Curtobacterium allii sp. nov., the actinobacterial pathogen causing onion bulb rot

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Abstract

A Gram positive, aerobic, and non-spore-forming bacterial strain, 20TX0166^T, was isolated from a diseased onion bulb in Texas, USA. Upon testing its pathogenicity on onion bulb, it produced pathogenic response which makes it first species of pathogen belonging to the phylum actinobacteria detected in onion. Phylogenetic analysis of the 16S rRNA gene sequence revealed that the strain belonged to the genus *Curtobacterium* and was most similar to *Curtobacterium flaccumfaciens* LMG 3645^T (100%), *C. pusillum* DSM 20527^T (99.5%), and *C. oceanosedimentum* ATCC 31317^T (99.5%). The orthologous ANI (orthoANIu), ANI based on blast (ANIb), and dDDH values between the novel strain and the closest relative, *C. flaccumfaciens* LMG 3645^T, were 95.7%, 95.4%, and 63.3%, respectively. These values were below the recommended species cut-off threshold of 96% (ANI) and 70% (dDDH), suggesting the strain may be a novel species. The estimated genome size of the novel species was 3.98 Mbp with a G + C content of 70.8%. Physiologic and phenotypic characters of this novel strain were also unique when compared with the closely related species. The major cellular fatty acids of this strain were $C_{15:0}$ anteiso and $C_{17:0}$ anteiso. Using a polyphasic approach based on phenotypic and genotypic analyses, strain 20TX0166^T represents a novel species of the genus *Curtobacterium*, and the name *Curtobacterium allii* sp. nov. is proposed. The type strain is 20TX0166^T (= LMG 32517^T, =CIP112023^T, =NCIMB 15427^T).

Introduction

Curtobacterium was first introduced as a new genus of coryneform bacteria under phylum actinobacteria by Yamada and Komagata in 1972 (Yamada and Komagata 1972). Bacteria in this genus are aerobic, Gram positive, non-spore-forming, filamentous, and rod-shaped. *Curtobacterium* spp. are ubiquitous, cosmopolitan, and associated with diverse ecosystems around the world, including soil, water, and plant microbiomes (Aizawa et al. 2007; Chase et al. 2016; Li et al. 2016; Nascimento et al. 2020). Studies conducted to examine the microbial communities of leaf litter found *Curtobacterium* was the dominant genus, which was highly diverse within itself (Behrendt et al. 2002; Chase et al. 2017; Matulich et al. 2015). Numerous studies on beneficial strains have highlighted their importance as plant growth promoters and biological plant disease control agents (Bulgari et al. 2014; Khan et al. 2019; Lacava et al. 2007; Mayer et al. 2019; Silambarasan et al. 2019; Vimal et al. 2019). Although rare, *Curtobacterium* strains are also associated with human infections (Francis et al. 2011; Funke et al. 2005; Rivera et al. 2012). *Curtobacterium flaccumfaciens* (Cf) is a common plant pathogen with the potential to cause significant disease in many legumes, including beans, soybean, and cowpea (Gonçalves et al. 2017; Osdaghi et al. 2018a; Sammer and Reiher 2012). However, no bacterium of this genus or entire phylum actinobacteria were ever reported before as a pathogen of onion (*Allium cepa*). Due to the extent of plant diseases caused by Cf, many taxonomic studies using rep-PCR, MLSA, and whole genome analyses conducted have revealed high phenotypic and genotypic diversity, and authors from these studies have suggested that many strains previously assigned to this species could potentially be new species (Agarkova et al. 2012; Chen et al. 2021; Gonçalves et al. 2019; Osdaghi et al. 2018b).

In this study, a bacterial strain 20TX0166^T, pathogenic to onion was isolated from rotting onion bulb and characterized using polyphasic approach of taxonomy. Based on phenotypic and genotypic analyses, it is presumably a novel species of *Curtobacterium*.

