of inoculum into the base of the pseudostem and root scarification prior to adding 10 ml inoculum to the soil (1 liter of soil per pot) surface (drenching inoculation). Seven strains (six phylotype II/4NPB strains and one phylotype II non-sequevar 3, 4, or 6 strain) were inoculated on Cavendish and plantain bananas, whereas four other strains (one phylotype II/4NPB strain and three phylotype II non-sequevar 3, 4,

TABLE 2. Distributions of the different phylotypes in the collections of *Ralstonia solanacearum* strains from Martinique based upon DNA typing employing the Pmx- and Mmx-PCR tests

Collection (yr) (reference)	No. (%) of strains of collection			Total no. of strains
	Phylotype I	Phylotype II subcluster/ sequevar		
		A/Non- <i>Musa</i>	B/4NPB	
Prior and Steva (1990) (36)	4 (66.7)	2 (33.3)	0 (0)	6
SPV (1989–2002)	18 (21.2)	12 (14.1)	55 (64.7)	85
CIRAD survey (2002–2003)	40 (28.8)	35 (25.2)	64 (46)	139
Total no. of strains	62 (26.7)	51 (22)	119 (51.3)	232

or 6 strains) were inoculated on Cavendish bananas only. Six plants were inoculated with each strain (three plants by each inoculation technique), and six control plants were inoculated with sterile water. All 48 plantain and 72 Cavendish plants were maintained on plastic trays 1 m above the ground in a randomized experimental design at 29°C ± 6°C in a closed quarantine greenhouse, watered as needed with tap water, and observed every 2 days for 50 to 54 days. Finally, plants were uprooted, and microbiological isolations were done on three sections of each plant: roots, pseudocorm, and the top of the pseudostem (at the insertion of the leaf blades). Pseudostem and pseudocorm pieces were surface sterilized by dipping the pieces in 95% ethanol and flamed; roots were first thoroughly washed under tap water, then surface sterilized by dipping the roots in ethanol, and dried in a laminar hood on sterile filter paper. Plant material was then chopped and crushed with a pestle in 5 ml Tris buffer (pH 7.2) and then macerated for 15 min. Afterwards, macerates were streaked (50 µl per petri dish) on MGS medium and SMSA (5) and incubated 28°C for 2 or 3 days to allow development of characteristic *R. solanacearum* colonies (mucoid egg-shaped colonies, with pink-reddish center). Typical colonies, chosen at random, were identified as *R. solanacearum* by Pmx-PCR.

The pathogenicity of 10 phylotype II/4NPB strains chosen at random (Table 1) was tested on solanaceous commercial varieties, namely, tomato cv. Heat Master (Seminis), sweet pepper cv. Narval (Tezier), and eggplant cv. Kalenda (Technisem). Plants were inoculated at the two to three expanded leaf stage in a complete block design with three blocks and 10 plants per block. Inoculation was done by drenching inoculation by adding 10 ml of inoculum to the soil (10⁸ CFU · ml⁻¹) of each plant, after the roots had been scarified with a sterile scalpel. Incubation was carried out in a greenhouse (29°C ± 6°C), and plants were monitored for wilting symptoms every 2 or 3 days for 41 days. Disease incidence was assessed by the final wilting rate. Disease severity was estimated by the area under the disease progression curve (AUDPC) (39), calculated with the formula:

$$\text{AUDPC} = \sum_{i=1}^n [(x_i + x_{i-1})/2](t_i - t_{i-1})$$

where n is the number of times the plants were evaluated, x_i is the wilting rate at each evaluation time, and $(t_i - t_{i-1})$ is how long wilting lasted (39).

The virulence/aggressiveness of strains that represent phylotypes I (CIR03-076), IIA (RS25), and IIB/4NPB (CIR03-077, CFBP6783, and CFBP7013) on tomato plants was assessed on a range of cultivars carrying some degree of genetic resistance to bacterial wilt (38, 43): Roma (susceptible control, Technisem), R3034 (University of the Philippines, Los Baños), CRA84 (AVRDC, Taiwan), Hawaii7996 (University of Florida), and BF-Okitsu (NIVOT, Japan). Plants were inoculated as described above at the two or three fully expanded leaf stage in a complete randomized design with two replicates and 10 plants per replicate. Incubation was carried out in a climatic chamber with a 14-h photoperiod (28°C day/24°C night), and wilt symptoms were monitored every 2 or 3 days for 30 days. Disease incidence was assessed by the final wilting rate, and disease severity was estimated by the AUDPC (38).

Data analysis. Bliss transformation (arcsin \sqrt{x}) was applied to wilt rate data prior to statistical analysis. Analysis of variance (ANOVA) was used to determine the effects of isolate, species, and isolate-species interactions on the final wilting incidence and AUDPC scores. Statistical analysis was performed with the software STATBOX Pro 2.5 (Grimmersoft, Paris, France), and the ANOVA

requirements (homogeneity of the intratreatment variances, normality of the residuals, and independence of the residuals) were fulfilled for both arcsin-transformed wilting data and AUDPC. When the ANOVA assumptions were not validated for AUDPC, AUDPC values were transformed by the function \sqrt{x} . The chi-square test (Microsoft Excel 2000) was applied to analyze the distribution of the different phylotypes within the different host groups (members of the family *Solanaceae*, *Anthurium*, and cucurbits) and the distribution of the different host groups for the different types of strains (phylotypes I, II, and II/4NPB).

