Erwinia papayae sp. nov., a pathogen of papaya (*Carica papaya*)

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Bacterial canker of papaya (*Carica papaya*) emerged during the 1980s in different islands of the Caribbean. Nineteen strains of Gram-negative, rod-shaped, non-spore-forming bacteria isolated from papaya were compared to 38 reference and type strains of phytopathogenic *Enterobacteriaceae* and related bacteria. Phylogenetic analysis of 16S rRNA gene sequences showed that the papaya strains belonged to the genus *Erwinia*. The DNA G+C content of strain CFBP 5189^T, 52.5 mol%, is in the range of the genus *Erwinia*. The 19 papaya strains were all pathogenic to papaya and were differentiated clearly from type or reference strains of phytopathogenic enterobacteria and related bacteria by phenotypic tests. The papaya strains constituted a discrete DNA hybridization group, indicating that they belonged to a unique genomic species. Thus, strains pathogenic to papaya belong to a novel species for which the name *Erwinia papayae* sp. nov. is proposed, with the type strain CFBP 5189^T (=NCCPB 4294^T).

Papaya (*Carica papaya*) is an economically important tropical cash crop for export, as well as a significant vegetable crop in small farming systems worldwide. Early in the 20th century, a disease caused by '*Bacillus papaya*' in Java (von Rant, 1931) was identified; this bacterium was further typed to the genus *Erwinia* by Magrou (1937). Nelson & Alvarez (1980) described a purple stain of *Carica papaya*, due to *Erwinia herbicola*. More recently, Trujillo & Schroth (1982) reported a bacterial decline of papaya caused by two *Erwinia* species in the Mariana Islands. Finally, reports from Webb (1985) in the US Virgin Islands, from Frossard *et al.* (1985) and Prior *et al.* (1985) in the French West Indies and from Guevara *et al.* (1993) in Venezuela, all consistently described this disease, which was named 'bacterial stem canker'. Typical symptoms of bacterial stem cankers on Solo-type cultivars include greasy, water-soaked lesions and spots on leaves, which evolve into foliar, angular lesions.

Firm, water-soaked cankers develop on the stem, sometimes leading to the destruction of papaya trees (Prior *et al.*, 1985; Webb, 1985).

Preliminary identification of this novel bacterium by classical phenotypic tests led Trujillo & Schroth (1982), Frossard *et al.* (1985), Prior *et al.* (1985) and Webb (1985) to place this bacterium in the genus *Erwinia* and in the Amylovora group according to Dye (1968). In order to clarify the taxonomic status of this phytopathogenic bacterium, we developed a polyphasic taxonomic study.

Gram-negative bacteria were isolated from water-soaked lesions and were typically slow-growing, giving colonies of 2–3 mm diameter after 3–4 days incubation. Colonies were hyaline on YBGA (0.7 % yeast extract, 0.7 % bactopectone, 0.7 % glucose and 1.5 % agar, pH 7.2) or on King's medium B at 25 °C, with a mucoid aspect and white to creamy white colour after 2–3 days. A non-diffusible blue pigment was observed on King's medium B.

This study included a selection of 19 strains from a collection of 72 strains that were isolated in 1982–1991 from *Carica papaya* in different islands of the Caribbean (Table 1) and 38 type or reference strains of phytopathogenic *Enterobacteriaceae* that belong to the genera *Erwinia*, *Enterobacter*, *Brenneria*, *Pantoea*, *Pectobacterium* and *Samsonia*.

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Abbreviations: ML, maximum-parsimony; ML, maximum-likelihood; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of CFBP 5189^T is AY131237.

A full phylogenetic tree is available as supplementary material in IJSEM Online.

Table 1. Strains of *Erwinia* sp. isolated from *Carica papaya* lesions used in this study

