Curtobacterium uspiensis sp. nov., an endophytic bacterium isolated from Citrus sinensis (sweet orange) in Brazil

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Abstract

Endophytic bacteria were isolated from Citrus plants and based on the similarity of 16S rRNA gene sequence, 20 isolates were included in the genus *Curtobacterium* in the family *Microbacteriaceae*, class Actinobacteria. Amplified Fragment Length Polymorphism (AFLP) analysis indicated that these strains formed four clusters with low variability that were separated from *Curtobacterium flaccumfaciens*. The isolates were Gram-positive, non-motile, non-spore forming, pale-yellow to orange-pink colonies. Analysis of eleven isolates showed that the major fatty acids of these strains were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$ and MK-9 and MK-8 as the major isoprenoid quinone, supporting the affiliation into *Curtobacterium* genus. The similarity of 16S rRNA gene between these isolates ranged from 99.9 to 100%, suggesting that this endophytic population present a low variability and similarity to species with validity published names within this genus, forming a distinct group in the phylogenetic tree. The DNA–DNA relatedness values to closest species were less than 48% for ER1/6^T, suggesting that this strain does not belong to already described species. Analysis of the ER1/6^T genome detected genes predicted to be involved in secondary metabolites synthesis, such as siderophore Desferrioxamine-like, bacteriocin Lactococcin 972-like, and terpene C50 carotenoid-like. Based on genome sequencing, the G + C content of the strain ER1/6^T was 72.2%. Therefore, the polyphasic taxonomic characterization demonstrated that the strains ER1/6^T, ER1.4/2, SR4/1 and SR4/8 dominant in the citrus tissues, represent a new species of the genus *Curtobacterium*, for which the name *Curtobacterium uspiensis* sp. nov. is proposed, with strain ER1/6^T = CBMAI 2131 as the type strain.

Introduction

The endophytic environment is an important habitat for the *Curtobacterium* species, which may interact with host plants and their indigenous microbiomes. This genus was first described by Yamada and Komagata (1972) and includes eight recognized species. *Curtobacterium* spp. have been isolated from many environments and host plants, including *Curtobacterium citreum* from rice plants (Yamada and Komagata 1972; Komagata and Iizuka 1964), *Curtobacterium herbarum* from litter layer (Behrendt et al. 2002), *Curtobacterium luteum* and *Curtobacterium albidum* from Chinese rice paddies (Yamada and Komagata 1972; Komagata and Iizuka 1964), *Curtobacterium pusillum* from oil brines (Yamada and Komagata 1972; Iizuka et al. 1965), *Curtobacterium ammoniigenes* from plants inhabiting acidic swamps (Aizawa et al. 2007), and *Curtobacterium ginsengisoli* from soil of ginseng crop (Kim et al. 2008). A phytopathogenic *Curtobacterium flaccumfaciens* species has also been identified (Collins and Jones 1983), which present six subspecies: *C. f.* subsp. *flaccumfaciens, C. f.* subsp. *betae, C. f.* subsp. *poinsettiae, C. f.* subsp. *oortii, C. f.* subsp. *beticola, and C. f.* subsp. *basellae.* Because it is difficult to discriminate between *Curtobacterium* species and *C. f.* subspecies using phenotypic features, most of the strains have been identified as *Curtobacterium* sp. or affiliated with *C. flaccumfaciens* (Behrendt et al. 2002). Unfortunately, 16S rRNA sequence analysis is also not appropriate for identifying endophytic strains at the species or subspecies level.

Endophytic *Curtobacterium* strains isolated from *Citrus sinensis* (sweet Orange) and *C. reticulata* (ponkan tangerine) were identified by Fatty Acid Methyl Ester (FAME – MIDI) as *C. flaccumfaciens* (Araújo et al. 2002). Interaction between these endophytic bacteria and the causal agent of CVC, *Xylella fastidiosa*, has also been proposed (Araújo et al. 2002; Lacava et al. 2007) since *Curtobacterium* is generally isolated from CVC resistant plants (Araújo et al. 2002). In another study, Lacava et al. (2007) showed that this endophytic bacterium can reduce the CVC symptoms in *Catharanthus roseus*, a CVC model plant, thereby reinforcing the belief that this bacterium could interacts with *X. fastidiosa.*

Endophytic *Curtobacterium* species have been isolated from many host plants including citrus (Araújo et al. 2002; Araújo et al. 2001), red clover (Sturz and Christie 1998), rice (Elbeltagy et al. 2000), potato (Sturz and Matheson 1996), yam (Tor et al. 1992), and prairie plants (Zinniel et al. 2002). They have also been associated with the control of diseases in tobacco and cucumber (Raupach and Kloepper 2000) and potato plants (Sturz and Matheson 1996), which suggests that the diversity and/or plasticity of this genus is high, allowing it to colonize different host plants. The identification of these endophytic strains is currently based on 16S rRNA gene sequencing, even though this method is unable to characterize the different species in this genus. The whole genome of strain ER1/6^T was previously sequenced and annotation data was deposited at DDBJ/EMBL/GenBank under the accession number MJAK00000000 (17). This sequence was also used to annotate genes related to secondary metabolite biosynthesis. Therefore, in the present work, we completed the polyphasic taxonomic analysis of the endophytic strains ER1.4/2, ER1/6^T, SR4/1 and SR4/8 isolated from *Citrus sinensis* stems and we are formally proposing the new species *Curtobacterium uspiensis*, with strain ER1/6^T as the type strain.

Materials And Methods

Bacterial strains and growth conditions

Strains used in the present study (Table 1) were stored at -80^{o}C and grown on Tryptic Soy (TS - Merck) agar plates or *Corynebacterium* - agar (10 g Casein peptone, tryptic digested; 10 g Yeast extract; 5 g glucose; 5 g NaCl in 1000 mL of water) at 28^{o}C under aerobic conditions and checked for purity.

