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16S Ribosomal DNA Characterization of Nitrogen-Fixing Bacteria Isolated from Banana (*Musa* spp.) and Pineapple (*Ananas comosus* (L.) Merril)

LEONARDO MAGALHÃES CRUZ,[1] EMANUEL MALTEMPI DE SOUZA,[1] OLMAR BALER WEBER,[2] JOSÉ IVO BALDANI,[3] JOHANNA DÖBEREINER,[3] AND FÁBIO DE OLIVEIRA PEDROSA[1]*

Departamento de Bioquímica, Universidade Federal do Paraná, 81.531-990 Curitiba, Paraná,[1] Centro Nacional de Agroindústria Tropical-Empresa Brasileira de Pesquisa Agropecuária, 60.511-110 Fortaleza, Ceará,[2] and Centro Nacional de Pesquisa de Agrobiologia-Empresa Brasileira de Pesquisa Agropecuária, 23.851-970 Seropédica, Rio de Janeiro,[3] Brazil

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Nitrogen-fixing bacteria isolated from banana (*Musa* spp.) and pineapple (*Ananas comosus* (L.) Merril) were characterized by amplified 16S ribosomal DNA restriction analysis and 16S rRNA sequence analysis. *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, *Burkholderia brasilensis,* and *Burkholderia tropicalis* were identified. Eight other types were placed in close proximity to these genera and other alpha and beta *Proteobacteria*.

Associative nitrogen-fixing bacteria such as *Azospirillum brasilense*, *Herbaspirillum seropedicae,* and *Acetobacter diazotrophicus* may benefit their host plants as N biofertilizers and plant growth promoters. The latter two organisms were the first nitrogen-fixing bacteria suggested to be endophytes (1, 4). Several new classified and as-yet-unclassified diazotrophic bacteria have been isolated from economically important mono- and dicotyledonous plants (3, 5), including banana and pineapple (17).

Thirty-eight nitrogen-fixing bacteria isolated from stems, leaves, roots, and fruits of pineapple and banana cultivars from Bahia (BA) and Rio de Janeiro (RJ) States, Brazil, including 14 isolates previously described (17), were analyzed following DNA sequencing and PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene (amplified 16S ribosomal DNA restriction analysis [ARDRA]) to define their phylogenetic positions. Reference strains Z67, Z78, and M2 for *H. seropedicae*, M4 for *Herbaspirillum rubrisubalbicans*, M130 for *Burkholderia brasilensis*, and Ppe8 for *Burkholderia tropicalis* (1) were from our collection (Table 1). All strains were grown overnight in NFbHPN (8) medium at 30°C at 120 rpm, diluted (1:10), boiled for 5 min, and cooled on ice, and the DNA was amplified (7) in an OmniGene thermocycler from Hybaid Ltd., Teddington, United Kingdom. The primers used were Y1 (5′-TGGCTCAGAACGAACGCTGGCGGC-3′) (19) (positions 20 to 43 of the *Escherichia coli* 16S rRNA gene) and Y3 (5′-TACCTTGTTACGACTTCACCCCAGTC-3′) (J. P. W. Young, personal communication) (positions 1482 to 1507 of the *E. coli* 16S rRNA gene) (2), complementary to the ends of the 16S rDNA. The DNA templates extracted from all of the strains produced a single band of approximately 1,500 bp.

Y1-Y3 PCR products (10 μl) were digested with *Alu*I, *Hae*III, *Hin*fI, or *Rsa*I (5 U) as specified by Life Technologies, and the fragments were separated on a 2.5% agarose gel and stained with ethidium bromide (0.5 μg/ml). Three to seven fragments and 5 to 10 unique restriction patterns were produced by each endonuclease.