CFBP no.	Host plant	Geographical origin	Year of isolation
2109	<i>Carica papaya</i>	Guadeloupe, France	
2110	<i>Carica papaya</i>	Guadeloupe, France	
2111	<i>Carica papaya</i>	Guadeloupe, France	
2462	<i>Carica papaya</i>	Guadeloupe, France	1985
2464	<i>Carica papaya</i>	Guadeloupe, France	1985
2465	<i>Carica papaya</i>	Martinique, France	1985
5164	<i>Carica papaya</i> cv. <i>Solo</i>	Grenada	1994
5168	<i>Carica papaya</i> cv. <i>Local</i>	Saint Lucia	1994
5174	<i>Carica papaya</i> cv. <i>Local</i>	Saint Vincent	1994
5184	<i>Carica papaya</i> cv. <i>Local</i>	Sainte Croix	1995
5185	<i>Carica papaya</i> cv. <i>Local</i>	Guadeloupe, France	1993
5189 ^T	<i>Carica papaya</i> cv. <i>Local</i>	Martinique, France	1995
5288	<i>Carica papaya</i> cv. <i>Local</i>	Guadeloupe, France	1988
5292	<i>Carica papaya</i> cv. <i>Local</i>	Guadeloupe, France	1988
5300	<i>Carica papaya</i>	Grenada	1991
5301	<i>Carica papaya</i> cv. <i>Tainung</i>	Dominica	1991
6528	<i>Carica papaya</i>	Grenada	1991
6529	<i>Carica papaya</i>	Grenada	1991
6530	<i>Carica papaya</i>	Grenada	1991

Pathogenicity of papaya isolates was assessed on Solo-type cultivars following inoculation with a sterile needle; this consisted of wound inoculation at the apex of young seedlings with 50 µl calibrated suspension at 10^8 c.f.u. ml⁻¹. Infected plants were grown under greenhouse conditions (25–30 °C in the day/23–25 °C at night and a photoperiod of 6 h).

We used 121 phenotypic tests, including 22 conventional tests and assimilation of 99 carbon sources by using Biotype 100 strips (bioMérieux), as described previously (Gardan *et al.*, 2002). A distance matrix was calculated by using the Jaccard coefficient and cluster analysis was done by using UPGMA (Sneath & Sokal, 1973). Discriminative tests were selected by using a diagnostic ability coefficient that was deduced from the numerical analysis (Descamp & Véron, 1981).

Selection of related sequences was performed according to previous phylogenetic analyses of a database of 62 000 already aligned bacterial 16S rRNA gene sequences and BLAST searches against the latest release of EBI (European Bioinformatics Institute). The new sequence was aligned manually by using SeaView (Galtier *et al.*, 1996). In a preliminary analysis, 150 sequences were selected. A phylogenetic tree of 32 sequences was then built, including a sequence for each type strain of species of the genera *Erwinia* and *Pantoea* that were available, as well as representatives of the genera *Brenneria*, *Pectobacterium* and *Samsonia*. Sequences from three *Pantoea* species that do not yet have validly published names ('*Pantoea oleae*', '*Pantoea cedenensis*' and '*Pantoea toletana*') were also included for completeness of the analysis. Phylogenetic trees were then constructed by using three different methods: neighbour-joining (NJ) by using BIONJ (Gascuel, 1997), maximum-likelihood (ML)

and maximum-parsimony (MP). For the BIONJ analysis, distance matrices were calculated by using Kimura's two-parameter correction. ML and MP were from PHYLIP (Phylogeny Inference Package, version 3.573c; distributed by J. Felsenstein, Department of Genetics, UW, Seattle, WA, USA). Almost-entire sequences that corresponded to positions 74–1436 of strain CFBP 5189^T were used for these analyses (shown in Fig. 1 and Supplementary Fig. A in IJSEM Online). Phylogenetic trees were drawn by using NJPLOT (Perrière & Gouy, 1996). The topology shown is that of the bootstrap tree, as it has been demonstrated that this topology is often better than that of a simple tree (Berry &

Fig. 1. Rooted tree, subset of a larger tree (available as supplementary material in IJSEM Online), resulting from a neighbour-joining bootstrap analysis (1000 replications). Bootstrap percentages are indicated only for branches that were also retrieved by MP (strict consensus of six equally parsimonious trees) and ML at $P < 0.01$, therefore indicating robust clades. For ML analysis, ln(likelihood) was -6492 and 4370 trees were examined.

Phenons

Fig. 2. UPGMA-based dendrogram of phenotypic characteristics of the 57 strains (numbers indicate CFBP accession numbers). Distance, 1–Jaccard coefficient.

Gascuel, 1996). There is no distance bar in Fig. 1; it would be meaningless as (i) distances are corrected (see above) and (ii) this is a bootstrap tree.