Materials And Methods

Isolation and Pathogenicity assay

The bacterial strain 20TX0166^T was isolated from symptomatic tissue of a rotting onion bulb collected in Texas, USA (Fig. S1). For isolation, a 5 mm^2 piece of tissue along the margin of the rotting symptom was cut from the bulb, washed with sterile water, and crushed in 100 µL sterile water. The resulting suspension was streaked on nutrient agar medium and incubated at 25°C. After successive culturing of single colonies 3 times, the pure culture was stored at -80°C in 15% aqueous glycerol (v/v). The isolate was tested for pathogenicity by inoculating detached fleshy scales from a red onion bulb with a suspension of the bacterium, and by injecting the bacterial suspension into a yellow onion bulb. For the scale assay, a 10 µl suspension (10^8 CFU/ml) of the bacterium was placed over a wound made at the centre of the piece of scale (~ 3 cm x 4 cm) with a sterile needle, and the inoculated scale pieces were incubated at 25°C for 10 d. For the bulb assay, a 0.5 ml aliquot of the bacterial suspension (10^8 CFU/ml) was injected into the upper shoulder of the bulb, and the bulb incubated at 25°C for 12 d. Each assay was conducted with three replicate samples (detached bulb scale pieces or whole bulbs) and the experiment was repeated. A known strain of *Pantoea ananatis* (PNA 97-1R) pathogenic to onion was used as a positive control treatment, whereas sterile phosphate-buffer saline (PBS) solution was used as a negative control treatment (Stice et al. 2018).

16S rRNA analysis and phylogeny

Genomic DNA of 20TX0166^T was extracted using the DNeasy Power Soil kit (Qiagen, MD, USA) and stored at -20 ^{0}C. The universal primers 27F (5'- AGAGTTTGATCMTGGCTCAG – 3'), 534R (5'- CGGTTACCTTGTTACGACTT – 3'), and 1492R (5'- CGGTTACCTTGTTACGACTT – 3') were used to amplify and sequence the 16S rRNA gene (Walker et al. 2015; Webster et al. 2006; Weisburg et al. 1991; Wilson et al. 1990). Sequences generated by the three primers were trimmed and assembled to make a consensus sequence of 1,424 nucleotides (nt) using Geneious Prime 2020 (https://www.geneious.com/). The partial 16S rRNA gene sequence of 20TX0166^T was deposited and made publicly available (GenBank OK275102). The gene sequence of the novel strain was compared with those of the type strains of *Curtobacterium* species using reference RNA sequences (refseq_rna) database in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990). To examine the phylogenetic relationship of 20TX0166^T with previously documented species, the 16S rRNA gene sequences of eight *Curtobacterium* species were downloaded from GenBank and aligned using MUSCLE v3.8.425 (Edgar 2004) in Geneious Prime 2020. Using the aligned sequences (1,388 nt), a maximum likelihood

phylogenetic tree was constructed using PhyML 3.0 (Guindon et al. 2010) and visualized using MEGA v10.2.6 (Kumar et al. 2018). In PhyML 3.0, the HKY85 substitution model with 1,000 bootstrap replicates was used for the phylogenetic analysis (Lefort et al. 2017).

Whole genome analysis and phylogeny

Using the genomic DNA, the genome of the novel species was sequenced and assembled at CD Genomics (Shirley, NY, USA). Pair-end sequencing with an average sequencing depth of 100X was performed using the Illumina NovaSeq 6000 platform. The genome was compiled after trimming and assembling sequences from the raw fastq files using the default settings of Unicycler v0.4.8 (Wick et al. 2017) and SPAdes v3.13.0 (Bankevich et al. 2012). The genome was annotated functionally using PROKKA v1.14.5 (Seemann 2014). The annotated genome was used to generate a graphical illustration of the circular genome using DNAplotter v18.1.0 (Fig. S2) (Carver et al. 2009). The genome sequence of the novel strain was compared with that of eight validly published *Curtobacterium* species to determine the average nucleotide identity (ANI) using the Orthologous ANI Tool (OAT software v0.93.1) based on OrthoANIu algorithm (Yoon et al. 2017) and pairwise ANI based on BLAST (ANIb) (Goris et al. 2007) using Python-based software - pyANI (Pritchard et al. 2016). Similarly, the digital DNA-DNA hybridization (dDDH) values were calculated using Type (Strain) Genome Server-TYGS (https://tygs.dsmz.de/) (Meier-Kolthoff and Göker 2019). A phylogenetic tree based on whole genome sequence was created in the TYGS webserver, comparing the novel strain with the same eight species mentioned earlier. The Genome BLAST Distance Phylogeny method (GBDP) was used to calculate intergenomic distances. The GBDP method utilized the d5 distance formula and 100 bootstrap replicates for each bacterial strain (Meier-Kolthoff et al. 2013). A phylogenetic tree was estimated using FASTME 2.1.6.1 followed by Subtree Pruning and Regrafting post-processing. To resolve the species delineation of 35 strains deposited as *C. flaccumfaciens* in the NCBI Genome database, we used an approach adapted from Zhao *et al*. (Zhao et al. 2021), in which the ANI and dDDH values were computed after comparing with the *C. flaccumfaciens* type strain LMG 3645^T and the proposed *C. allii* type strain 20TX0166^T.