A combination of the restriction digests produced 12 unique banding patterns or ARDRA types (Table 1). Isolates BA153 and X8 shared the same pattern as *H. seropedicae* type strains Z67, Z78, and M2. Isolates AB7, BA10, BA11, BA12, BA14, BA15, BA16, BA17, BA134, BA149, and BA161 had the same pattern as *H. rubrisubalbicans* strain M4. Isolate BA124 showed the same pattern as *B. brasilensis* strain M130. Finally, AB98 and AB147 had the same pattern as *B. tropicalis* strain Ppe8. The remaining 22 isolates produced eight new ARDRA types, types 5 to 12.

Restriction analysis with endonucleases *Alu*I and *Hae*III was sufficient to allocate the strains into the 12 types. Moreover, *Hae*III alone was capable of resolving the most types (10 types), followed by *Alu*I (7 types), and *Hin*fI and *Rsa*I (5 types) (Table 1).

ARDRA types 1 (*H. seropedicae*) and 2 (*H. rubrisubalbicans*) shared all but one DNA fragment in the *Alu*I restriction pattern. Types 10 and 12 were differentiated by only two *Alu*I restriction fragments, and types 6 and 8 were differentiated only by the *Hae*III restriction pattern. A dendrogram, constructed from restriction patterns by using the TreeCon program (15), illustrated these tight relationships and showed three major clusters (Fig. 1). The first was formed by types 1 and 2 and included *H. seropedicae* and the *H. rubrisubalbicans* reference strains, the second was formed by types 3 to 9 and included *Burkholderia* reference strains M130 (type 3) and Ppe8 (type 4), and the third was formed by types 10 to 12 and was distant from the other two. Type 5 was separated from the other types in the cluster formed by types 3 to 9 and separated the *Burkholderia* and *Herbaspirillum* clusters (Fig. 1).

The Y1-Y3 PCR products were purified using Nucleon QC (Amersham Pharmacia Biotech) and sequenced using dye ter-

* Corresponding author. Mailing address: Departamento de Bioquímica, Universidade Federal do Paraná (UFPR), C. Postal 19046, 81.531–990, Curitiba, Paraná, Brazil. Phone: 55 41 3664398. Fax: 55 41 2662042. E-mail: fpedrosa@bio.ufpr.br.

TABLE 1. Characterization of nitrogen-fixing bacteria isolated from *Musa* spp. and *Ananas comosus* (L) Merril cultivated in Brazil, as revealed by ARDRA of Y1-Y3-amplified fragments and Y1-Y2 sequence of the 16S rDNA