Nucleotide sequence accession numbers. The sequences from newly described strains were deposited into the GenBank database (Table 1).

RESULTS

Strain characterization. Pmx-PCR showed that all biovar 3 strains belonged to phylotype I and biovar 1 strains belonged to phylotype II (Table 1). Mmx-PCR showed that strains isolated from cucurbit and anthurium hosts predominantly belonged to phylotype II sequevar 4 and displayed the 351-bp amplicon only. Strains with this pattern were designated phylotype II/4NPB (not pathogenic to banana) as explained in the introduction. The phylotyping results for the 232 *R. solanacearum* strains from Martinique are reported in Table 2. Strains from the collection of Prior and Steva (36) which were collected in the 1980s belonged to phylotype I or phylotype II (Table 1), but the strains belonging to phylotype II did not produce amplicon when used as the template in the Mmx-PCR, indicating that they did not belong to sequevar 3, 4, or 6. Within strains collected in Martinique from 1989 to early 2002 (SPV collection) and late 2002 to 2003 (CIRAD collection), three groups of strains were identified: phylotype I; phylotype II non-sequevar 3, 4, or 6; and phylotype II/4NPB. In the SPV collection, 64.7% strains were phylotype II/4NPB, 21.2% were phylotype I, and 14.1% were phylotype II non-sequevar 3, 4, or 6 strains. In the CIRAD collection, 46% were phylotype II/4NPB strains, 28.8% were phylotype I, and 25.2% were phylotype II non-sequevar 3, 4, or 6 strains.

Relationship of *R. solanacearum* genotype to host. In Martinique, *R. solanacearum* strains were mostly isolated from the family *Solanaceae*, *Anthurium andreaeanum*, and the family *Cucurbitaceae* (Fig. 1). Strains isolated from *Solanaceae* were 50% phylotype I, approximately 28% phylotype II non-sequevar 3, 4, or 6, and 22% phylotype II/4NPB. In contrast, strains isolated from anthurium were 5% phylotype I, 18% phylotype II non-sequevar 3, 4, or 6, and 77% phylotype II/4NPB. Strains from cucurbits were 4.5% phylotype I, 4.5% phylotype II (not sequevar 3, 4, or 6), and 91% phylotype II/4NPB. The fre-

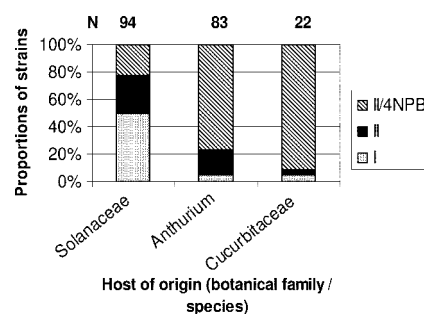


FIG. 1. Distribution of phylotypes I, II, and II/4NPB of *Ralstonia solanacearum* in Martinique as related to the host of origin of the strains.

TABLE 3. Recovery of *Ralstonia solanacearum* from plantain (AAB) and Cavendish (AAA) bananas

Strain, host, and phylotype/sequence	Tissue or organ ^a	Bacterial recovery ^b from banana type by inoculation technique			
		Plantain		Cavendish	
		Injection	Drenching	Injection	Drenching
CIR02-005(1), <i>Anthurium andreaum</i> , II/4NPB	Roots	+	–	–	–
	Pseudocorm	+	+	–	–
	Pseudostem	+	–	–	–
CFBP6783, <i>Heliconia caribea</i> , II/4NPB	Roots	+	+	–	+
	Pseudocorm	+	–	–	–
	Pseudostem	+	–	–	–
CIR02-080, <i>A. andreaum</i> , II/4NPB	Roots	–	–	–	–
	Pseudocorm	+	–	–	–
	Pseudostem	+	+	–	–
CFBP6781, <i>Cucumis sativus</i> , II/4NPB	Roots	+	+	–	–
	Pseudocorm	+	+	–	–
	Pseudostem	+	+	–	–
CFBP7012, <i>Cucurbita moschata</i> , II/4NPB	Roots	+	+	–	–
	Pseudocorm	+	+	–	–
	Pseudostem	+	+	–	–
CIR03-077, <i>Lycopersicon esculentum</i> , II/4NPB	Roots	+	+	–	–
	Pseudocorm	+	+	–	–
	Pseudostem	+	+	+	–
CFBP6779, <i>Canna indica</i> , II	Roots	–	–	–	–
	Pseudocorm	+	–	–	–
	Pseudostem	–	–	–	–
CIR02-136(4), <i>A. ferrierense</i> , II	Roots	NT	NT	–	–
	Pseudocorm	NT	NT	–	–
	Pseudostem	NT	NT	–	–
CIR02-204, <i>L. esculentum</i> , II	Roots	NT	NT	–	–
	Pseudocorm	NT	NT	–	–
	Pseudostem	NT	NT	–	–
CIR02-141, <i>L. esculentum</i> , II	Roots	NT	NT	–	–
	Pseudocorm	NT	NT	–	–
	Pseudostem	NT	NT	–	–
CFBP6784, ^c <i>A. andreaum</i> , II/4NPB	Roots	NT	NT	–	–
	Pseudocorm	NT	NT	–	–
	Pseudostem	NT	NT	–	–
None (water control)	Roots	–	–	–	–
	Pseudocorm	–	–	–	–
	Pseudostem	–	–	–	–

^a Tissue or organ from which the bacteria were isolated.