Table 1
Curtobacterium species, hosts, and origin of used strains.

Strains	Species	Plant interaction	Host	Country	References
AR 1/2, AR 4/15, AR 1.4/7, AF2/7, EF 1/6, AR 1.4/11, AR 1.5/3, AR 4/6, ER 1.4/2, ER 1.4/8, ER 1/11, ER 1/5, ER 1/6, ER 1/7, ER 1/9, ER 4/15, SR 1/6, SR 1/8, SR 2/1, SR 3/13, SR 3/8, SR 4/1, SR 4/8, AF 2/5, SF 2/17, SR 1.3/6, SR 1.4/9, SR 3/19, SR 4/7	*Curtobacterium* sp.	Endophyte	*Citrus sinensis*	Brazil	(8)
PR 1.1/8, PR 1/10, PR 1/11, PR 1/8, PR 2/2, PR 2/3, PR 3/2, PR 4/3, PR 1.4/8, PR1.4/1	*Curtobacterium* sp.	Endophyte	*C. reticulata*	Brazil	(8)
IPO 237, IPO 238	*C. flaccumfaciens*	Phytopathogen	*Phaseolus vulgaris*	Netherlands	ND*
1290, 1336, 1337	*C. f.* pv. *flaccumfaciens*	Phytopathogen	*P. vulgaris*	Brazil	ND
Feij-2500, Feij-2502, Feij-2634, Feij-2910, Feij-2912	*C. f.* pv. *flaccumfaciens*	Phytopathogen	*P. vulgaris*	Brazil	(40)
315	*C. f.* pv. *betae*	Phytopathogen	*Beta vulgaris*	Brazil	ND
SG041	*Curtobacterium* sp.	Endophyte	*Bouteloua curtipendula*	USA	(15)
20035C, 2A-Lt, 2344	*C. f.* pv. *flaccumfaciens*	Phytopathogen	*Glycine max*	USA	(15)
CB101	*C. f.* pv. *betae*	Phytopathogen	*B. vulgaris*	USA	(15)
CO101	*C. f.* pv. *oortii*	Phytopathogen	*Spathodea* sp.	USA	(15)
CA 1, CA 2, CA 3, CA 5	*C. flaccumfaciens*	Endophyte	*Trifolium* sp.	Canada	(11)
CA 4	*C. luteum*	Endophyte	*Trifolium* sp.	Canada	(11)

***ND:** not determined

Cell morphology

Cell morphology was determined by light microscopy using a Nikon microscope at 1000x magnification, with 3-day-old cells at 28°C on TS-agar and *Corynebacterium*-agar medium. Gram staining was performed using crystal violet–iodine staining method.

Diversity of endophytic Curtobacterium *by AFLP technique*

Genomic DNA was extracted from bacteria with the bead beater strategy (Araújo et al. 2002), and the DNA concentration and quality were estimated using agarose gels. All strains of endophytic *Curtobacterium* were submitted to AFLP analysis. To test the reproducibility of the AFLP analysis, the genomic DNA was extracted twice, and two independent analyses were carried out. The PCR reactions were performed in a GeneAmp 9700 thermocycler (Applied Biosystems), and the *Eco*RI and *Mse*I adaptors and primers were synthesized by Invitrogen (Brazil).

DNA digestion was performed for 2 h at 37°C. Each reaction was carried out in 1X reaction buffer (50 mM Tris acetate, pH 7.5; 50 mM Mg/acetate; 250 mM K/acetate), 5 U of each *Eco*RI and *Mse*I enzymes (Invitrogen, Brazil), and 200 ng DNA. Enzyme denaturation was performed at 70°C for 10 min, and the adapters for *Eco*RI and *Mse*I were linked to the fragments using the T4 DNA ligase at 23°C for 3 h. After ligation, DNA fragments were amplified using three primer sets (*Eco*RI + A / *Mse*I + C, *Eco*RI + C / *Mse*I + G, and *Eco*RI + A / *Mse*I + T). Amplification was performed with 26 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min.

The amplification products were mixed with an equal volume (8 µl) of formamide dye (98% formamide, 10 mM EDTA, pH 8.0, with bromophenol blue and xylene cyanol as tracking dyes), heat-inactivated at 95°C for 5 min, chilled on ice, and further detected by electrophoresis on a 6% polyacrylamide gel in 1x TBE buffer with a sensor using 80 W per gel at a maximum of 50°C. Finally, the gel was fixed on glass and silver stained (Creste et al. 2001).

AFLP experiments were repeated at least once and DNA fingerprints from each isolate were first converted to either 0 (absence) or 1 (presence) directly from the stained gel. A dendrogram was constructed based on the Jaccard coefficient (S_j) using band positions (for each pattern, S_j divides the number of corresponding bands by the total number of bands in both patterns) and UPGMA (Unweighted Pair Group Method with Averages) cluster analysis. Consensus tree was obtained using Winboot software with the bootstrap replicate number test at 1,000.

Phylogenetic analysis

The 16S rRNA gene (27–1401, according to *E. coli* position) of citrus endophytic strains representing the four AFLP groups was amplified using primers P027F and R1387 (Lane et al. 1985) and sequenced using internal primers. For this, the PCR products generated by primers P027 and R1387 were purified using the PCR purification kit PowerCleanTM DNA Clean-Up Kit (MoBio Laboratories, USA). DNA fragments were sequenced with internal primers in an automated sequencer. For identification, the obtained sequences were subjected to similarity analysis using $Blast_n$ analysis at GenBank (http://www.ncbi.nlm.nih.gov). In addition, the phylogenetic relationship with closely related species was determined by using MEGA version 4.0 (Tamura et al. 2007). Evolutionary distances were computed as described previously (Jukes and Cantor 1969) and the phylogenetic trees were constructed using neighbor-joining method (Kluge and Farris 1969). The reliability of the tree topologies was evaluated by bootstrap analysis with 1000 replicates (Felsenstein 1985).

