

## *Liberibacter crescens* gen. nov., sp. nov., the first cultured member of the genus *Liberibacter*

Jennie R. Fagen,<sup>1</sup> Michael T. Leonard,<sup>1</sup> Janelle F. Coyle,<sup>1</sup> Connor M. McCullough,<sup>1</sup> Austin G. Davis-Richardson,<sup>1</sup> Michael J. Davis<sup>2</sup> and Eric W. Triplett<sup>1</sup>

Correspondence  
Eric W. Triplett  
ewt@ufl.edu

<sup>1</sup>Microbiology and Cell Science Department, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL, USA

<sup>2</sup>Plant Pathology Department, Citrus Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Lake Alfred, FL, USA

The Gram-stain-negative, rod-shaped bacterial isolate BT-1<sup>T</sup> is the closest relative to the genus '*Candidatus Liberibacter*' cultured to date. BT-1<sup>T</sup> was recovered from the phloem sap of a defoliating mountain papaya in Puerto Rico. The BT-1<sup>T</sup> 16S rRNA gene sequence showed that strain BT-1<sup>T</sup> is most closely related to members of the genus '*Ca. Liberibacter*' sharing 94.7% 16S rRNA gene sequence similarity with '*Ca. Liberibacter americanus*' and '*Ca. Liberibacter asiaticus*'. Additionally, average nucleotide identity, 16S rRNA gene sequences and conserved protein sequences supported inclusion of the previously described species of the genus '*Ca. Liberibacter*' in a genus with BT-1<sup>T</sup>. The prominent fatty acids of isolate BT-1<sup>T</sup> were C<sub>18:1</sub>ω7c (77.2%), C<sub>16:0</sub> OH (4.8%), C<sub>18:0</sub> (4.4%) and C<sub>16:0</sub> (3.5%). Both physiological and genomic characteristics support the creation of the genus *Liberibacter*, as well as the novel species *Liberibacter crescens* gen. nov., sp. nov. with type strain BT-1<sup>T</sup> (=ATCC BAA-2481<sup>T</sup>=DSM 26877<sup>T</sup>).

The candidate genus '*Candidatus Liberibacter*' was first described as a member of the class *Alphaproteobacteria* (Jagoueix *et al.*, 1994). The genus includes pathogens affecting economically important crops such as potato, tomato and citrus (Hansen *et al.*, 2008; Jagoueix *et al.*, 1994; Liefting *et al.*, 2009; Teixeira *et al.*, 2005). *Ca. Liberibacter* infection is confined to the phloem of the plant leading to symptoms indicative of a restricted nutrient supply, such as yellowing of the leaves and diminished fruit quality (Kapur *et al.*, 1978). Previously described pathogens of the genus '*Ca. Liberibacter*' are vectored by members of the *Psyllidae* family of insects and are introduced into the plant phloem during feeding (Capoor *et al.*, 1967; Pelz-Stelinski *et al.*, 2010). All previously documented species of the genus '*Ca. Liberibacter*' are fastidious and have yet to be cultured reproducibly in the laboratory. This has led to a dearth of physiological data and as such, each species of the genus '*Ca. Liberibacter*' has been labelled '*Candidatus*' (Murray & Stackebrandt, 1995).