^b Symbols: +, successful recovery of *R. solanacearum* on at least one plant; –, no recovery of *R. solanacearum*. NT, not tested.

^c Reference strain of reference 34.

quency distributions of each host group within the different phylotypes were significantly different from their expected values (χ^2 test, $P = 2.21 \times 10^{-9}$ for *Solanaceae*, $P = 7.67 \times 10^{-6}$ for anthurium, and $P = 0.0018$ for cucurbits). The frequency distributions of phylotype I strains within the different host groups were significantly different from their expected values (χ^2 test, $P = 1.54 \times 10^{-8}$), much higher within the strains isolated from the *Solanaceae* and much lower within the strains isolated from anthurium and cucurbits. The frequency distri-

butions of phylotype II/4NPB strains were also significantly higher than expected (χ^2 test, $P = 6.03 \times 10^{-6}$) within the strains derived from anthurium and cucurbits and significantly lower than expected within the *Solanaceae* strains. Thus, these data suggested that phylotype I strains were predominantly isolated from *Solanaceae*, whereas phylotype II/4NPB strains were predominantly isolated from anthurium and cucurbits.

Pathogenicity to *Musa* spp. No Moko disease or any external symptom of disease was observed on Cavendish and plantain

TABLE 4. Test for pathogenicity to members of the family *Solanaceae*

Strain (phylogroup/sequence)	Pathogenicity on:					
	Tomato		Sweet pepper		Eggplant	
	Mean AUDPC ^a	% Wilting at day 41 ^b	Mean AUDPC	% Wilting at day 41	Mean AUDPC	% Wilting at day 41
CFBP6783 (II/4NPB)	29.5 A	90.0 A	29.8 A	100.0 A	8.6 AB	30.0 A
CIR02-080 (II/4NPB)	27.7 AB	83.0 A	16.8 AB	60.0 B	9.0 AB	47.0 A
CIR02-005 (II/4NPB)	27.6 AB	87.0 A	30.2 A	100.0 A	7.6 ABC	33.0 A
CFBP6781 (II/4NPB)	26.3 AB	87.0 A	16.5 AB	87.0 AB	3.8 ABCD	20.0 A
CFBP6780 (II/4NPB)	26.0 AB	80.0 A	13.6 AB	60.0 B	6.9 ABC	33.0 A
CFBP7017 (II/4NPB)	24.7 AB	73.0 A	20.1 AB	80.0 AB	3.9 ABC	23.0 A
CFBP7012 (II/4NPB)	18.3 AB	60.0 A	20.1 AB	67.0 AB	2.3 BCD	20.0 A
CFBP6777 (II/4NPB)	17.3 AB	57.0 A	24.4 A	80.0 AB	13.5 A	53.0 A
CIR03-077 (II/4NPB)	16.6 AB	57.0 A	23.6 A	93.0 AB	5.4 ABC	23.0 A
CIR02-045 (I)	14.1 ABC	47.0 A	25.5 A	93.0 AB	9.7 AB	47.0 A
CFBP6778 (II/4NPB)	10.7 ABC	40.0 A	8.5 B	53.0 B	0.4 CD	20.0 A
CIR02-035 (I)	8.7 BC	30.0 A	9.8 B	67.0 AB	1.3 BCD	17.0 A
None (control) (water)	0.0 C	0.0 B	0.0 C	0.0 C	0.0 D	0.0 B
Mean AUDPC ^c	19.0 A		18.4 A		5.6 B	
Mean % wilting ^c		61.0 B		71.0 A		28.0 C

^a Mean AUDPC, mean area under the disease progression curve ($n = 3$). See the text for details on its calculation. Values followed by the same letter in the same column are not significantly different (Newman-Keuls test [$P = 0.05$] done on square root-transformed values).

^b Mean percentage of wilted plants ($n = 3$ replicates, 10 plants per replicate) assessed on the 41st day after inoculation. Values followed by the same letter in the same column are not significantly different (Newman-Keuls test [$P = 0.05$] done on arcsin [square root]-transformed values).

^c Values followed by the same letter in the same row are not significantly different (Newman-Keuls test, $P = 0.05$).

bananas 51 and 54 days after inoculation with any strain. After the plants were cut into pieces, no bacterial oozing was observed, although occasionally purple discoloration of the vascular tissues within the pseudocorm was observed. No bacterium of any genus was recovered from the control plants (inoculated with water). On plantain banana, following injection inoculation, all the phylotype II/4NPB strains were recovered from pseudocorm, pseudostem, and roots, except CIR02-080 which was recovered from pseudostem and pseudocorm only. In contrast, strain CFBP6779 (a phylotype II non-sequevar 3, 4, or 6 strain) was recovered only from pseudocorm, near the inoculation point (Table 3). Following drenching inoculation, recoveries were more variable, even though three out of six strains (CFBP6781, CFBP7012, and CIR03-077) were recovered from all points on the plant, indicating that they invaded the plant from roots to leaf top. On Cavendish banana, *R. solanacearum* recovery was observed only on plants inoculated by injection with strain CIR03-077 and by drenching inoculation with strain CFBP6783. Both strains were phylotype II/4NPB. No recovery of *R. solanacearum* was found for reference strain CFBP6784 (ANT307 (34) inoculated on Cavendish banana from any banana plant. The only successful recovery of a phylotype II non-sequevar 3, 4, or 6 strain from an inoculated plant was obtained from the pseudocorm of one plantain plant inoculated with strain CFBP6779 from *Canna indica*. With the exception of this case, no phylotype II non-sequevar 3, 4, or 6 strain was ever recovered from any infected banana plant.