Species and strain[a]	Host	Cultivar	Tissue	Geographic origin	Restriction pattern[b]	Type	
						ARDRA[c]	Y1-Y2 sequence[d]
H. seropedicae							
Z67					aaaa	1	I
Z78					aaaa	1	NE[f]
M2					aaaa	1	NE
BA153	Banana	Marmelo	Fruit	Itaguaí, RJ	aaaa	1	1
X8	ND[e]	ND	ND	ND	aaaa	1	II
H. rubrisubalbicans							
M4					baaa	2	III
AB7	Pineapple	Alenquer	Leaf	Cruz das Almas, BA	baaa	2	I
BA10	Banana	Butuhan	Stem	Cruz das Almas, BA	baaa	2	I
BA11	Banana	Butuhan	Leaf	Cruz das Almas, BA	baaa	2	I
BA12	Banana	Yangambi	Root	Cruz das Almas, BA	baaa	2	I
BA14	Banana	Yangambi	Stem	Cruz das Almas, BA	baaa	2	I
BA15	Banana	Prata Anã	Root	Cruz das Almas, BA	baaa	2	I
BA16	Banana	Prata Anã	Stem	Cruz das Almas, BA	baaa	2	I
BA17	Banana	Butuhan	Stem	Cruz das Almas, BA	baaa	2	NE
BA134	Banana	Maçã	Stem	Itaguaí, RJ	baaa	2	I
BA149	Banana	Maçã	Leaf	Itaguaí, RJ	baaa	2	NE
BA161	Banana	Maçã	Root	Itaguaí, RJ	baaa	2	I
B. brasilensis							
M130					cbbb	3	IV
BA124	Banana	Prata Manteiga	Stem	Macaé, RJ	cbbb	3	NE
B. tropicalis							
Ppe8					dccb	4	V
AB98	Pineapple	Pérola	Fruit	Macaé, RJ	dccb	4	V
AB147	Pineapple	Smooth cayenne	Stem	Quissamã, RJ	dccb	4	V
Unknown							
O1	Banana	Yangambi	ND	Cruz das Almas, BA	geed	5	VI
BA22	Banana	Prata Anã	Leaf	Cruz das Almas, BA	geed	5	VI
BA23	Banana	Yangambi	Stem	Cruz das Almas, BA	geed	5	VI
BA25	Banana	Prata Anã	Stem	Cruz das Almas, BA	geed	5	NE
BA27	Banana	Yangambi	Leaf	Cruz das Almas, BA	geed	5	VI
BA88	Banana	Maçã	Leaf	Itaguaí, RJ	geed	5	NE
BA104	Banana	Prata Anã	Stem	Itaguaí, RJ	geed	5	NE
BA106	Banana	Maçã	Leaf	Itaguaí, RJ	geed	5	NE
BA128	Banana	Prata	Fruit	Itaguaí, RJ	geed	5	NE
BA136	Banana	Prata	Leaf	Itaguaí, RJ	geed	5	VI
AB117	Pineapple	Smooth Cayenne	Root	Quissamã, RJ	gfcc	6	VII
AB120	Pineapple	Pérola	Stem	Macaé, RJ	gfcc	6	VII
BA123	Banana	Prata Manteiga	Root	Macaé, RJ	dgce	7	VIII
BA126	Banana	D'água	Root	Itaguaí, RJ	dgce	7	VIII
AB48	Pineapple	Perolera	Root	Cruz das Almas, BA	ghcc	8	IX
AB71	Pineapple	Perolera	Stem	Cruz das Almas, BA	ghcc	8	IX
AB119	Pineapple	Pérola	Leaf	Macaé, RJ	dibb	9	NE
Ala	ND	ND	ND	ND	fddc	10	X
BA131	Banana	D'água	Leaf	Itaguaí, RJ	bjf–	11	XI
A2a	ND	ND	ND	ND	eddc	12	NE
A3b	ND	ND	ND	ND	eddc	12	NE
A8b	ND	ND	ND	ND	eddc	12	NE

[a] The reference strains analyzed were Z67 and M2 from *H. seropedicae*, M4 from *H. rubrisubalbicans*, Ppe8 from *B. tropicalis*, and M130 from *B. brasilensis*.
[b] Restriction pattern obtained for the endonuclease *Alu*I, *Hae*III, *Hin*fI, and *Rsa*I. The four-letter code refers to the restriction patterns produced by the four restriction endonucleases. Each letter defines a common pattern for a given endonuclease.
[c] The ARDRA genotype represents the combination of the four endonucleases.
[d] The Y1-Y2 sequenced region covers approximately 300 bp in the 5′ end of the 16S rDNA molecule. Numbers represent genotypes with 100% identical sequences.
[e] ND, no data.
[f] NE, not examined.

minator chemistry and an ABI PRISM 310 sequencer (Applied Biosystems). Primers Y1 and Y2 (5′-CCCACTGCTGCCTCC CGTAGGAGT-3′) (19) were used to sequence both strands of the variable region (approximately 300 bp) located at the 5′ end of the 16S rRNA gene. The length of the Y1-Y2 region varied from 286 to 290 bp for types I to IX (see below), as reported for beta *Proteobacteria* (11), and was 260 and 259 bp for types X and XI, respectively, as reported for alpha *Proteobacteria* (16, 19).