Physiological and chemotaxonomic analyses

Phenotypic analyses were performed following the protocols described by Schaad *et al*. (Schaad et al. 2001) unless otherwise stated. A 24-h-old culture of strain 20TX0166^T was tested for the Gram reaction using the standard Gram staining technique and KOH test (Suslow 1982). Cells of the strain were stained with malachite green to evaluate whether the strain formed spores. Spore-forming bacteria stain green while non-spore-forming bacteria stain red. Cultures were grown for 7 d in nutrient agar tubes in the absence of oxygen using a layer of mineral oil, to determine the facultative aerobic status of the strain. Motility was observed by culturing the bacteria in a semi-solid growth motility test medium containing tryptone, sodium chloride, and agar. Catalase activity was tested with 3% hydrogen peroxide and oxidase activity was tested using Oxistrips and Oxidrops (Hardy Diagnostics, CA, USA). Imaging was performed

using cultures that were grown for 24 h at 28°C in yeast extract peptone glucose (YPG) broth and visualized using calibrated magnification with a 15C CCD camera on a JEOL 1200Ex transmission electron microscope (TEM) operated at 100kV at the Texas A&M Microscopy and Imaging Centre. A pectolytic assay to assess potato soft rotting capacity was conducted using flame-sterilized, peeled potato slices, and the tobacco hypersensitivity response was tested *in planta* on a leaf of each of three replicate tobacco plants along with relevant positive and negative control strains of bacteria and buffer.

To determine optimum growth conditions, cultures were incubated in nutrient broth yeast agar (NBYA), yeast extract peptone glucose agar (YPGA), yeast extract dextrose calcium carbonate agar, and nutrient agar media as described by Schaad *et al*. (Schaad et al. 2001) for at least 3 d at 4, 15, 20, 22, 25, 28, 30, 33, 35, 37, 39, and 41°C. The optimal pH of the isolate was determined by incubating bacterial cells for 5 d at 25°C in yeast extract peptone glucose (YPG) broth cultures adjusted to a pH of 3, 4, 5, 6, 7, 8, 9, and 10. Salt tolerance was determined by culturing the strain on YPGA medium with NaCl concentrations ranging from 0 to 10%, at intervals of 1% for 7 d at 25°C.

Biolog GenIII microplates, API 20NE strips (bioMérieux), and API Coryne strips (bioMérieux) were utilized to assess various biochemical and physiological characteristics using a fresh 24-h-old culture grown in NBYA medium at 30°C per the manufacturer's instructions. For Biolog testing, an IF-A suspension [optical density (OD_{600}) of 0.022] was prepared and dispensed into the GenIII microplates, which were incubated at 30°C. Results were read after 24, 48, and 72 h. For the API Coryne test, a bacterial suspension (turbidity greater than that of the McFarland #6 standard) was prepared using a 24-h-old culture, dispensed into the test strips, incubated at 33°C, and results read after 24 and 48 h. For the API 20NE assay, a bacterial suspension (OD_{600} of 0.09) was prepared in 0.85% sterile saline, dispensed into the test strips, incubated at 30°C, and results read after 24 and 48 h. The OD_{600} was measured using a Fluostar Omega microplate reader (BMG LABTECH Inc., NC, USA). These three tests were carried out a minimum of 3 times each, and results were compared with the results of type strains of the six closely related species of *Curtobacterium*. Among these species, *C. flaccumfaciens* is a known plant pathogen, though not known to be pathogenic to onion. The remaining five species are not known to be plant pathogens. The fatty acid methyl ester (FAME) profile of this strain was determined using the Sherlock Microbial Identification System at ESML Analytical Inc. (Cinnaminson, NJ, USA) (Sasser 1990).