Pathogenicity on members of the family *Solanaceae*. Ten phylotype II/4NPB strains were tested for pathogenicity on tomato, sweet pepper, and eggplant, and the results were compared to plants inoculated with two phylotype I strains (Table 4). All phylotype II/4NPB strains from Martinique were pathogenic to *Solanaceae*, irrespective of the host from which they were initially isolated. Based on both AUDPC and the final

wilting rate, sweet pepper and tomato were the most susceptible species, eggplant being less affected (Table 4). Virulence within strains belonging to phylotype II/4NPB varied according to strain and host, but these variations were not statistically significant. Surprisingly, the phylotype I strains were not ranked as the most aggressive strains on their respective hosts of origin (sweet pepper for CIR02-035, eggplant for CIR02-045).

Among tomato cultivars tested with phylotype II/4NPB strains CIR03077, CFBP7013, and CFBP6783, phylotype II non-sequevar 3, 4, or 6 strain RS25, and phylotype I strain CIR03076, Roma was the most affected cultivar, followed by Hawaii7996, R3034, CRA84, and BF-Okitsu. Phylotype II/4NPB strains were able to wilt all five cultivars, whereas phylotype II non-sequevar 3, 4, or 6 strains and phylotype I strains were unable to cause disease on Hawaii7996, CRA84, and BF-OKITSU and did not confer wilting (Table 5) or stem colonization (data not shown). The three phylotype II/4NPB were significantly more aggressive than the phylotype II non-sequevar 3, 4, or 6 strains and phylotype I strains, inducing a final wilting rate of 50 to 60% on the universal resistant reference strain Hawaii7996.

Phylogenetic analysis. Partial *egl* and *mutS* gene sequences were generated from a set of representative strains from Martinique, including phylotype II non-sequevar 3, 4, or 6 strains and phylotype II/4NPB strains, and compared to different reference strains covering the known genetic diversity within the four phylotypes shown in both the endoglucanase (*egl*) (9) and the *mutS* (35) phylogenetic trees. Trees constructed with maximum likelihood, maximum parsimony, and distance NJ phylogenetic approaches were totally congruent; therefore, only NJ phylogenetic trees, carrying bootstrap values, are presented (Fig. 2 and 3). NJ trees from *egl* and *mutS* gene sequences were in general agreement, showing a similar branching pattern

TABLE 5. Test for virulence to tomato

Strain	Phylotype	Virulence on tomato line											
		% Wilting at day 30 ^a						Mean AUDPC ^b					
		Roma	R3034	Hawaii7996	CRA84	BF-Okitsu	Mean	Roma	R3034	Hawaii7996	CRA84	BF-Okitsu	Mean
CIR03077	II/4NPB	70.0 A	50.0 A	60.0 A	30.0 AB	55.0 A	53.0 A	16.0 A	10.7 A	12.2 A	6.5 AB	12.1 A	11.4 A
CFBP7013	II/4NPB	60.0 A	25.0 B	50.0 A	50.0 A	20.0 B	42.0 A	12.7 A	6.0 A	8.6 A	10.0 A	4.6 B	9.0 A
CFBP6783	II/4NPB	57.0 A	30.0 B	50.0 A	45.0 A	25.0 B	42.0 A	14.1 A	7.0 A	8.2 A	9.8 A	5.0 B	9.2 A
RS25	II	44.0 A	20.0 B	0.0 B	0.0 B	0.0 B	13.0 B	9.6 AB	3.6 A	0.0 B	0.0 B	0.0 B	2.6 B
CIR03076	I	24.0 B	10.0 B	0.0 B	0.0 B	0.0 B	7.0 B	3.7 B	2.9 A	0.0 B	0.0 B	0.0 B	1.2 B
Mean ^c		53.0 A	27.0 B	32.0 B	25.0 B	20.0 B		11.2 A	6.0 B	6.6 B	5.2 B	4.3 B	

^a Mean percentage of wilted plants ($n = 2$ replicates, 10 plants per replicate) Assessed on the 30th day After inoculation. Values followed by the same letter in the same column Are not significantly different (Newman-Keuls test [$P = 0.05$], done on arcsin [square root]-transformed wilt values and on AUDPC values).

^b Mean AUDPC, mean area under the disease progression curve ($n = 2$). See the text for details on its calculation. Values followed by the same letter in the same column are not significantly different (Newman-Keuls test [$P = 0.05$], done on arcsin [square root]-transformed wilt values and on AUDPC values).

^c Values followed by the same letter in the same row are not significantly different (Newman-Keuls test [$P = 0.05$]).