The 30 bacterial isolates examined were allocated into 11

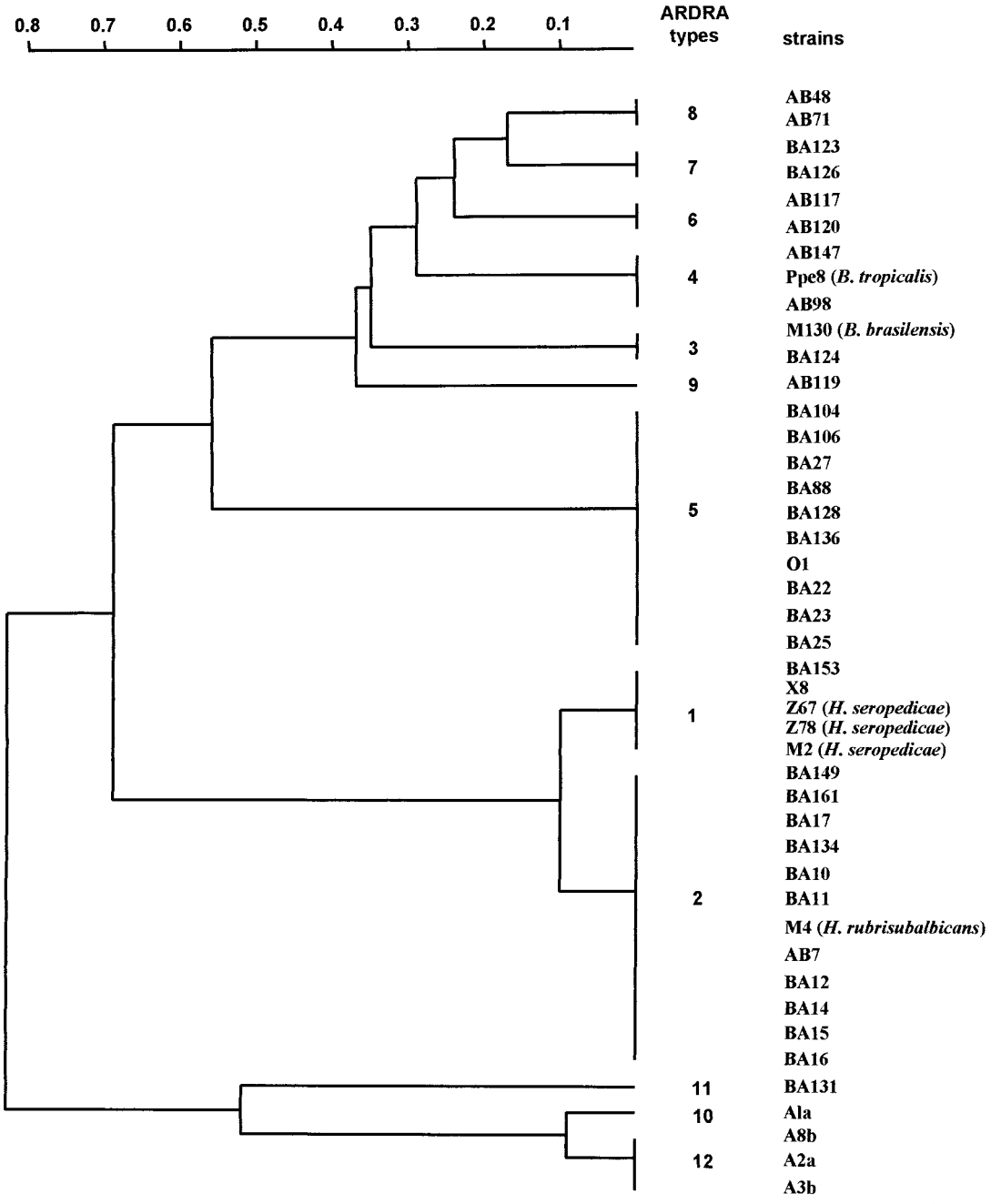

FIG. 1. Dendrogram inferred from *Alu*I, *Hae*III, *Hin*fI, and *Rsa*I restriction pattern data from Y1-Y3 16S rDNA PCR-amplified fragments obtained for the types shown in Table 1. Distances were calculated for all pairwise patterns with Nei-Li coefficient (9), and the dendrogram was established by the unweighted pair-group method with arithmetic average (12).