Results And Discussion

Pathogenicity assay

In both, scale and bulb pathogenicity assays, the bacterial strain 20TX0166^T caused a pathogenic response, inducing necrosis of the fleshy scales (Fig. S3) and whole bulbs (Fig. 1). Two isolates (20TX0166b from fleshy scale and 20TX0166c from bulb) with similar morphology to the original isolate were re-isolated from each of the two pathogenicity tests. When these re-isolated daughter strains were tested further using the scale and bulb assays, the symptoms they produced were identical to that of the

original strain. After sequencing the 16S rRNA gene, the daughter strains (GenBank OM863553 and OM863552) were confirmed as identical to the original strain.

Phylogenetic analysis based on 16S rRNA

Upon searching on BLAST, the 16S rRNA gene sequence (GenBank accession number OK275102) of the strain 20TX0166^T shared 100% similarity with *Curtobacterium flaccumfaciens* LMG 3645^T (Table 1). The novel strain sequence also had > 99% similarity with the sequences of type strains of six other species of *Curtobacterium*. Such a high level of similarity makes it difficult to assign 20TX0166^T as one of the previously documented species based on 16S rRNA gene similarity. In the phylogenetic tree created using 16s rRNA sequences, the novel strain did not produce separate branching from the *C. flaccumfaciens* LMG 3645^T, indicating the strain was most closely related to this species (Fig. 2). However, previous studies on *Brevundimonas* and *Streptomyces* have reported that multiple bacterial species with distinct ecological niches can share identical 16S rRNA gene sequences (Antony-Babu et al. 2017; Jaspers and Overmann 2004). Also, in a taxonomical classification study conducted using 1142 actinobacterial type strain genomes (Nouioui et al. 2018), it was suggested that similarity based on a single gene sequence cannot be fully relied for species identification. Thus, the genome of the novel strain isolated from rotting onion was analysed further to confirm the species identity.

Table 1
Comparison of 16s rRNA gene similarity, digital DNA-DNA hybridization (dDDH), and average nucleotide identity (ANI) of *Curtobacterium allii* 20TX0166^T, obtained from a rotting onion bulb in Texas, USA with the most similar species of *Curtobacterium*.

Strain	16S rRNA similarity (%)†	dDDH (d4, %)‡	OrthoANIu (%)§	ANIb (%)¶
Curtobacterium flaccumfaciens LMG 3645^T	100.0	63.3	95.7	95.4
Curtobacterium pusillum DSM 20527^T	99.5	28.3	84.9	84.4
Curtobacterium oceanosedimentum ATCC 31317^T	99.5	26.4††	83.3††	83.0††
Curtobacterium ammoniigenes NBRC 101786^T	99.3	20.1	75.6	76.4
Curtobacterium luteum DSM 20542^T	99.3	25.9	83.3	82.8
Curtobacterium citreum DSM 20528^T	99.3	26.3	83.4	83.1
Curtobacterium albidum DSM 20512^T	99.1	26.4	83.6	83.2
Curtobacterium herbarum DSM 14013^T	98.3	25.3	82.4	82.2

†, ‡, §, ¶ Values were computed using BLAST, TYGS webserver, OAT software v0.93.1, and pyANI, respectively.

†† The dDDH and ANI values were calculated using the genome of *C. oceanosedimentum* strain NS263, as genome for the type strain was not available.