clearly partitioning the four phylotypes as previously described in the literature (Fig. 2 and 3): reference strains from phylotypes I, III, and IV clustered separately with high bootstrap values. Sequences of strains that were Pmx-PCR typed phylotype II resolved in two major and robust branches previously described by Fegan and Prior (8): (i) phylotype IIA, containing strains that were isolated from diverse hosts, and (ii) phylotype IIB, the Moko disease-causing strains from sequevar 3 (MLG24) and 4 (MLG25) and the highland temperate potato brown rot strains (biovar 2/race 3) from sequevars 1 and 2. Phylotype II strains from Martinique clustered in both phylotypes IIA and IIB. The strains that clustered into phylotype IIA were isolated from tomato, potato, heliconia, and anthurium (including CFBP7016), and none of these strains produced amplicons with the Mmx-PCR and were therefore phylotype II non-sequevar 3, 4, or 6 strains. The 21 strains isolated from various hosts in Martinique, which were typed as phylotype II/4NPB based on Mmx-PCR, clustered into phylotype IIB regardless of the *egl* or *mutS* sequences analyzed. Phylogenetically, the 20 phylotype II/4NPB strains were undistinguishable from the Moko disease-causing strains UW70, UW129 and UW162 that were included as reference for sequevar 4 (MLG25). Interestingly, Brazilian strains ISBSF1503 from *Cucumis sativus* and ISBSF1454 from *Cucurbita pepo* also clustered in phylotype IIB sequevar 4. From both the *egl* and *mutS* phylogenetic trees, phylotype IIB sequevar 4 strains had identical sequences, with the exception of strain CFBP7014 which varied by 10 nucleotide positions, while the closely related strain CFBP7014, isolated from anthurium in Trinidad, was distinguishable from sequevar 4 strains, forming a new branch with high bootstrap support (83% and 56% in *mutS* and *egl* trees, respectively). The phylogenetic position of strain ISBSF1712 isolated from *Pelargonium* in Brazil was not congruent between the *egl* and *mutS* phylogenetic trees. Based on the *egl* gene sequence, ISBSF1712 was closely related to phylotype IIB sequevars 1 and 2, in a group that contains biovar 2/race 3 strains that cause potato brown rot and is listed in the United States as a bioterrorism select agent (22). Based on the *mutS* gene sequence, ISBSF1712 resolved into a distinct and robust cluster (89% bootstrap value). Based on the *egl* gene sequence analysis of Fegan and Prior (8), the sequences of Brazilian strains ICMP6782 and ICMP 9600 were included as

reference sequences for phylotype II subcluster A sequevar 24, a previously unrecognized group of *R. solanacearum* Moko disease-causing strains. *Musa* strain IBSBF1900 was the only other strain found to belong to this group (90% bootstrap value), and no Martinique strain was assigned to it.

DISCUSSION

Ralstonia solanacearum strains causing bacterial wilt on solanaceous crops and ornamental plants are found in all four phylotypes of the *R. solanacearum* species complex defined by Fegan and Prior (9). Conversely, strains that have ecologically adapted to a particular host, for example, Moko disease-causing strains pathogenic to banana, have much narrower genetic diversity. *R. solanacearum* strains causing Moko disease have been resolved into distinct subclusters within phylotype II: sequevar 3 (MLG24), sequevar 4 (MLG25), sequevar 6 (MLG28), and sequevar 24 (8). We report here a new group of strains which were predominantly isolated from diseased anthurium and cucurbit species (Fig. 1 and reference 45) and pathogenic on solanaceous crops as well. Bacterial wilt of anthurium caused by *R. solanacearum* has been reported in Singapore (46), Reunion Island (31), and Mauritius Island (7) and was caused by biovar 3 strains; in Taiwan (42), it was caused by biovar 4 strains. The positions of these strains in the phylotyping scheme (11) have not been determined, except for the anthurium strains from Reunion Island that have been found to belong to phylotype I (31); however, these strains most likely belong to phylotype I. In Martinique, the *R. solanacearum* strains from anthurium were shown to belong to phylotype II sequevar 4 according to the DNA molecular tools developed by Prior and Fegan (34). As phylotype II sequevar 4 contains strains that cause Moko disease (10, 35), this finding potentially constituted a great threat to the banana industry in the French West Indies. This paper describes the genetic diversity and pathogenicity of these strains, as well as the phylogenetic position of this new group of *R. solanacearum* strains in Martinique, which have been named phylotype II/4NPB.

R. solanacearum strains in the French West Indies have previously been isolated from solanaceous plants and ornamental banana (*Musa ensete*) and classified as biovar 3 and biovar 1, with a dominance of biovar 3 strains (36), whereas