Extraction and purification of DNA were performed as described by Brenner *et al.* (1982). Native DNA of strain CFBP 5189^T was labelled *in vitro* by nick-translation with tritium-labelled nucleotides (Amersham Biosciences). The S1 nuclease/trichloroacetic method was used for DNA–DNA hybridization (Crosa *et al.*, 1973). DNA reassociation was performed at 65 °C.

The DNA G+C content of strain CFBP 5189^T was determined by the thermal denaturation method of Marmur & Doty (1962) and calculated by using the equation of Owen & Lapage (1976).

Isolates that gave a positive reaction induced water-soaked, greasy spots around the inoculation point, 5–7 days after inoculation. These water-soaked lesions developed into stem cankers, mainly due to secondary invaders. Death occurred about 3 weeks after inoculation. Thus, all symptoms observed under field conditions were reproduced and the original bacteria could be reisolated consistently as pure cultures from water-soaked necroses.

A dendrogram of phenotypic distances among the 57 strains is shown in Fig. 2. At a distance of 0.419, six phenons and four isolated strains were delineated. Phenon 4 corresponded to the 19 strains isolated from papaya and *Erwinia mallotivora* CFBP 2503^T. Phenons 1, 2, 3, 5 and 6, each of which contained between two and nine strains,

Table 2. Phenotypic characteristics that differentiate phenons and strains

+, 90–100 % of strains are positive; –, 90–100 % of strains are negative; D, 11–89 % of strains are positive (percentage of positive strains is given in parentheses).

Characteristic	Phenon no.*						Type strain†				
	1	2	3	4	5	6	7	8	9	10	11
Utilization of:											
Aesculin	+	+	+	–	D (67)	–	–	+	–	+	–
L-Serine	+	D (25)	+	–	D (33)	D (50)	–	+	–	–	–
Maltotriose	–	–	+	–	–	–	–	+	–	–	–
D-Alanine	–	–	+	–	–	–	–	+	–	–	–
Maltose	–	–	+	–	D (33)	–	–	+	–	–	–
L-Aspartate	+	+	+	–	+	–	–	+	–	+	+
α-L-Rhamnose	D (85)	+	+	–	–	–	–	–	–	–	–
2-Keto-D-gluconate	D (23)	–	+	–	–	–	–	+	–	–	–
D-Glucuronate	D (77)	D (25)	+	–	–	–	–	+	–	–	–
L-Alanine	D (31)	–	+	–	–	D (50)	–	+	–	–	–
Melibiose	D (77)	–	+	–	D (33)	D (50)	–	–	–	+	+
Liquefaction of calcium polygalacturonate	+	D (25)	–	–	–	–	–	–	–	–	–
Growth at 36 °C	+	D (50)	D (73)	–	D (67)	–	–	–	–	–	+
L-(+) Arabinose	+	D (75)	+	+	+	+	–	–	+	+	+
D-Glucosamine	+	+	D (82)	+	+	D (50)	–	+	+	+	+
Mannitol	+	+	+	–	+	+	+	+	–	+	+
DL-Lactate	D (69)	D (50)	+	–	–	–	+	+	–	–	–
10-Methyl-β-galactopyranoside	D (85)	–	D (82)	–	–	–	–	–	–	–	+
D-(+)-Raffinose	D (77)	–	D (45)	–	–	–	–	+	–	+	+
D-(+)-Trehalose	D (31)	+	+	+	+	+	+	+	–	–	+
D-(–)-Arabinose	D (38)	D (25)	+	–	D (67)	–	–	+	–	–	+
Sucrose-reducing compounds	D (23)	–	–	–	+	+	+	–	+	+	–

*Reference or type strains (strain numbers are given in Fig. 2) in each phenon: 1, *Pectobacterium chrysanthemi* (nine biovars), *Pectobacterium carotovorum* (four subspecies); 2, *Samsonia erythrinae*, *Pectobacterium carotovorum* subsp. *wasabiae*, *Erwinia psidii*, *Pectobacterium cacticida*; 3, *Pantoea agglomerans*, *Pantoea agglomerans* pv. *milletiae*, *Pectobacterium cypripedii*, *Erwinia herbicola* pv. *gypsophilae*, *Pantoea ananatis* pv. *ananatis*, *Pantoea ananatis* pv. *uredovora*, *Pantoea stewartii* subsp. *indologenes*, *Erwinia rhapontici*, *Erwinia persicina*, *Enterobacter pyrinus*, *Enterobacter cancerogenus*; 4, *Erwinia papayae* (19 strains) without *Erwinia mallotivora*; 5, *Brenneria rubrifaciens*, *Brenneria alni*, *Brenneria quercina*; 6, *Erwinia amylovora*, *Erwinia pyrifoliae*.