different groups (types I to XI [Table 1]), with each group consisting of isolates with the identical sequence. These sequences defined types which agreed well with the ARDRA-defined types, showing no apparent polymorphism within the types. However, two disagreements were observed with *Herbaspirillum* types: ARDRA type 1 contained the sequence-defined types I and II, and ARDRA type 2 contained the sequence-defined types I and III. While reference strains of *H. seropedicae* and *H. rubrisubalbicans* had distinct ARDRA (types 1 and 2) and sequence (types I and III) types, 11 isolates had the same *H. rubrisubalbicans* ARDRA type while showing 100% sequence identity to *H. seropedicae* in the Y1-Y2 region (Table 1). These isolates failed to hybridize with an *H. seropedicae* 23S rDNA species-specific probe (17), and the present molecular data support that these may constitute a new *Herbaspirillum* cluster.

A phylogenetic tree was constructed using the type I to XI sequences plus 47 sequences of 16S rDNAs of alpha and beta *Proteobacteria* available in the GenBank database (Fig. 2). The sequences were aligned with the ClustalX program (13), and

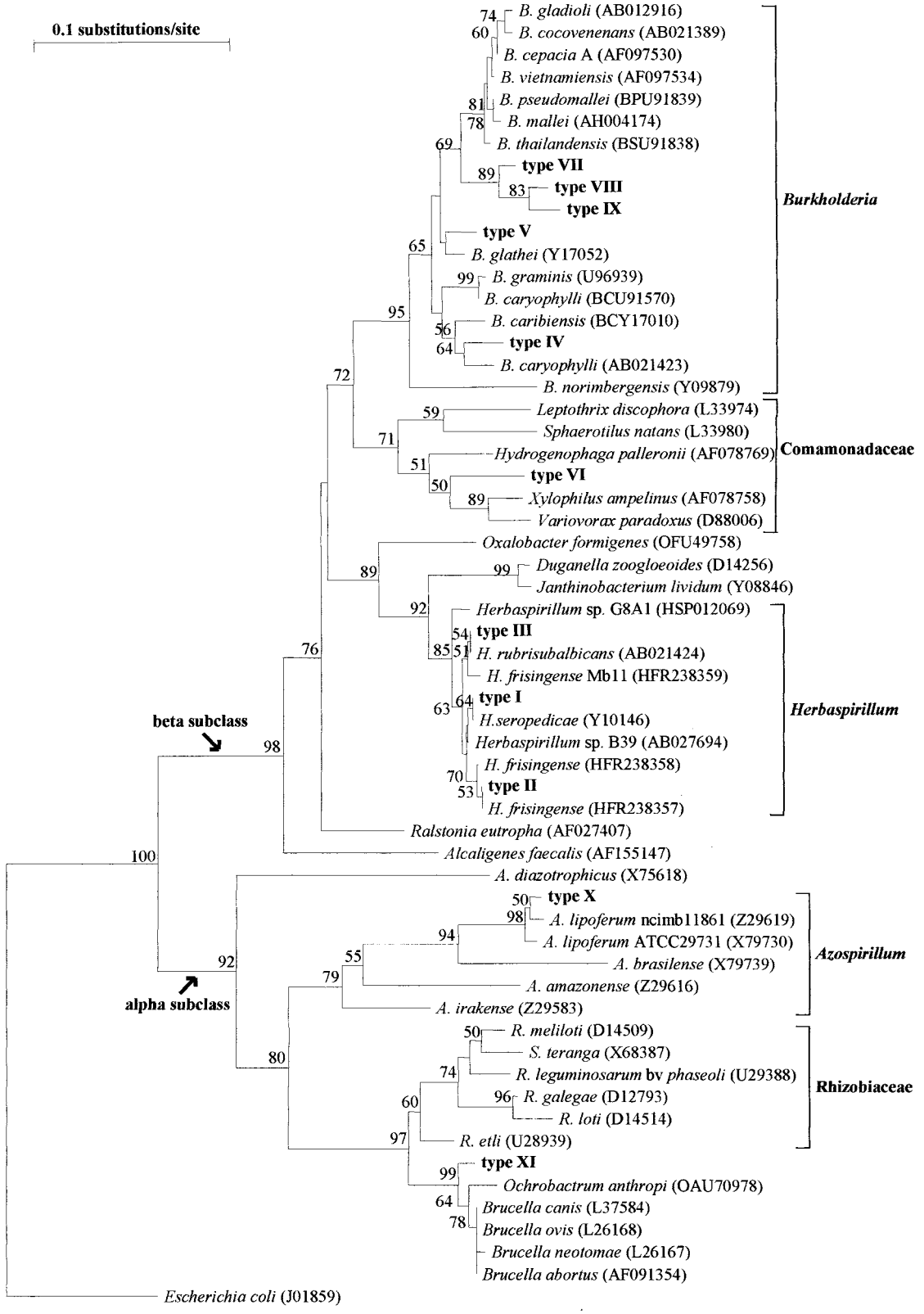

FIG. 2. Phylogenetic tree inferred for genotype sequences I to X and representative organisms of the alpha and beta subclasses of *Proteobacteria* available in the GenBank database (accession numbers are in parentheses). The Y1-Y2 region was used to reconstruct the tree by the neighbor-joining method (10) from distances calculated by the method of Jukes and Cantor (6). A bootstrap analysis with 100 repetitions was performed, and only values above 50 are shown. The sequence of *E. coli* (a member of the gamma subclass of *Proteobacteria*) was used to root the tree.