Further, a phylogenetic tree based on 16S *rRNA* gene sequences, available in RDP query and NCBI database, was generated, having shown the relationship between *Curtobacterium uspiensis* sp. nov. ER1/6^T and *Curtobacterium* species using Kimura model. For this, *Leifsonia xyli, Leifsonia soli, Cryobacterium psychrophilum, and Clavibacter michiganensis* were used as outgroup.

DNA-DNA hybridization

DNA-DNA hybridization (DDH) was performed at the Laboratorium voor Microbiologie – Universiteit Gent (UGent) under supervision of Dr. Ilse Cleenwerck (BCCM/LMG). DNA-DNA similarity was examined between ER1/6^T, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (LMG 3645^T), *Curtobacterium citreum* (LMG8786^T), *Curtobacterium luteum* (LMG8787^T), *Curtobacterium pusilum* (LMG 8788^T), and *Curtobacterium herbarum* (LMG19917^T). For this, DNA was extracted according to modification of the procedure of Gevers et al, (2001) and hybridizations were performed in the presence of 50% formamide at 52^{o}C according to a modification (Goris et al. 1998; Cleenwerck 2002) of the method previously described (Ezaki et al. 1989). Reciprocal reactions (A x B and B x A) were performed and the variation was within the limits of the experiment.

Chemotaxonomic analysis

The whole fatty acid methyl ester (FAME) was determined by gas chromatography (Agilent 6850 unit) using an automatic injector and a Flame Ionization Detector (FID) detector (Agilent model 7683A automatic sampler). For this, fatty acid methyl esters were obtained from 40 mg cells scraped from Petri dishes by saponification, methylation, and extraction (Miller et al. 1982; Kuykendall et al. 1988). The output data were organized into a chromatogram and the identification report was prepared using the Microbial Identification System software (Sherlock TSBA40 library; MIDI Inc., Newark, DE, USA). The similarity of 0.70 with hits in the database was used to classify strains at species level.

Analyses of respiratory quinones/polar lipids were carried out by the identification service, DSMZ, Braunschweig, Germany (https://www.dsmz.de), as described on the website. Briefly, respiratory quinones were extracted from 100 mg of frozen dried cells using the two stage method of methanol:hexane (Tindall 1990a; Tindall 1990b), followed by phase separation into hexane thin layer chromatography on silica gel, using hexane:tert-butyl methyl ether (9:1 v/v) as solvent. UV absorbing bands corresponding to the different quinone classes (menaquinones, ubiquinones, etc.) were removed from the plate and further analyzed on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel, 2 mm x 125 mm, 3 µm, RP18) using methanol:heptane 9:1 (v/v) as the eluent and detected at 269 nm.

Phenotypic characterizations

The tests of sole carbon source utilization by *Curtobacterium uspiensis* sp. nov. ER1/6 were performed using the Biolog GEN III. For this, the cells were prepared according to BIOLOG (Hayward, CA, USA) instructions that include the preparation of a standardized liquid suspension (*GN/GP inoculating Fluid*) based on turbidity (95% T) and inoculation of one GEN III MicroPlate. After incubation (at 28^{o}C for 48 h), the MicroPlates were read on an OmniLog System BIOLOG plate reader.

Further biochemical characterization of the strains ER1.4/2, ER1/6^T, SR4/1 and SR4/8 was carried out using API ZYM strips (API System, Bio Merieux) according to the manufacturer's instructions. Briefly, the cells of a pure culture were suspended in sterile water and the concentration was adjusted to McFarland barium sulfate turbidity standard six. The cell suspension was inoculated into the test kits and incubated at 37^{o}C for 4 h, and reagents were added according to manufacturer's instructions. The enzymatic activity was recorded as positive if a score > 1 was obtained after comparing the color intensity of the reaction tubes with the manufacturer's color chart.

Genome analysis and secondary metabolic gene clusters

The first version of a whole-genome shotgun of the citrus endophytic *Curtobacterium* strain ER1/6 was deposited at DDBJ/EMBL/GenBank under the accession number MJAK00000000 (Garrido et al. 2016) and was used in the present study. Genes clusters potentially associated with the synthesis of secondary metabolites were identified based on similarity searches against the non-redundant protein database at GenBank by BLASTp or by antiSMASH 2.0 (Blin et al. 2013), and manually annotated using Geneious software (https://www.geneious.com). The metabolic pathways were constructed using BioCyc Database Collection (Caspi et al. 2016).

Results And Discussion

Morphological characterization

Citrus endophytic strains were cultured on TSB and *Corynebacterium*-agar at 30^{o}C, and yielded pale-yellow to orange-pink, circular colonies, while phytopathogenic strains presented yellow-ivory colonies. The cells of all evaluated strains were Gram-positive and rod shaped.

Diversity of endophytic Curtobacterium *by AFLP technique*

AFLP analysis was used as the main genome fingerprinting method to assess the overall genotypic similarity of the strains. The reproducibility of the results was tested at least once and less than 1.5% of the bands presented variation and were discarded from the present analysis. The analysis of endophytic *Curtobacterium* strains, *C. luteum* and subspecies of *C. flaccumfaciens* resulted in 147 bands for the three primer combinations, with 132 polymorphic bands (89.8%) which were used to determine genetic distances. The result of the comparative analysis of the AFLP profile of the citrus endophytic *Curtobacterium* strains, endophytic strains from other host plants, *C. luteum*, and *C. flaccumfaciens* reveled, at 50% similarity, that the evaluated isolates grouped into six clusters (Fig. 1). The phytopathogenic *C. flaccumfaciens* isolates clustered in a divergent group that shared only 36% of the bands with the other five clusters. The citrus endophytic *Curtobacterium* grouped into four clusters, which were separate from all other strains with similarity in < 29% of the bands.