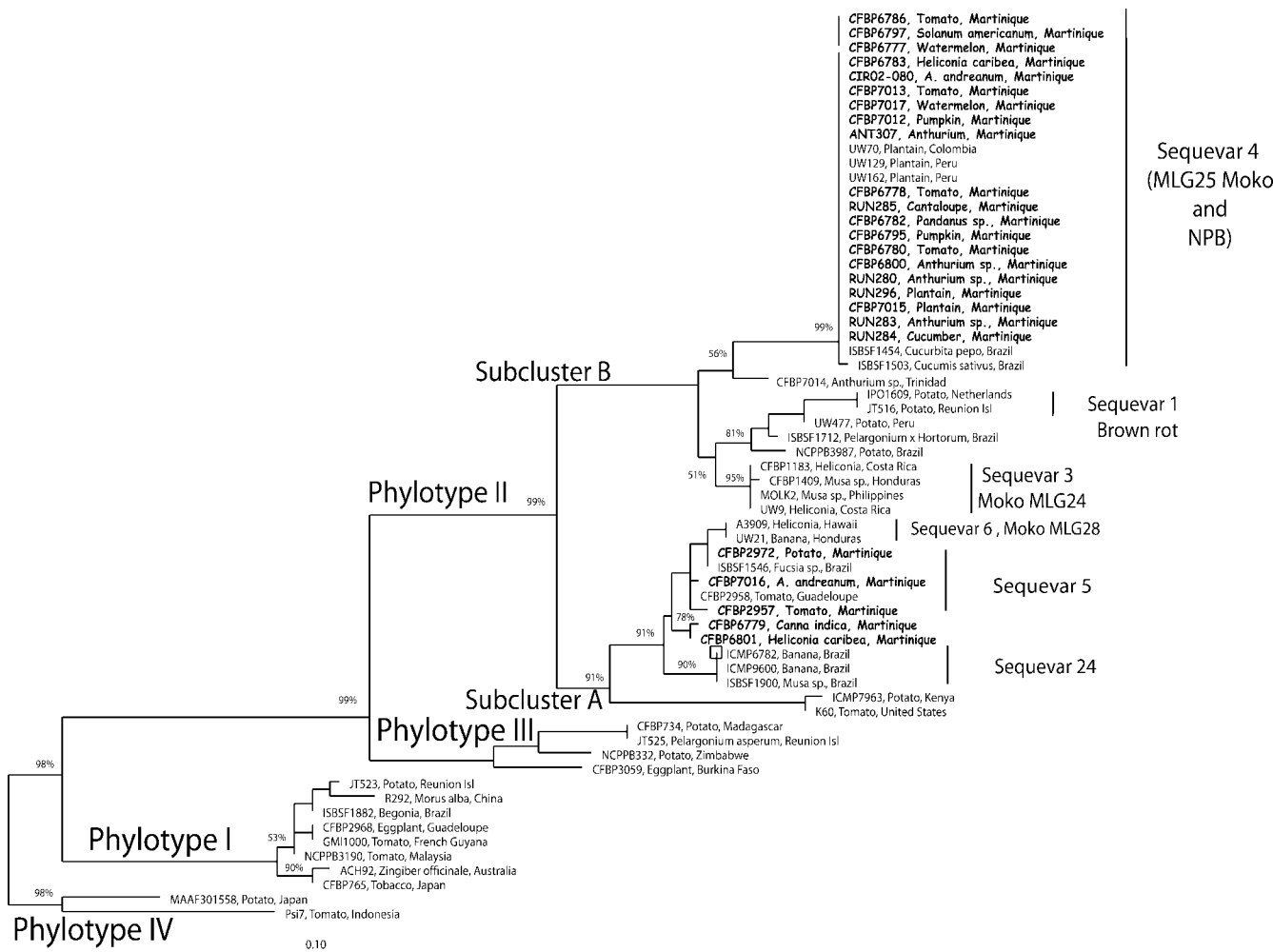


FIG. 2. Phylogenetic NJ tree based on the partial endoglucanase (*egl*) gene sequences of strains from Martinique and reference strains for the *R. solanacearum* species complex. Strains originating from Martinique are written in bold type. The number at each node is the bootstrap value (5,000 resamplings), and significant bootstrap values less than 100% are indicated at each node. The scale bar represents 1 nucleotide substitution per 10 nucleotides. Reunion Isl, Reunion Island.

Moko disease had never been observed in Martinique. By application of the Pmx- and Mmx-PCR tests in this study on the Prior and Steva strains (36), we confirmed that biovar 3 strains belong to phylotype I and that biovar 1 strains are phylotype II non-sequevar 3, 4, and 6 strains, as would have been expected. The recent (1999 to 2003) extensive survey of isolates of *R. solanacearum* from Martinique has revealed that the strains belonged to phylotype I, phylotype II non-sequevar 3, 4, and 6, and phylotype II sequevar 4 based on Pmx- and Mmx-PCR-based identification tools. Since the identification of the first phylotype II sequevar 4 strain in Martinique in 1998 from tomato, this group of *R. solanacearum* strains has been regarded as an emerging group in Martinique. The well-established phylotype I and phylotype II non-sequevar 3, 4, and 6 strains that have been present in Martinique for decades are regarded as the historical population.

Bacterial wilt caused by *R. solanacearum* biovar 1 strains has been previously reported for summer squash, zucchini summer squash, and cucumber in Brazil, but the phylotype affiliation of the strains involved was not determined (28, 40). This study has

shown that some Brazilian strains isolated in 1999 from *Cucurbita pepo* (ISBSF1454) and *Cucumis sativus* (ISBSF1503) also clustered into phylotype II/4. As these Brazilian strains were genotyped as phylotype II/4NPB by the Mmx-PCR, we may consider that they would be more closely related to the strains from Martinique than to Moko disease-causing strains. Furthermore, Moko disease-causing strains have never been reported to be pathogenic to cucurbits. However, this hypothesis remains to be validated by pathogenicity tests on banana. While being phylogenetically undistinguishable from other members of phylotype II/4, the pathogenicities of the strains from Martinique within this cluster are clearly different from the Moko disease-causing strains of sequevar 4 (MLG25). Moko disease-causing strains of phylotype II/4 cause wilting of plantain and banana (3, 4, 11, 19). However, the phylotype II/4 strains from Martinique are unable to wilt Cavendish banana (AAA genotype *Musa* sp.) and plantain (AAB genotype *Musa* sp.) and were thus designated not pathogenic to banana (NPB) and designated phylotype II/4NPB. These phylotype II/4NPB strains were unable to spread from the inoculation point within