Phylogenetic analysis based on Whole genome

The genome (GenBank accession number JAIWKR000000000) of the novel strain was estimated to be 3,986,426 nt (35 contigs, N50 = 502,963 nt, and the longest contig = 865,032 nt) with a G + C content of 70.8%. The draft genome consisted of 3,762 coding DNA sequences (CDS), 55 tRNA genes, 3 rRNA genes, and 1 tmRNA gene. The othoANIu, ANIb and dDDH values (Table 1) between the novel strain and *C. flaccumfaciens* LMG 3645^T were 95.7%, 95.4%, and 63.3%, respectively, which were lower than the minimum threshold values of 96% (ANI) and 70% (dDDH) for species determination (Auch et al. 2010; Ciufo et al. 2018; Wayne et al. 1987). Although the ANI values between the novel strain and *C. flaccumfaciens* LMG 3645^T were closer to the species delineation threshold, the dDDH value between them was well below the threshold, which was a scenario similar to Sawada *et al*. (Sawada et al. 2021). Also, the novel species formed a separate branching away from *C. flaccumfaciens* LMG 3645^T on the

phylogenetic tree, supported with a 100% bootstrap value, indicating 20TX0166^T is a novel species (Fig. 3).

Upon comparing the 35 strains deposited as *C. flaccumfaciens* in the NCBI Genome database (Table 2), the 15 strains (CFBP 3418, BRIP 70607, DSM 20129, BRIP 70606, BRIP 70601, BRIP 70614, BRIP 70615, VKM Ac-1386, CFBP 8820, P990, VKM Ac-1795, BRIP 70624, CFBP 8825, CFBP 8821, and 208) had dDDH and ANI values greater than the species cut-off threshold of 70% and 96%, respectively, compared with *C. flaccumfaciens* LMG 3645^T, suggesting the 15 strains are all *C. flaccumfaciens* strains. However, the dDDH and ANI values of 6 strains (Cff1037, CFBP 1384, CFBP 8822, CFBP 2402, CFBP 3401, and CFBP 3423), compared with *C. allii* 20TX0166^T were greater than the species cut-off threshold of 70% and 96%, respectively, suggesting the species assignations should be moved from *C. flaccumfaciens* and reclassified as *C. allii*. While the remaining 14 strains (BRIP 70610, CFBP 3417, CFBP 2403, MEB126, CFBP 3422, UCD-AKU, CFBP 3415, S5.26, CFBP 8818, CFBP 8819, CFBP 8823, CFBP 8824, CFBP 3400, and Jub65) had dDDH and ANI values lesser than the species cut-off threshold compared to both *C. flaccumfaciens* LMG 3645^T and *C. allii* 20TX0166^T, indicating these 14 strains may potentially be new species. A phylogenetic tree was created using the TYGS web server to show the relationships among these strains (Fig. S4). The 14 strains branched into 5 species clusters, which indicated these could be classified potentially as five new species.

Table 2
Genomic assessment of bacterial strains designated as *Curtobacterium flaccumfaciens* in NCBI, compared with the novel strain 20TX0166^T obtained from a rotting onion bulb in Texas, USA, and *C. flaccumfaciens* LMG 3645^T.

	dDDH (d4, %)†		**OrthoANIu (%)‡**		
Strain	**20TX0166^T**	**LMG 3645^T**	**20TX0166^T**	**LMG 3645^T**	Curtobacterium **species status**
Cff1037$^\Psi$	**83.7**	64.5	**98.3**	95.9	*C. allii*
CFBP 1384	**82.2**	63.5	**98.1**	95.7	*C. allii*
CFBP 8822	**80.5**	63.9	**97.9**	95.8	*C. allii*
CFBP 2402	**80.4**	63.9	**97.9**	95.7	*C. allii*
CFBP 3401	**80.4**	63.8	**97.8**	95.7	*C. allii*
CFBP 3423	**77.0**	65.0	**97.5**	95.9	*C. allii*
CFBP 3418	63.2	**100.0**	95.6	**99.9**	*C. flaccumfaciens*
BRIP 70607	62.9	**77.2**	95.3	**97.3**	*C. flaccumfaciens*
DSM 20129	62.8	**77.0**	95.4	**97.4**	*C. flaccumfaciens*
BRIP 70606	62.9	**76.3**	95.6	**97.5**	*C. flaccumfaciens*
BRIP 70601	63.0	**76.2**	95.6	**97.4**	*C. flaccumfaciens*
BRIP 70614	62.9	**76.2**	95.6	**97.4**	*C. flaccumfaciens*
BRIP 70615	62.8	**76.1**	95.6	**97.5**	*C. flaccumfaciens*
VKM Ac-1386	61.8	**75.6**	95.3	**97.3**	*C. flaccumfaciens*
CFBP 8820	63.2	**75.5**	95.6	**97.3**	*C. flaccumfaciens*
P990$^\Phi$	63.0	**75.4**	95.6	**97.3**	*C. flaccumfaciens*
VKM Ac-1795	62.6	**75.3**	95.6	**97.2**	*C. flaccumfaciens*
BRIP 70624	63.4	**75.1**	95.7	**97.2**	*C. flaccumfaciens*
CFBP 8825	63.4	**75.0**	95.6	**97.3**	*C. flaccumfaciens*
CFBP 8821	63.4	**73.9**	95.6	**97.1**	*C. flaccumfaciens*