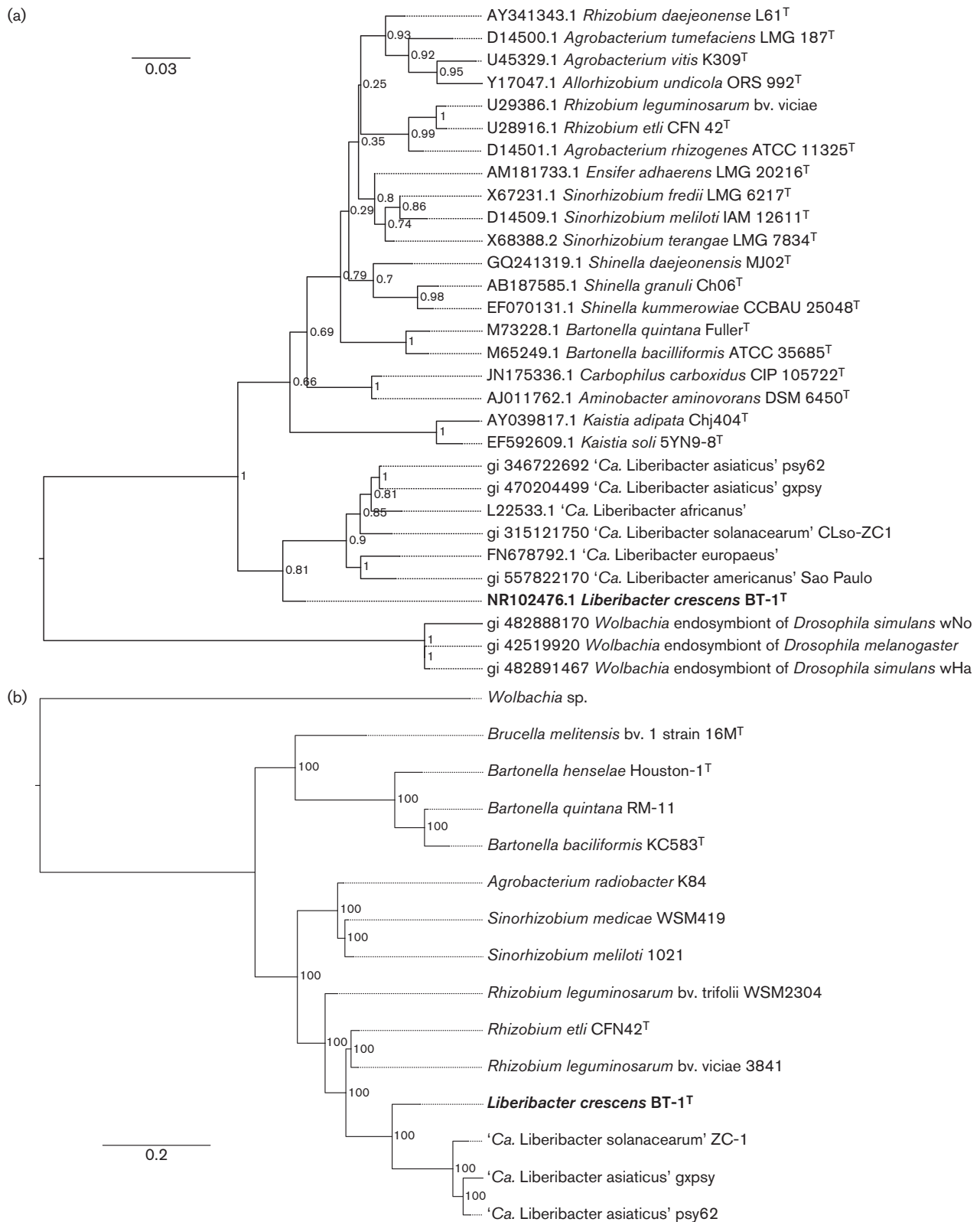
A novel bacterium, BT-1<sup>T</sup>, was isolated from expressed sap of a Babaco papaya, *Carica pentagona* Heilborn, family *Caricaceae*, during an investigation of Papaya Bunchytop Disease (Davis *et al.*, 2008). Transmission electron microscopy imaging (Leonard *et al.*, 2012) showed a rod-shaped

bacterium of 0.5 × 1.75 μm. BT-1<sup>T</sup> exhibiting polar division consistent with that described in other members of the order *Rhizobiales* (Brown *et al.*, 2012). Fimbriae were abundant, but no other outstanding surface features were observed.

To investigate further the phylogenetic placement of strain BT-1<sup>T</sup>, the genome of isolate BT-1<sup>T</sup> was sequenced (Leonard *et al.*, 2012; NCBI Reference Sequence NC\_019907.1). The 16S rRNA gene sequence derived from the completed genome was compared to those of species of the genus '*Ca. Liberibacter*' as well as related members of the order *Rhizobiales*. The BT-1<sup>T</sup> 16S rRNA gene sequence was aligned against the NCBI non-redundant nucleotide database using Megablast. The alignment showed the 16S rRNA gene of BT-1<sup>T</sup> shares 94.7% sequence similarity with the 16S rRNA genes of '*Ca. Liberibacter americanus*' and '*Ca. Liberibacter asiaticus*', 94.0% similarity with '*Ca. Liberibacter solanacearum*', and 93.4% similarity with '*Ca. Liberibacter africanus*'.