†Type strains: 7, *Erwinia mallotivora* CFBP 2503^T; 8, *Brenneria nigrifluens* CFBP 3613^T; 9, *Erwinia tracheiphila* CFBP 2355^T; 10, *Brenneria salicis* CFBP 802^T; 11, *Pantoea stewartii* subsp. *stewartii* CFBP 3620^T.

Table 3. Levels of DNA reassociation among strains of *Erwinia papayae* and reference or pathotype strains of the genera *Samsonia*, *Pectobacterium*, *Brenneria* and *Pantoea*

Values are relative binding (% with SD in parentheses) with labelled DNA from *Erwinia papayae* CFBP 5189^T.

Taxon	DNA binding
<i>Erwinia papayae</i> :	
CFBP 5189 ^T	100
CFBP 6529	100
CFBP 5300	95 (10)
CFBP 6528	94 (8·7)
CFBP 5301	91 (10·4)
CFBP 5164	87 (9·9)
CFBP 5292	86 (4·2)
CFBP 6530	85 (0·7)
CFBP 2110	84 (5·6)
CFBP 2109	80 (7·9)
CFBP 5184	80 (2·2)
CFBP 5168	76 (8·5)
CFBP 2111	76 (7·9)
CFBP 2465	76 (2·8)
CFBP 2464	75 (9)
CFBP 2462	69 (5)
CFBP 5185	68 (2·8)
CFBP 5174	67 (7·4)
<i>Erwinia mallotivora</i> CFBP 2503 ^T	44 (4·5)
<i>Erwinia pyrifoliae</i> CFBP 4172 ^T	18
<i>Erwinia psidii</i> CFBP 3627 ^T	15
<i>Erwinia persicina</i> CFBP 3622 ^T	7
<i>Erwinia rhapontici</i> CFBP 3618 ^T	7
<i>Erwinia herbicola</i> pv. <i>gypsophilae</i> CFBP 4341	6
<i>Erwinia amylovora</i> CFBP 1232 ^T	5
<i>Erwinia alni</i> CFBP 3923 ^T	3
<i>Erwinia tracheiphila</i> CFBP 2355 ^T	2
<i>Enterobacter pyrinus</i> CFBP 4168 ^T	4
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i> CFBP 2046 ^T	3
<i>Pectobacterium carotovorum</i> subsp. <i>atrosepticum</i> CFBP 1526 ^T	3
<i>Pectobacterium carotovorum</i> subsp. <i>betavascularum</i> CFBP 2112 ^T	3
<i>Pectobacterium carotovorum</i> subsp. <i>odoriferum</i> CFBP 1878 ^T	2
<i>Pectobacterium carotovorum</i> subsp. <i>wasabiae</i> CFBP 3304 ^T	3
<i>Pectobacterium cacticidum</i> CFBP 3628 ^T	3
<i>Pectobacterium cypripedii</i> CFBP 3613 ^T	7
<i>Pectobacterium chrysanthemi</i> CFBP 2048 ^T	9
<i>Samsonia erythrinae</i> CFBP 5236 ^T	3
<i>Brenneria quercina</i> CFBP 3617 ^T	4
<i>Brenneria rubrifaciens</i> CFBP 3619 ^T	2
<i>Brenneria salicis</i> CFBP 802 ^T	4
<i>Brenneria nigrifluens</i> CFBP 4008 ^T	2
<i>Brenneria paradisiaca</i> CFBP 4178 ^T	3
<i>Pantoea stewartii</i> CFBP 3167 ^T	8
<i>Pantoea stewartii</i> subsp. <i>indologenes</i> CFBP 3614 ^T	7
<i>Pantoea agglomerans</i> pv. <i>milletiae</i> CFBP 3615	6
<i>Pantoea agglomerans</i> CFBP 2240 ^T	6
<i>Pantoea ananatis</i> pv. <i>ananatis</i> CFBP 3612	5