†, ‡ Values were computed using TYGS web server and OAT software v0.93.1. dDDH and OrthoANIu values > 70% and > 96%, respectively are highlighted in bold font.

$^\Psi$ (O'Leary and Gilbertson 2020). $^\Phi$ (Chen et al. 2021). $^\delta$ (Flanagan et al. 2013).

	dDDH (d4, %)†		OrthoANIu (%)‡		
208	63.5	**73.2**	95.8	**97.0**	*C. flaccumfaciens*
BRIP 70610	58.3	61.6	94.9	95.3	Potential new species
CFBP 3417	50.7	54.5	93.3	94.1	Potential new species
CFBP 2403	50.5	53.1	93.3	93.9	Potential new species
MEB126	50.5	53.1	93.4	93.9	Potential new species
CFBP 3422	50.8	53.0	93.3	93.9	Potential new species
UCD-AKU$^{\delta}$	50.7	53.0	93.3	93.8	Potential new species
CFBP 3415	50.3	53.0	93.3	93.8	Potential new species
S5.26	50.8	52.9	93.4	93.8	Potential new species
CFBP 8818	50.4	52.5	93.3	93.8	Potential new species
CFBP 8819	50.4	52.5	93.3	93.7	Potential new species
CFBP 8823	50.1	52.4	93.2	93.7	Potential new species
CFBP 8824	50.4	51.0	93.4	93.4	Potential new species
CFBP 3400	32.7	32.9	87.4	87.4	Potential new species
JUb65	28.8	28.8	85.4	85.5	Potential new species

†, ‡ Values were computed using TYGS web server and OAT software v0.93.1. dDDH and OrthoANIu values > 70% and > 96%, respectively are highlighted in bold font.

$^{\Psi}$ (O'Leary and Gilbertson 2020). $^{\Phi}$ (Chen et al. 2021). $^{\delta}$ (Flanagan et al. 2013).

Physiological and chemotaxonomic analyses

The cells of 20TX0166^{T} were Gram positive (Fig. S5), non-spore-forming rods (Fig. S6), obligately aerobic, non-motile, catalase positive, and oxidase negative. The TEM images showed that bacterial cells were rod-shaped with an absence of flagella, 1.5-2.0 µm long x 0.6-1.0 µm wide (Fig. 4). No soft rotting of potato slices was observed after 24 h at 22°C, indicating no pectolytic activity by strain 20TX0166^{T}. The tobacco hypersensitivity test was negative, in which the strain did not elicit a hypersensitive response, indicating the bacterium is non-pathogenic to tobacco.

Growth was observed at 4 to 39°C but not at 41°C on all media tested. The optimum temperature for growth was 25°C and, although growth was observed at 4°C, the rate of growth was slow as it took 5–7 d

to form visible colonies at that temperature. After 24 h of incubation at 25°C on YPGA and NBYA media, the bacterium produced 0.5-1.0 mm wide, smooth, creamy, round, and entire colonies. Colony size increased to 2–3 mm in 3 d, and became somewhat mucoid with light yellow pigmentation (Fig. S7). Bacterial growth was observed at a pH of 5–10 with an optimal pH of 7. Growth was observed on YPGA medium with up to 9% NaCl, while optimum growth was observed at 0 to 1% NaCl.