Phylogenetic placement of BT-1<sup>T</sup> was visualized with relatedness trees of both the 16S rRNA gene (Fig. 1a) and 118 conserved protein sequences (Fig. 1b). The 16S rRNA gene sequence tree (Fig. 1a) was reconstructed using DNA sequences from the BT-1<sup>T</sup> genome and close relatives. These sequences were aligned using MUSCLE (Edgar, 2004);

Two supplementary tables are available with the online version of this paper.



**Fig. 1.** Maximum-likelihood phylogenetic trees reconstructed using (a) 16S rRNA gene sequences and (b) concatenated amino acid sequences of 118 BT-1<sup>T</sup> proteins and those of related members of the class *Alphaproteobacteria*. Bars, 0.03 substitutions per nucleotide position (a); 0.2 substitutions per amino acid position (b).

overhanging regions were manually removed; and the alignment was curated using Gblocks (Castresana, 2000). Phylogeny was determined with PhyML using the GTR substitution model with 100 bootstraps (Anisimova & Gascuel, 2006; Guindon & Gascuel, 2003). The resultant phylogenetic tree was generated with TreeDyn (Chevenet *et al.*, 2006). These tools were accessed through phylogeny.fr (Dereeper *et al.*, 2008). A second phylogenetic tree was reconstructed using the concatenated amino acid sequences of 118 shared proteins (Fig. 1b). Sequences were aligned by MUSCLE (Edgar, 2004), trimmed by Gblocks (Castresana, 2000), and the tree was reconstructed by RAxML (Stamatakis, 2006) using the WAG substitution model. Bootstrap values were based on 1000 iterations and were all reported at 100 %.

Average nucleotide identity (ANI) was determined across the BT-1<sup>T</sup> genome and those of two previously sequenced species of the genus ‘*Ca. Liberibacter*’ as well as close relatives from other genera. ANI was calculated in JSpecies using the MUMmer (ANIm) algorithm (Richter & Rosselló-Móra, 2009). In all cases, BT-1<sup>T</sup> exhibited highest similarity with ‘*Ca. Liberibacter solanacearum*’ and ‘*Ca. Liberibacter asiaticus*’ with an ANIm of 78.2 % and 77.4 %, respectively (Table 1).

A direct fatty acid profile was generated using the Sherlock Microbial Identification system according to the manufacturer’s specifications, and peaks were identified using the aerobic bacteria library TSBA6 (Microbial ID). BT-1<sup>T</sup> was grown for 4 days in BM7 broth (Fagen *et al.*, 2014). Cultures were maintained at 28 °C in a shaking incubator at 125 r.p.m. Cells were pelleted and frozen prior to analysis.

The major fatty acids of isolate BT-1<sup>T</sup> were C<sub>18:1</sub>ω7c (77.2 %), C<sub>16:0</sub> OH (4.8 %), C<sub>18:0</sub> (4.4 %), C<sub>16:0</sub> (3.5 %), C<sub>20:1</sub>ω7c (3.0 %) and C<sub>18:1</sub>ω7c 11-methyl (2.5 %). Retention times and percentages for less prominent fatty acids can be seen in Table S1 (available in the online Supplementary Material). BT-1<sup>T</sup> has many of the same primary cellular fatty acids as species of the genus *Bartonella* but its fatty acid profile is quite divergent from those of both *Sinorhizobium meliloti* and *Rhizobium etli* (Galibert *et al.*, 2001; Segovia *et al.*, 1993). The phospholipid profiles of

species of the genus ‘*Ca. Liberibacter*’ are not available for comparison due to the absence of a pure culture.

The phenotypic properties of strain BT-1<sup>T</sup> were investigated using cells grown to exponential phase on BM7 (Fagen *et al.*, 2014). Oxidase (1 % tetramethyl *p*-phenylenediamine), catalase (3 %, v/v, H<sub>2</sub>O<sub>2</sub>), indole (Kovac’s reagent) and urease tests were negative. Many of these traditional tests are not compatible with slow-growing, fastidious bacteria; for this reason, each reaction was confirmed using genomic evidence. The optimal growth conditions for BT-1<sup>T</sup> were at pH 6.5 and 27 °C.