corresponded to type and reference strains of phytopathogenic *Enterobacteriaceae*. Phenotypic characteristics that differentiate the papaya strains from other reference strains are shown in Table 2. Numerous tests are available to identify the papaya strains, as they used only some carbon sources in comparison with most other strains characterized in this study (an exception is *Erwinia tracheiphila*). Like *Erwinia amylovora*, this novel bacterium requires growth factors; Prior *et al.* (1985) supplemented growth media with yeast extract. The papaya strains are differentiated clearly from *E. mallotivora* by their ability to assimilate L-(+)-arabinose and D-glucosamine, but not mannitol or DL-lactate, and to reduce sucrose compounds; the reverse reactions were obtained for *E. mallotivora* (Table 2). Strain-dependent reactions for papaya strains were observed for utilization of *cis*-aconitate (four strains), mucate (five strains), galacturonate (11 strains) and citrate (five strains). Phenon 4, which contained the papaya strains, was split into two subphena by *E. mallotivora*. Strains of different origins (several islands) are clustered in the same subphenon and, conversely, strains of one origin (one island, e.g. Guadeloupe, France) are clustered in different subphena; thus, we did not observe a correlation between geographical origin and clustering in the two subphena. Papaya strains evolved all over the Caribbean, which is also the diversification centre for *Carica papaya*; such restricted environments are well-known to develop particular traits in terms of population genetics, but we have no likely hypothesis to explain why the papaya strains were split into two subphena.

Detailed scrutiny of the phylogenetic tree, which is the consensus of all three methods (NJ, MP and ML), showed that papaya strain CFBP 5189^T formed a robust clade with the type strain of *E. mallotivora* (all methods; bootstrap value, 94%; 98.6% sequence similarity; 20 nucleotide differences). Both strains were also grouped with all other *Erwinia* species in a robust clade (all methods; bootstrap value, 67%) with *Pectobacterium cypripedii* as a sister taxon. The three species without validly published names, '*Pantoea oleae*', '*Pantoea cedenensis*' and '*Pantoea toletana*', clearly belong to the genus *Erwinia* and should be named adequately. A sequence retrieved from GenBank as the type strain of *Erwinia herbicola* did not belong to this clade (see Supplementary Fig. A), but analysis of the literature (Gavini *et al.*, 1989) demonstrated that this species had to be reassigned as *Pantoea agglomerans*, as supported by our phylogenetic analysis. In conclusion, the novel bacterium belongs phylogenetically to the genus *Erwinia*; DNA–DNA hybridization data are necessary to decide whether or not it constitutes a distinct species.

DNA–DNA reassociation results are shown in Table 3. The 19 papaya strains were 67–100% related to strain CFBP 5189^T (mean, 82.3 ± 10.2%). *E. mallotivora* CFBP 2503^T was 44% related to strain CFBP 5189^T and was the most closely related organism to the papaya strains. The rest of the type and reference strains of phytopathogenic *Enterobacteriaceae* were 2–18% related to strain CFBP 5189^T.

Thus, the papaya strains constitute a homogeneous genomic group at the species level.

The DNA G + C content of strain CFBP 5189^T was 52.5 mol%. This G + C content is in the range of the genus *Erwinia* (49.8–54.1 mol%) (Hauben *et al.*, 1998).

All these results correspond to the definition of bacterial species according to Wayne *et al.* (1987) and we propose the name *Erwinia papayae* sp. nov. for this novel bacterium that is pathogenic to *Carica papaya*.

Description of *Erwinia papayae* sp. nov.

Erwinia papayae (pa.pa'yae, N.L. gen. n. *papayae* of papaya, the host plant from which the organism was isolated).

This species has the characteristics of the genus *Erwinia*. Colonies on YBGA are hyaline and 2–3 mm in size after 3–4 days incubation at 25 °C. A non-diffusible blue pigment is produced on King's medium B. Growth occurs at 28 °C, but not at 36 or 39 °C; sucrose-reducing compounds are produced. In addition to the characteristics mentioned in Table 2, β-D-(+)-fructose, D-(+)-galactose, D-(+)-mannose, sucrose, D-(–)-ribose, glycerol, *N*-acetylglucosamine and D-gluconate are utilized; strains do not assimilate 73 of 100 carbon sources of the Biotype 100 gallery (bioMérieux). DNA G + C content of the type strain is 52.5 mol%.

The type strain is CFBP 5189^T (= NCPPB 4294^T). Strains of *Erwinia papayae* cause bacterial canker of papaya.

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