In addition, the AFLP analysis, which included the *C. flaccumfaciens* pathovars, showed that the citrus endophytic strains grouped into different clusters and shared less than 35% similarity with these *C. flacumfaciens* pathovars, suggesting that these strains belong to another genotype. Previous studies using *Acinetobacter* species determined that a 50% similarity level was the threshold for the delineation of species by AFLP analysis (Nemec et al. 2001; Nemec et al. 2009). This AFLP analysis highlights the distinctness of the citrus strains from the other endophytic strains, *C. luteum*, and *C. flaccumfaciens.* This citrus population present 4 lineages (clusters) with high similarity in each cluster, suggesting that divergence is occurring in this bacterium and could be related to environmental selection. Representative citrus endophytic strains from the four clusters were further evaluated by 16S rRNA gene sequencing.

Phylogenetic analyses

The phylogenetic tree showed that all endophytic isolates belonged to the genus *Curtobacterium* within the family *Microbacteriaceae*. A high degree of 16S rRNA gene sequence similarity (≥ 98.3%) between all *Curtobacterium* species was observed with the 1030 bp sequence evaluated. The similarity between citrus endophytic strains ranged from 99.9 to 100%. The strains ER1/6^{T}, ER1.4/2, SR4/1 and SR4/8 presented 100% similarity in this 16S rRNA gene sequence. The similarity of these endophytic strains and *C. flaccumfaciens* pathovars was ≥ 99.9% (Fig. S1). The same similarity level (≥ 99.9%) was observed between citrus endophytic *Curtobacterium* strains, and although the similarity between the evaluated species was extremely high, the 16S rRNA gene sequence analysis, with type strains of valid species for this *Curtobacterium* genus, grouped the endophytic strains in a different cluster (Fig. 2). This analysis showed that these endophytic strains, belonged to the lineage containing members of the genus *Curtobacterium*, as evidenced by the high (97%) bootstrap value.

The similarity between citrus endophytic strains and *C. f.* pathovars was ≤ 99.4%, while the similarity between *C. f.* pathovars and *C. luteum* was ≥ 99.9%; between *C. f.* pathovars and *C. albidum, C. ammoniigenes, C. citreum, C. herbarum*, and *C. pusillum* was ≥ 99.6%; and the similarity between *C. f.* pathovars and *C. ginsengisoli* was 98.6%. However, the similarity between citrus endophytic strains and the type strains was ≤ 99.7% for *C. albidum, C. citreum*, and *C. pusillum*, ≤ 99.3% for *C. luteum*, ≤ 99.1% for *C. herbarum* and *C. ammoniigenes*, and ≤ 98.4% for *C. ginsengisoli.* Although these citrus endophytic strains of *Curtobacterium* have high degrees of 16S rRNA gene sequence similarity to the established species of the genus, they clustered in a separate group (Fig. S1), indicating that they may be a divergent genotype in this genus.

In the phylogenetic tree based on 16S rRNA gene sequencing, strains ER1/6^{T}, ER1.4/2, SR4/1 and SR4/8, which present 100% similarity in 16S rRNA sequence, grouped to the lineage containing members of the genus *Curtobacterium* as evidenced by the high bootstrap value, but the proximity to *C. herbarum* was not clear due to the low bootstrap value (Fig. 2). The DNA-DNA relatedness between *Curtobacterium uspiensis* sp. nov. ER1/6^{T} and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (LMG 3645^{T}), *Curtobacterium citreum* (LMG8786^{T}), *Curtobacterium luteum* (LMG8787^{T}), *Curtobacterium pusilum* (LMG 8788^{T}), and *Curtobacterium herbarum* (LMG19917^{T}) was 35%, 48%, 42%, 37%, and 35%, respectively. The result for DNA-DNA hybridization between *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (LMG 3645^{T}) and *Curtobacterium herbarum* (LMG19917^{T}) was 33%, having been similar to results previously reported (Behrendt et al. 2002). This result support the proposal of a novel species within the genus *Curtobacterium*, for which the name *Curtobacterium uspiensis* sp. nov. is proposed with ER1/6^{T} as the type strain.

Chemotaxonomic analysis

Major fatty acids of 11 citrus endophytic *Curtobacterium* strains belonging to different AFLP clusters are described in Table 2. The fatty acid profile of citrus endophytic isolates had similarities with *C.f.* pv. *flaccumfaciens, C. f.* pv. *Poinsettiae*, and *C. f.* pv. *betae/oortii*. The major cellular fatty acids of the strain ER1/6^{T} were anteiso-$C_{15:0}$ (48.01%), anteiso-$C_{17:0}$ (32.68%), and iso-$C_{16:0}$ (8.68%) as previously reported for members of the genera *Curtobacterium* and *Rathayibacter* (Kim et al. 2008). The strain ER1/6^{T} contained menaquinones MK-9 as the predominant (85%) isoprenoid quinone with a smaller amount (14%) of MK-8, supporting the affiliation of these 11 strains, including ER1/6^{T}, ER1.4/2, SR4/1 and SR4/8 to *Curtobacterium*genus.

Table 2
Fatty acid methyl ester analysis. Most similar *Curtobacterium* genotypes with citrus endophytic strains based on FAME-MIDI analysis, and the percentage of major fatty acids present in these strains.