BT-1<sup>T</sup> was challenged with the following laboratory antibiotics in liquid culture: ampicillin (25 µg ml<sup>-1</sup>), kanamycin (50 µg ml<sup>-1</sup>), nalidixic acid (10 µg ml<sup>-1</sup>), rifampicin (2.5 µg ml<sup>-1</sup>), chloramphenicol (30 µg ml<sup>-1</sup>), streptomycin (50 µg ml<sup>-1</sup>), spectinomycin (50 µg ml<sup>-1</sup>), neomycin (50 µg ml<sup>-1</sup>), tetracycline (15 µg ml<sup>-1</sup>), vancomycin (40 µg ml<sup>-1</sup>) and amikacin (100 µg ml<sup>-1</sup>). Of these, BT-1<sup>T</sup> was resistant to nalidixic acid, vancomycin and amikacin. A more complete description of the BT-1<sup>T</sup> antibiotic profile is given in Table S2.

Although strain BT-1<sup>T</sup> was originally isolated from a diseased plant, it is not known whether it is a plant pathogen. All previously described species of the genus ‘*Ca. Liberibacter*’ are transmitted through an insect vector of the family *Psyllidae* (Capoor *et al.*, 1967; Hansen *et al.*, 2008; Raddadi *et al.*, 2011). Whether BT-1<sup>T</sup> inhabits an insect host is unknown; however, the leafhopper *Empoasca papayae* is recommended for further study due to previous documentation of its association with phloem-limited bacteria in papaya (Pérez *et al.*, 2010).

Primers were designed for specific amplification of the chorismate synthase gene of BT-1<sup>T</sup> (LCF 5′-CGCTCTC-GATGGGATTGGAA-3′ and LCR 5′-CTGAGGTTTCTG-TCCCGTC-3′). A recent comparative genomics study showed that this gene is absent in the uncultured species of the genus ‘*Ca. Liberibacter*’ whose complete genomes are available (Fagen *et al.*, 2014). These primers amplify BT-1<sup>T</sup> sequences using the following thermal profile: 2 min at 94 °C; followed by 25 cycles of 94 °C for 20 s, 62 °C for 20 s, 72 °C for 30 s; and a final elongation at 72 °C for 5 min.

The 16S rRNA gene sequence similarities, conserved protein sequences and whole genome comparisons, as well as general attributes such as native ecology and reduced genome size (Table 2) show that isolate BT-1<sup>T</sup> is a novel member of the genus ‘*Ca. Liberibacter*’. Isolate BT-1<sup>T</sup> is divergent from the uncultured members of the genus, which cluster together in all comparisons presented in this work. This isolate is the first member of the genus to be maintained in culture and thereby allows for a complete description of the genus and removal of the *Candidatus* status. We propose that the name *Liberibacter* be retained for this genus and propose the name *Liberibacter crescens* sp. nov. to accommodate the BT-1<sup>T</sup> isolate.

**Table 1.** Shared average nucleotide identity (ANI) across the genomes of BT-1<sup>T</sup> and close relatives including two species of the genus *Bartonella*

	1	2	3	4	5
1. <i>Bartonella henselae</i>	–	86.8	76.48	76.61	76.98
2. <i>Bartonella quintana</i>	86.81	–	76.63	76.63	77.13
3. ‘ <i>Ca. Liberibacter solanacearum</i> ’	76.45	76.61	–	81.33	78.23
4. ‘ <i>Ca. Liberibacter asiaticus</i> ’	76.54	76.61	81.32	–	77.35
5. <i>Liberibacter crescens</i> sp. nov. BT-1 <sup>T</sup>	77.01	77.12	78.19	77.37	–