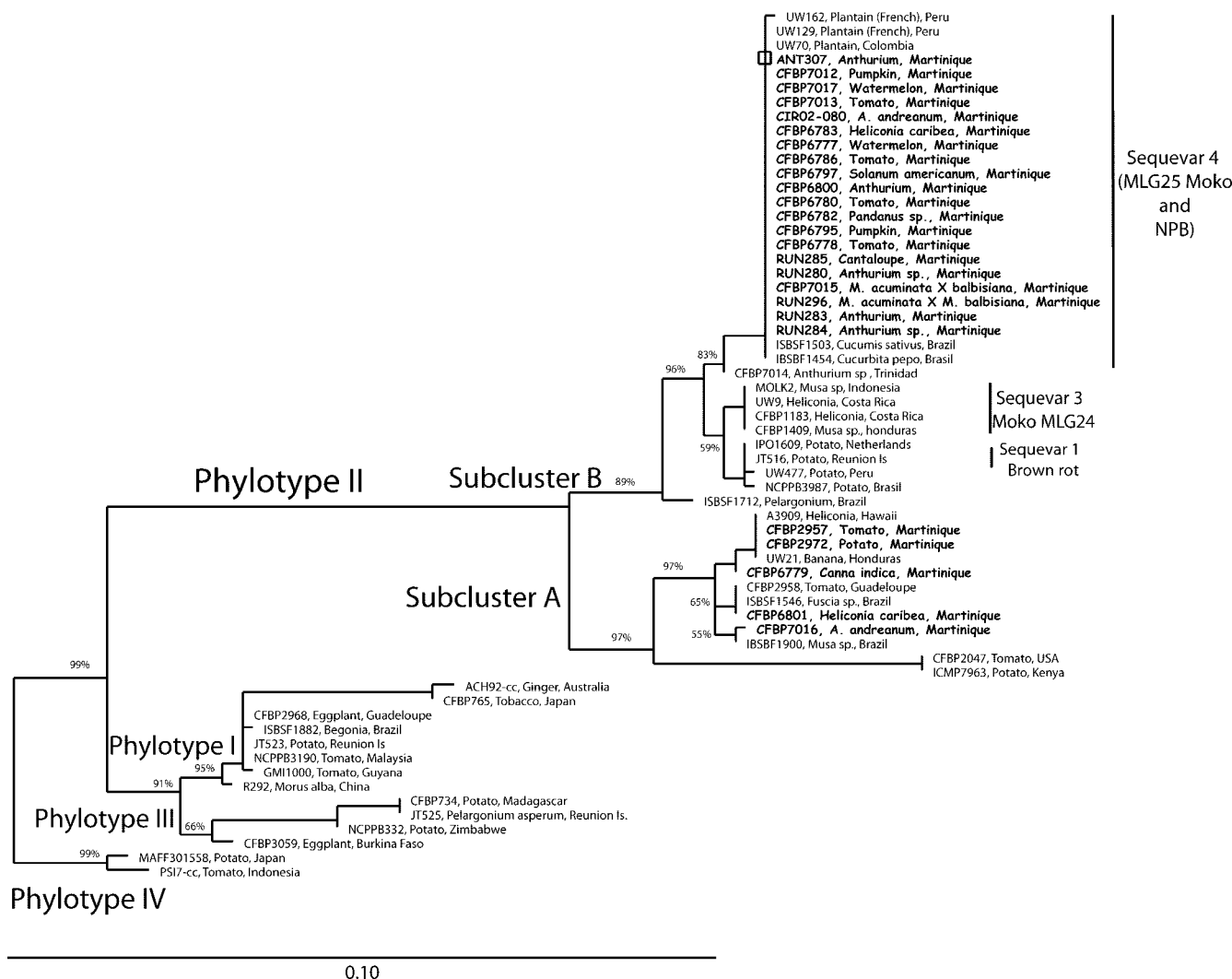


FIG. 3. Phylogenetic NJ tree based on partial sequences of the DNA repair *mutS* gene from strains from Martinique and reference strains for the *R. solanacearum* species complex. Strains originating from Martinique are indicated in bold type. The number at each node is the bootstrap value (5,000 resamplings), and significant bootstrap values less than 100% are indicated at each node. The scale bar represents 1 nucleotide substitution per 10 nucleotides. Reunion Is, Reunion Island.

the plant tissues of the Cavendish cultivar used, regardless of the inoculation method (pseudostem or roots). For example, reference strain CFBP6784 (34) was not pathogenic to and did not establish any latent infection in Cavendish banana (AAA genotype) tissues. Nevertheless, the phylotype II/4NPB strains, although not pathogenic to banana, had the capacity to establish and move within the vascular tissues of plantain (AAB genotype), whatever the point of entry into the plant. After being injected directly in the pseudostem, strains of *R. solanacearum* phylotype II/4NPB were shown to invade the tissues both upwards and downwards from the point of inoculation. Following soilborne contamination, these strains were able to enter the root system and spread throughout the plant to establish latent infections in asymptomatic plantain stalks. This feature needs to be confirmed on other plantain cultivars, but if confirmed, this may be a key epidemiological issue, as native plantains are distributed throughout the island of Martinique in Creole gardens, although plantain has never been

cultivated as a cash crop in the French West Indies as it has in Central America and especially in Colombia. The ability of phylotype II/4NPB strains to infect plantains was confirmed by the identification of naturally infected plants in 2004 in one of the few industrial plantain plantations (1). Strains CFBP7015 and CIR04-006/16F were isolated from the flower peduncle of a diseased plantain of cv. French in Martinique, whose terminal leaf growth was blocked and bunch was distorted, but showing no typical wilt symptoms. This may indicate the potential of phylotype II/4NPB strains to cause Moko disease. Also, as these strains were isolated from the flower peduncle, it may indicate that these strains were transmitted by insects, as are the Moko disease-causing strains of phylotype II/4 (MLG25). Fortunately, growers of Cavendish bananas on large banana plantations in the French West Indies remove the male bud on each stalk, thus preventing the aerial mode of entry of pathogen into the plant. The phenotype of this last case may lead us to consider these phylotype II/4NPB strains as similar

to ecotype "D," "isolated from *Heliconia* and causing leaf distortion and slow wilting of bananas" (11), or ecotype "H," "affecting plantains but not bananas." However, recent phylogenetic analysis has assigned these two ecotypes to sequevar 3 (34) and French and Sequeira (11) reported these ecotypes to be weakly pathogenic to eggplant and pepper, unlike the strains from Martinique.