Strains	Identity	Similarity	Fatty acids		
			$C_{15:0}$ antiso	$C_{17:0}$ antiso	$C_{16:0}$ iso
AF2/5	*C. f.* pv. poinsettiae	0.763	42.96	33.81	8.89
PR2/2	*C. f.* pv. poinsettiae	0.834	45.05	30.97	10.70
AR1/2	*C. f.* pv. betae/ortii	0.813	45.54	29.25	10.45
SR1/6	*C. f.* pv. poinsettiae	0.776	46.41	29.19	9.74
SR4/1	*C. f.* pv. poinsettiae	0.891	47.92	30.48	7.33
SR4/8	*C. f.* pv. poinsettiae	0.887	48.03	30.54	7.32
ER1/5	*C. f.* pv. poinsettiae	0.871	45.18	32.68	8.68
ER1/6	*C. f.* pv. poinsettiae	0.898	48.01	30.64	7.48
ER1.4/2	*C. f.* pv. poinsettiae	0.897	48.02	30.62	7.45
EF1/6	*C. f.* pv. poinsettiae	0.764	50.88	17.69	12.12
SF2/17	*C. f.* pv. flaccumfaciens	0.750	45.86	33.53	9.39

Phenotypic characterizations

The differences in phenotypic characteristics between the proposed *C. uspiensis* and closely related type strains of *Curtobacterium ammoniigenes* B55^T (Aizawa et al. 2007), C. *citreum* (Behrendt et al. 2002; Aizawa et al. 2007), C. *pusillum* (Behrendt et al. 2002; Aizawa et al. 2007), C. *luteum* (Behrendt et al. 2002; Aizawa et al. 2007), C. *albidum* (Behrendt et al. 2002; Aizawa et al. 2007), C. *flaccumfaciens pv. flaccumfaciens* (Behrendt et al. 2002; Aizawa et al. 2007), C. *ginsengisoli* (Kim et al. 2008), C. *herbarum* LMG 19917^T (Behrendt et al. 2002)d *plantarum* (Dunleavy 1989) are listed in Table 3. Based on the full characterization of eleven *C. uspiensis* strains, this species characterized by gram-positive, rod-shaped cells that form orange/pink-colored colonies that grow optimally at 28^{o}C. The enzymatic profiles of the endophytic strains were determined using a commercial system, API ZYM (API System, Bio Merieux) and analysis showed that these 11 endophytic strains are able to produce leucine arylamidase, α–galactosidase, β-galactosidase, α–glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, and α-manosidase. The endophytic strains produced variable results for esterase lipase (C8), cystine arylamidase, valine arylamidase, esterase (C4), and acid phosphatase, and they were unable to produce alkaline phosphatase, lipase (C14), trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, and α-fucosidase (Table 3).

Table 3
Physiological characteristics of *C. uspiensis* sp. nov. and closely related type strains of *Curtobacterium* species.

Characteristics [a]	Cam[b]	Cit	Cpu	Clu	Cal	Cff	Cgi	Che	Cpl	ER1/6^T	ER1.4/2	SR4/1	SR4/8
DNA G + C content (mol%)	68.8	72.1[c]	70.37 [c]	71.3[c]	ND	70.85[c]	65.8	71	76	72.2	ND	ND	ND
Colony color	Yellow	Yellow	Yellow	Yellow	Ivory	Yellow	Yellow	Orange	Yellow	Orange	Orange	Orange	Orange
Growth at 37°C	ND	W	W	W	W	W	ND	ND	W	W	W	W	W
Growth at 4°C	-	W	-	W	W	W	W	W	W	-	-	-	-
Motility	-	+	+	+	-	-	-	+	+	-	-	-	-
Hydrolysis of:													
Gelatin	-	-	+	-	+	+	ND	ND	+	-	-	-	-
L-Rhamnose	+	-	+	+	+	+	ND	ND	+	-	ND	ND	ND
N-Acetyl-D-glucosamine	-	-	+	+	-	W	-	ND	ND	+	ND	ND	ND
D-Trehalose	-	+	+	+	W	+	ND	ND	-	+	+	+	+
Assimilation of:													
Tween 40	-	W	-	W	+	W	ND	ND	ND	+	+	+	+
N-Acetyl-D-galactosamine	-	-	-	-	-	+	ND	ND	ND	-	ND	ND	ND
N-Acetyl-D-glucosamine	-	+	-	+	W	+	ND	ND	ND	+	ND	ND	ND
D-Arabitol	-	-	W	-	-	+	ND	ND	ND	-	ND	ND	ND
D-Cellobiose	-	+	-	+	+	+	ND	ND	-	+	+	+	+
L-Fucose	-	+	-	+	-	W	-	ND	ND	-	-	-	-
Gentiobiose	-	-	+	-	+	+	ND	ND	ND	+	ND	ND	ND
myo-Inositol	-	-	-	-	-	+	ND	ND	+	+	+	+	+
a-D-Lactose	+	+	-	+	+	+	ND	ND	-	+	-	+	-
D-Mannitol	+	-	+	-	W	+	-	+	d	+	+	+	+
D-Melibiose	+	+	-	+	W	+	-	+	ND	+	ND	ND	ND
Methyl b-D-glucoside	-	-	+	-	W	+	ND	ND	ND	+	ND	ND	ND
D-Raffinose	-	-	-	-	+	+	ND	ND	-	-	ND	ND	ND
D-Sorbitol	+	-	+	-	+	+	-	+	ND	+	+	+	+
Turanose	+	-	+	-	+	+	ND	ND	ND	+	ND	ND	ND
D-Gluconic acid	+	-	+	-	+	+	ND	ND	+	+	ND	ND	ND
D-Glucuronic acid	-	-	-	-	-	+	+	+	ND	-	ND	ND	ND
a-Ketobutyric acid	-	W	-	+	-	-	-	+	ND	-	ND	ND	ND

[a]Cam: *Curtobacterium ammoniigenes* B55^T (5); Cit: *C. citreum* (3, 5); Cpu: *C. pusillum* (3, 5); Clu: *C. luteum* (3, 5); Cal: *C. albidum* (3, 5); Cff: *C. flaccumfaciens pv. flaccumfaciens* (3, 5); Cgi: *C. ginsengisoli* (6); Che: *C. herbarum* LMG 19917^T (3); Cpl: *C. plantarum* (41).