the phylogenetic tree was reconstructed with the TreeCon program (15). A close relationship of types I, II, and III (Table 1) to the *Herbaspirillum* cluster was evident (Fig. 2). High bootstrap values supported types IV, V, VII, VIII, and IX being clustered within the *Burkholderia* genus. Type VI clustered within the *Comamonadaceae*, a family that originated from the *Pseudomonas* rRNA group III (14), as *Burkholderia* originated from *Pseudomonas* rRNA group II (18), and which also contains phytopathogenic species. Finally, types X and XI clustered within the alpha *Proteobacteria*, the former close to *Azospirillum lipoferum* and the latter close to *Ochrobactrum anthropi*.

The 14 isolates described by Weber et al. (17) used in this work were originally assigned to six groups related to *Herbaspirillum* and *Burkholderia*. Isolates AB48, AB98, AB119, AB120, AB147, BA123, BA124, and BA126, originally present within the same morphological and physiological group as strain M130 of *B. brasilensis* (17), clustered into six ARDRA groups (types 3, 4, 6, 7, 8, and 9), with only isolate BA124 being related to strain M130 (Table 1). Isolates AB98 and AB147 were similar to *B. tropicalis* Ppe8, while isolates AB48, AB119, AB120, BA123, and BA126 clustered within the *Burkholderia* genus. Isolates BA22 and BA23 failed to hybridize to oligonucleotide probes specific for *Azospirillum* spp., *Herbaspirillum* spp., *Burkholderia* spp., and *Acetobacter diazotrophicus* (17). The present results showed that these two isolates and eight new isolates, sharing the same ARDRA type, were related to *Comamonadaceae*.

In this paper we redefined 14 isolates described by Weber et al. (17) and the 24 new isolates into 12 genotypes. The discovery of eight new nitrogen-fixing bacterial genotypes, in addition to *H. seropedicae*, *H. rubrisubalbicans*, *B. brasilensis,* and *B. tropicalis*, in a few bacterial isolates from banana and pineapple revealed the great diversity of nitrogen-fixing bacteria associated with these fruit crops.

Nucleotide sequence accession numbers. The sequences of the Y1-Y2 region of the 16S rDNA have been deposited in the GenBank database under accession numbers AF164042 through AF164065 and AF213248.

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