Other strains with a genotype similar to those of the phylotype II/4NPB strains have been reported outside of Martinique. In 1998, two strains (P515 and P548) that were isolated in Florida from pothos (*Epipremnum aureum*) cuttings imported from Costa Rica (26) were reported to cluster with anthurium strains from Martinique in phylotype IIB/4 (35). However, these strains from pothos were described as weakly pathogenic to tomato (unlike the strains from Martinique) and mildly pathogenic to Cavendish banana, inducing "dark veins, leaf necrosis and chlorosis," but not wilting or death (26). More recently, *R. solanacearum* strains isolated from anthurium in Florida (27) were reported to induce chlorosis, necrosis, and wilting within 2 weeks on anthurium, tomato, and triploid banana, but their phylogenetic position in the phylotyping scheme has not yet been established. These reports and our study show that extensive comparative studies will be required to assess pathogenicity of strains belonging to phylotype II/4 on the available genetic diversity in triploid banana genomes (AAA, AAB, and ABB) and diploid banana.

Phylotype II/4NPB strains appear to represent a relatively new emerging pathogenic variant within phylotype II sequevar 4. Indeed, this genetic group was absent in *R. solanacearum* collections from the French West Indies until the first strain was isolated from a wilted tomato during a survey in Martinique in 1998. Furthermore, this group of strains carries a previously unknown host range, including solanaceous, cucurbit, and ornamental plants, while being not pathogenic to Cavendish banana, even in artificial infections. Cantaloupe, watermelon, and pumpkin were not considered hosts of *R. solanacearum* until the first report by Wicker et al. (45). None of the Moko disease-causing strains belonging to phylotype II/4 (MLG25) have been described as being naturally pathogenic for vegetable crops (11). However, strains can wilt and kill tomato when artificially inoculated into the stem (13). The only Moko disease-causing strains that have been isolated from naturally infected tomato and reported to be both pathogenic to solanaceous and banana plants belong to phylotype II sequevar 6 (MLG28) (34). Although extensively surveyed only in Martinique, it is possible that phylotype II/4NPB strains may be present throughout the entire Caribbean region and even South America. Two phylotype II/4NPB strains were also identified in 1998 from pothos imported from Costa Rica (26), and this study has shown that similar strains are present in Brazil and Trinidad. The factors that may explain the establishment of this new pathogenic variant in Martinique will be discussed in another paper focusing on the epidemiology of this group of strains.

The phylotype II/4NPB strains were also highly aggressive and virulent on tomato, significantly overcoming the resistance carried in tomato strain Hawaii7996 (50 to 60% wilt), whereas the phylotype I and phylotype II non-sequevar 3, 4, and 6 strains tested were unable to wilt this variety. It is therefore possible that phylotype II/4NPB strains thus constitute a new

challenge for tomato breeders to develop cultivars in this region; screening tests done recently tend to support this hypothesis (E. Wicker, unpublished 2005 and 2006 data).

The *egl* and *mutS* phylogenetic trees presented in this study were in agreement with previous work (8, 9, 13, 34, 35), and all sequences analyzed in this work clustered in previously described phylotypes and sequevars. However, strain CFBP 7014 from Trinidad and strain ISBSF1712 from Brazil did not cluster with other phylotype II/4NPB strains. According to the phylogenetic analysis, strain CFBP7014, isolated from anthurium in Trinidad, resolved in a cluster distinct from the sequevar 4 cluster within subgroup IIB (Fig. 2 and 3), whereas this strain was genotyped as phylotype II/4NPB by Pmx- and Mmx-PCRs. Both *egl* and *mutS* sequences from this particular strain placed it in a unique single-isolate cluster, expanding the known genetic diversity within strains of *R. solanacearum*. Further investigations are needed, particularly for extended knowledge of the *R. solanacearum* populations and of the epidemiological context in Trinidad. The Brazilian strain ISBSF1712 that was isolated in 2002 from pelargonium also gave inconsistent clusters in *egl* and *mutS* phylogenetic trees. Further investigations will be necessary to infer its degree of genetic relatedness with the pelargonium strains reported in the United States (18).

The use of the phylotyping scheme proposed by Fegan and Prior (9) and the PCR-based molecular tools described by Fegan and Prior (9) and Prior and Fegan (34) has allowed the identification and characterization of group of *R. solanacearum* strains with a novel pathogenicity. We propose that strains described in this paper, belonging to phylotype II/4NPB, should be considered a new pathogenic variant within the phylotype II/4 phylogenetic group. This variant has been recognized in Martinique since 1998, and similar strains have also been isolated in Brazil, Costa Rica, and Trinidad. Many questions remain to be addressed on the epidemiology of this pathogenic variant. It remains unclear whether this variant arose in Martinique or elsewhere and how widespread it may be on the island of Martinique. Further work is required to determine how widespread this pathogenic variant of *R. solanacearum* has become.

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