[b]ND: not determined; - : no growth; +: strong growth observed; W: weak growth, d: 11–89% of the strains were positive

[c]Sequenced genomes deposited at GenBank (NCBI).

Characteristics [a]	Cam[b]	Cit	Cpu	Clu	Cal	Cff	Cgi	Che	Cpl	ER1/6^T	ER1.4/2	SR4/1	SR4/8
DL-Lactic acid	+	W	-	W	-	+	ND	ND	+	-	-	-	-
Bromosuccinic acid	-	W	-	-	-	+	ND	ND	ND	-	ND	ND	ND
L-Alanine	-	-	-	-	W	+	-	ND	ND	-	-	-	-
L-Glutamic acid	-	+	-	-	-	+	ND	ND	ND	+	+	+	+
Lactate	+	W	-	W	-	+	-	ND		+	+	+	+
Acetic acid	ND	+	+	+	+	+	ND	ND	+	+	+	+	+
Malic acid	ND	+	-	+	-	+	ND	ND	d	-	-	-	-
a-Ketoglutaric acid	ND	+	-	-	-	D	ND	ND	+	-	ND	ND	ND
Citric acid	ND	+	-	-	+	+	ND	ND	d	-	-	-	-
Propionic acid	ND	-	+	-	-	-	ND	ND	+	-	-	-	-
Arginine	ND	-	-	-	-	-	ND	ND	-	-	-	-	-
Glucose	ND	W	W	+	W	W	ND	ND	+	+	+	+	+
Fructose	ND	W	W	+	W	W	ND	ND	+	+	+	+	+
Mannose	ND	W	+	+	-	W	ND	ND	+	+	+	+	+
Galactose	ND	W	+	-	-	-	ND	ND	+	+	+	+	+
Sucrose	ND	-	-	-	-	-	ND	ND	-	+	+	+	+
Maltose	ND	W	W	-	W	W	ND	ND	+	+	+	+	+
Glycerol	ND	-	-	-	-	-	ND	ND	-	+	+	+	+

[a]Cam: *Curtobacterium ammoniigenes* B55^T (5); Cit: *C. citreum* (3, 5); Cpu: *C. pusillum* (3, 5); Clu: *C. luteum* (3, 5); Cal: *C. albidum* (3, 5); Cff: *C. flaccumfaciens pv. flaccumfaciens* (3, 5); Cgi: *C. ginsengisoli* (6); Che: *C. herbarum* LMG 19917^T (3); Cpl: *C. plantarum* (41).

[b]ND: not determined; - : no growth; +: strong growth observed; W: weak growth, d: 11–89% of the strains were positive

[c]Sequenced genomes deposited at GenBank (NCBI).

Genome analysis and secondary metabolic gene clusters

Based on genome sequencing, the DNA G + C content of the type strain is 72.2%. The endophytic bacterium *C. uspiensis* ER1/6^T reduces symptoms caused by *Xylella fastidiosa* in *Catharanthus roseus* (Lacava et al. 2007) and *in vitro* (Lacava et al. 2004), and produces, based on ESI-MS/MS fragmentation profile, phospholipids including the classes of glycerophosphocholine, glycerophosphoglycerol, and glycerophosphoinositol as well as several fatty acids (Araújo et al. 2018). Therefore, we screened the genome sequence for the presence of genes encoding for the biosynthesis of phospholipids, antibiotics, and other metabolites contributing to plant disease suppression. We detected three clusters encoding secondary metabolites (Table 4), including a bacteriocin similar to cell wall-active bacteriocin lactococcin 972 like (Martínez et al. 2008), the putative pathway for the desferrioxamines (Fig. S2 and S3), a siderophore belonging to hydroxamate group (Ronan et al. 2018), and the rare C50 carotenoid decaprenoxanthin (Fig. S4 and S5), a pigment used for coloration of food, feed and beverages (Henke et al. 2017).

Table 4
Identified clusters of secondary metabolites in *Curtobacterium uspiensis* ER1/6^T genome (NZ_MJAK01000000.1).

Genbank acession	Location	Identified cluster of secondary metabolite
NZ_MJAK01000001.1	22,753 – 28,012 nt	siderophore Desferrioxamine-like
NZ_MJAK01000003.1	220,629 – 226,575 nt	terpene C50 carotenoid-like
NZ_MJAK01000008.1	69,090 – 69,623 nt	bacteriocin Lactococcin 972 like

Table 5. Description of *Curtobacterium uspiensis* sp. nov. according to Digital Protologue TA00526 assigned by the www.imedea.uib.es/dprotologue website.

Taxonumber	*TA00526*
Species name	*Curtobacterium uspiensis*
Genus name	*Curtobacterium*
Specific epithet	*uspiensis*
Species status	sp. nov.
Species etymology	us.pi.en'sis. N.L. n. *uspiensis* from USP, named in reference to USP – University of São Paulo - where this bacterium has been studied
Authors	Araújo WL, Belmonte UCF, Dourado MN, Garrido LM, Yara R, Azevedo, JL
Title	*Curtobacterium uspiensis* sp. nov., an endophytic bacterium isolated from *Citrus sinensis* (sweet orange) in Brazil
Corresponding author	Welington Luiz Araújo
E-mail of the corresponding author	wlaraujo@usp.br
Submitter	Welington Luiz Araújo
E-mail of the submitter	wlaraujo@usp.br
Designation of the type strain	ER1/6
Strain collection numbers	
16S rRNA gene accession number	
Genome accession number [RefSeq]	MJAK00000000
Genome status	Draft
Genome size	3,368.952 kbp
GC mol%	72.2
Country of origin	Brazil
Region of origin	São Paulo
Date of isolation	1997
Source of isolation	*Citrus sinensis* branch
Sampling date	1997
Geographic location	Novais City
Number of strains in study	20
Source of isolation of non-type strains	Citrus branches
Growth medium, incubation conditions [temperature, pH, and further information] used for standard cultivation	Tryptic Soy (TS) agar plates at 28^{o}C under aerobic conditions.
Alternative medium 1	Corynebacterium - agar (10 g Casein peptone, tryptic digest; 10 g Yeast extract; 5 g glucose; 5 g NaCl in 1000 mL of water) at 28oC under aerobic conditions
Conditions of preservation	stored at -80oC in glycerol 20% and lyophilized.
Gram stain	Positive
Cell shape	Rod
Motility	Non-motile
Colony morphology	pale yellow to orange-pink with irregular borders and smooth surface
Temperature optimum	25 to 30
pH optimum	6,8
pH category	Neutrophile
Salinity optimum	<1
Salinity category	halotolerant