**Table 2.** Distinguishing features of strain BT-1<sup>T</sup> compared with close relatives

Taxa: 1, strain BT-1<sup>T</sup>; 2, '*Ca. Liberibacter asiaticus*' (data from Shokrollah *et al.*, 2010; Zhang *et al.*, 2011); 3, '*Ca. Liberibacter solanacearum*' (Liefting *et al.*, 2009); 4, *Bartonella henselae* (Chenoweth *et al.*, 2004; Welch *et al.*, 2002); 5, *Bartonella quintana* (Alsmark *et al.*, 2004); 6, *Sinorhizobium meliloti* (Galibert *et al.*, 2001); 7, *Rhizobium etli* (Jarvis & Tighe, 1994; Segovia *et al.*, 1993); 8, *Wolbachia pipientis* (Hertig, 1936; Werren, 1997). NA, data not available.

Characteristic	1	2	3	4	5	6	7	8
Family	<i>Rhizobiaceae</i>	<i>Rhizobiaceae</i>	<i>Rhizobiaceae</i>	<i>Bartonellaceae</i>	<i>Bartonellaceae</i>	<i>Rhizobiaceae</i>	<i>Rhizobiaceae</i>	<i>Anaplasmataceae</i>
Lifestyle	Papaya phloem, commensal	Insect vectored, citrus pathogen	Insect vectored, potato/tomato pathogen	Insect vectored, human intracellular pathogen	Insect vectored, feline intracellular pathogen	Legume symbiont		Insect endosymbiont
Cell Shape	Rod	Elongated rod	Elongated rod	Bacilli	Rod	Rod	Rod	Cocci
Flagellum	Not observed	Not observed	Not observed	Not observed	Not observed		Yes	
Primary fatty acids	C <sub>18:1</sub> ω7c, C <sub>16:0</sub> OH, C <sub>18:0</sub>	NA	NA	C <sub>18:1</sub> ω7, C <sub>18:0</sub> , C <sub>16:0</sub>	C <sub>18:1</sub> ω7, C <sub>18:0</sub> , C <sub>16:0</sub>	Summed feature 1*, C <sub>19:0</sub> cyclo, summed feature 2†	C <sub>19:0</sub> cyclo ω8c, C <sub>18:0</sub> , C <sub>18:1</sub> ω7c/ω9t/ω12t, C <sub>18:1</sub> ω7c/ω9c/ω12t, C <sub>16:0</sub>	NA
Genome size	1.5 Mb	1.23 Mb	1.26 Mb	1.93 Mb	1.58 Mb	6.7 Mb	6.53 Mb	1.27 Mb
DNA G+C content	35.3 mol%	36.5 mol%	35.2 mol%	38.2 mol%	39–40.0 mol%	62.0 mol%	60.5 mol%	34.0 mol%
rRNA operons	3	3	3	2	2	3	3	3

\*Summed feature 1 is composed of C<sub>18:1</sub>ω7, C<sub>18:1</sub>ω9 and C<sub>18:1</sub>t fatty acids.

†Summed feature 2 is composed of C<sub>12:0</sub> aldehyde(?), iso-C<sub>16:1</sub>, C<sub>14:0</sub> 3-OH fatty acids and an unknown compound with an equivalent chain length (ECL) value of 10.928.

## Description of *Liberibacter* gen. nov.

*Liberibacter* (Li.be.ri.bac'ter. L. adj. *liber* free; N.L. n. *bacter* rod; N.L. masc. n. *Liberibacter* free rod).

Cells are Gram-stain-negative aerobic rods. Non-motile and restricted to the phloem of the host plant. Cells exhibit a reduced genome of approximately 1.5 Mb or less with a DNA G+C content of 31–37%, similar to many insect symbionts. The type species is *Liberibacter crescens*.

## Description of *Liberibacter crescens* sp. nov.

*Liberibacter crescens* (cres'cens. L. part. adj. *crescens* growing, thriving).

Aerobic, Gram-stain-negative rods approximately 1.75 µm × 0.5 µm. Cream-coloured colonies with entire margins are visible 8–10 days after inoculation on solid medium. Negative for the presence of catalase, oxidase and urease, as well as for the production of indole from tryptophan.

The type strain is BT-1<sup>T</sup> (=ATCC BAA-2481<sup>T</sup>=DSM 26877<sup>T</sup>), isolated from expressed sap of a Babaco papaya.

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