Relationship to O_2	Aerobe
O_2 conditions for strain testing	Aerobiosis
Carbon source used [class of compounds]	Sugars, amino acids
Catalase	Positive
Positive tests with BIOLOG (BIOP)	Tween 40, N-acetyl-D-glucosamine, D-cellobiose, gentiobiose, D-lactose, D-mannitol, D-melibiose, methyl β-D-glucoside, D-sorbitol, turanose, D-gluconic acid, L-glutamic acid, acetic acid, lactate, D-fructose, D-glucose, D-galactose, D-mannose, maltose, sucrose, glycerol,
Negative tests with BIOLO (BION)	N-Acetyl-D-galactosamine, D-arabitol, L-fucose, myo-inositol, D-raffinose, D-glucuronic acid, α-ketobutyric acid, DL-lactic acid, bromosuccinic acid, L-alanine, malic acid, α-ketoglutaric acid, citric acid, propionic acid and arginine. The type strain was negative for gelatin hydrolysis
Positive tests with API (APIP)	leucine arylamidase α–galactosidase, β-galactosidase, α–glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-manosidase
Negative tests with API (APIN)	alkaline phosphatase, lipase (C14), trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, α-fucosidase
Variable tests with API (APIV)	esterase lipase (C8), cystine arylamidase, valine arylamidase, esterase (C4), acid phosphatase
Quinone type (QUIN)	MK-9 (85%), MK-8 (14%)
Major fatty acids (FAME)	anteiso-C15:0 (47.28%), anteiso-C17:0 (32.45%), iso-C16:0 (9.37%)
Biosafety level	1
Habitat	portion of plant tissues
Biotic relationship	Endophyte
Symbiosis with the host	Citrus plants
Known pathogenicity	None

Conclusion

Taxonomic analyses of citrus endophytic *Curtobacterium* strains revealed a phylogenetic high diverse assemblage, and based on polyphasic analysis, we suggest *Curtobacteriuim uspiensis* sp. nov. as a new species in *Curtobacterium* genus. In addition, we hypothesized that members of this species could be further evaluated for certain applications such as the biocontrol of plant diseases, including *Xylella fastidiosa* in citrus plants.

Description of Curtobacterium uspiensis *sp. nov.*

Curtobacterium uspiensis (us.pi.en'sis. N.L. n. *uspiensis* from USP, named in reference to USP – University of São Paulo - where this bacterium has been studied).

The morphological and physiological characteristics are summarized in Table 3. Cells are Gram-positive, non-motile rods, aerobic, non-spore-forming. Colonies grown on tryptic soy agar plates at 28-37^{o}C for 3 days are pale yellow to orange-pink with irregular borders and smooth surface, at pH 5.0, 6.0 and 7.0. Growth on culture medium with 1 and 4% NaCl, but not with 8% NaCl. Assimilation of Tween 40, N-acetyl-D-glucosamine, D-cellobiose, gentiobiose, D-mannitol, D-melibiose, methyl β-D-glucoside, D-sorbitol, turanose, D-gluconic acid, L-glutamic acid, acetic acid, lactate, D-fructose, D-glucose, D-galactose, D-mannose, maltose, sucrose, and glycerol, but not N-Acetyl-D-galactosamine, D-arabitol, L-fucose, *myo*-inositol, D-raffinose, D-glucuronic acid, α-ketobutyric acid, DL-lactic acid, bromosuccinic acid, L-alanine, malic acid, α-ketoglutaric acid, citric acid, propionic acid, and arginine, negative for gelatin hydrolysis an variable for D-lactose assimilation. The major fatty acids of the type strain are anteiso-$C_{15:0}$ (47.28%), anteiso-$C_{17:0}$ (32.45%), and iso-$C_{16:0}$ (9.37%). The predominant menaquinones are MK-9 (85%) and MK-8 (14%). Based on genome sequencing, the DNA G + C content of the type strain is 72.2%.

The formal proposal of the new species *Curtobacterium uspiensis* sp. nov. (Type strain ER1/6^{T} = CBMAI 2131^{T}), isolated from inner tissues of *Citrus sinensis* (sweet orange) in São Paulo State, Brazil, is given in Table 4 with the Taxonumber TA00526.

Declarations

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Authors' contributions

WLA and UCFB conceived and directed the studies. LMG participated in identification of secondary metabolites gene clusters and genome analysis. MND performed the BIOLOG analysis. The first draft of the manuscript was written by WLA, and discussed and corrected by UCFB, LMG, MND, RY and JLA. The funding for this work was provided by WLA and JLA. All authors read and approved the final manuscript.

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Figures

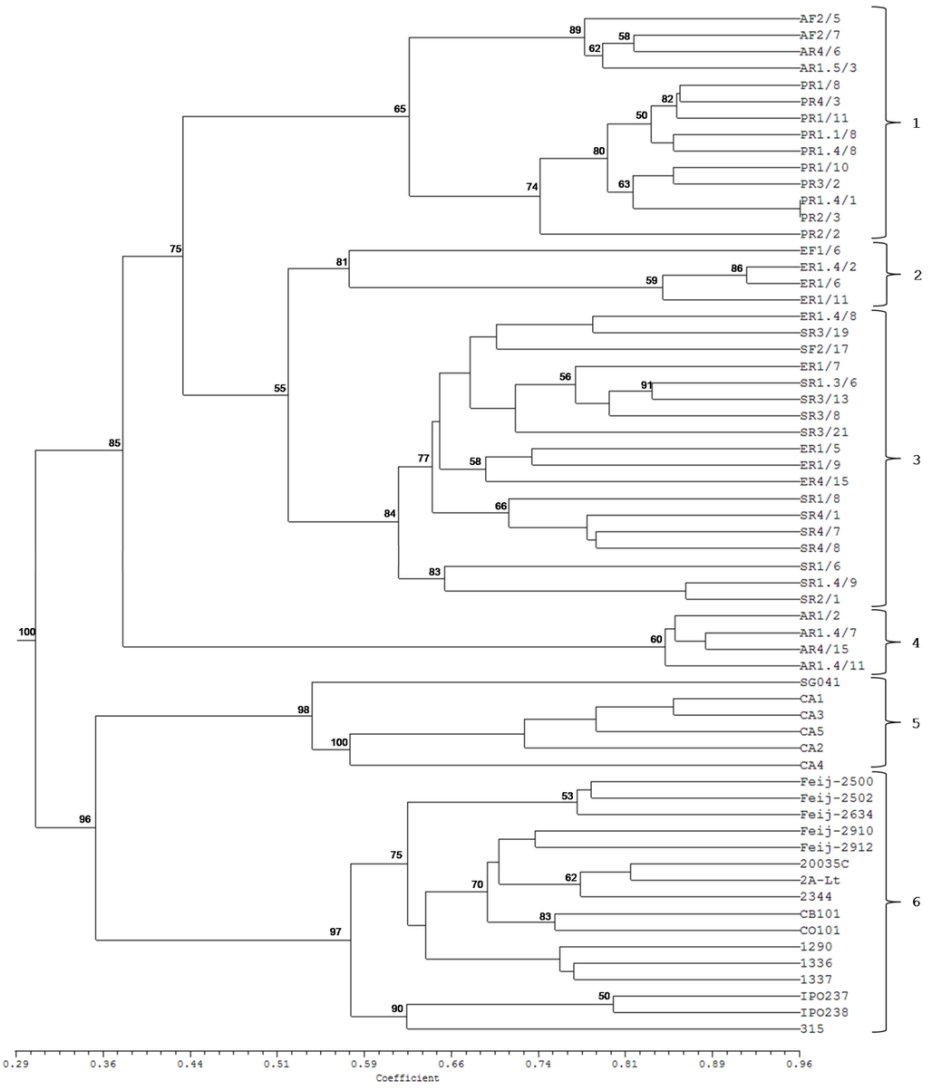

Figure 1

Dendrogram of cluster analysis of AFLP fingerprints of 40 citrus endophytic strains, 6 endophytic strains from other host plants, 1 *C. luteum* strain, and 21 strains of *C. flaccumfaciens* pathovars.

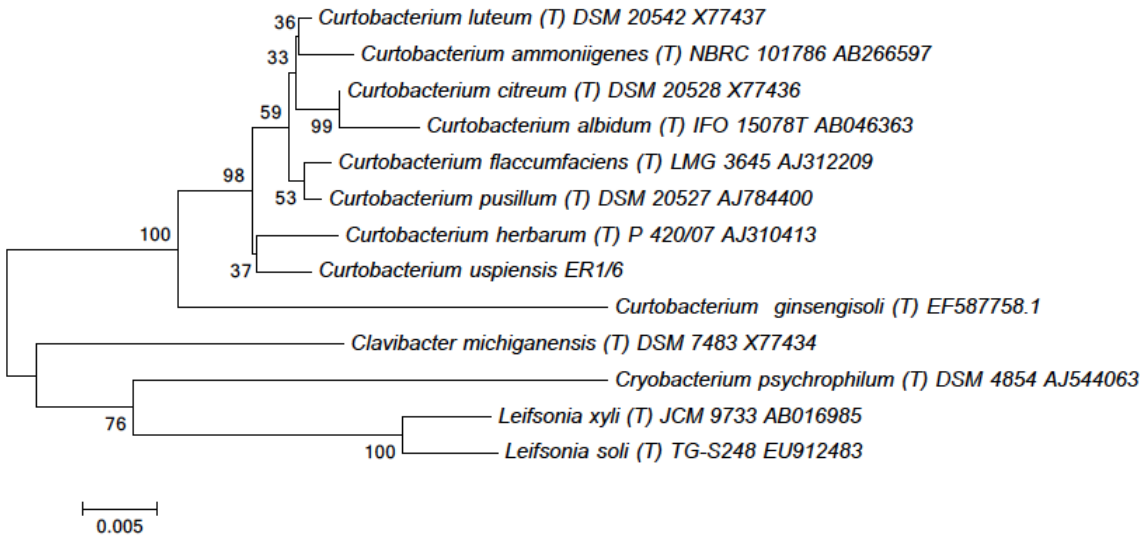

Figure 2

Phylogenetic tree showing the relationship between *Curtobacterium uspiensis* sp. nov. ER1/6^{T} and *Curtobacterium* species based on 16S *rRNA* gene sequences available in RDP query and NCBI database using Kimura model. Bootstrap values are expressed as percentages of 1000 replications at branch points. There were a total of 1420 nucleotide positions in the final dataset, and *Leifsonia xyli, Leifsonia soli, Cryobacterium psychrophilum* and *Clavibacter michiganensis* served as outgroup.

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