Significant differences were noted in the Biolog, API Coryne, and API 20NE test reactions between the novel strain and *C. flaccumfaciens* LMG 3645^T, as well as the type strains of other related species (Table 3). These results further support that the onion strain is a novel species of *Curtobacterium*. The comprehensive results of the Biolog, API Coryne, and API 20NE of the novel strain and the closely related species are provided in Table S1. The major fatty acids profile from FAME analysis were $C_{15:0}$ anteiso and $C_{17:0}$ anteiso (Table 4). Other fatty acids detected in lower amounts were $C_{15:0}$ iso and $C_{16:0}$ iso. The cellular fatty acid content of the novel strain was different from that of *C. flaccumfaciens* JCM 9670^T and the type strains of other related species, supporting the evidence that strain 20TX0166^T is a novel species of *Curtobacterium*.

Table 3

Comparison of selected physiological and phenotypic characteristics of strain 20TX0166^T of *Curtobacterium* obtained from a rotting onion bulb in Texas, USA, and of closely related *Curtobacterium* type strains.

Reactions	**Strains of *Curtobacterium*†**						
	1‡	**2‡**	**3§**	**4§**	**5§**	**6§**	**7§**
Catalase	+¶	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-
Motility	-	-	+	+	+	-	-
Optimum pH	7	7	7	7	7	7	4
Biolog							
N-Acetyl-DGalactosamine	+	+	-	-	-	-	-
D-Arabitol	+	+	w	-	-	-	-
myo-Inositol	+	+	-	-	-	-	-
L-Alanine	+	+	-	-	-	w	-
D-Glucuronic Acid	+	+	-	-	-	-	-
L-Malic Acid	-	+	ND	ND	ND	ND	ND
Bromo-Succinic Acid	-	+	-	w	-	-	-
API Coryne							
Pyrazinamidase	+	+	ND	-	-	ND	ND
Alkaline Phosphatase	+	-	ND	ND	ND	ND	ND
Gelatin hydrolysis/protease	-	-	+	-	-	+	-
L-ribose (Fermentation)	+	-	+	+	+	-	+
D-mannitol (Fermentation)	+	-	ND	ND	ND	ND	ND
D-maltose (Fermentation)	-	-	+	+	+	+	ND
D-lactose (Fermentation)	+	-	ND	ND	ND	ND	ND

† Strains: 1, 20TX0166^T; 2, *C. flaccumfaciens* LMG 3645^T; 3, *C. pusillum* JCM 1350^T; 4, *C. citreum* JCM 1345^T; 5, *C. luteum* JCM 1480^T; 6, C. albidum NBRC 15078^T; 7, *C. ammoniigenes* B55^T.

‡ Data are from this study.

§ Data are from (Aizawa et al. 2007).

¶ +, Positive reaction; –, negative reaction; w, weakly positive reaction; ND, No data available.

Reactions	Strains of *Curtobacterium*†						
	1‡	2‡	3§	4§	5§	6§	7§
glycogen (Fermentation)	+	-	-	+	+	-	-
API 20NE							
Gelatin hydrolysis/protease	-	-	+	-	-	+	-
D-mannitol (Assimilation)	-	+	ND	+	-	ND	ND
N-acetyl-glucosamine (Assimilation)	-	+	+	+	-	-	-
Potassium gluconate (Assimilation)	+	+	-	-	-	-	+
Malic acid/Malate (Assimilation)	-	+	ND	+	+	ND	ND
† Strains: 1, 20TX0166^T; 2, *C. flaccumfaciens* LMG 3645^T; 3, *C. pusillum* JCM 1350^T; 4, *C. citreum* JCM 1345^T; 5, *C. luteum* JCM 1480^T; 6, C. albidum NBRC 15078^T; 7, *C. ammoniigenes* B55^T.							
‡ Data are from this study.							
§ Data are from (Aizawa et al. 2007).							
¶ +, Positive reaction; –, negative reaction; w, weakly positive reaction; ND, No data available.							

Table 4

Comparison of fatty acid composition (%) of strain 20TX0166^T of *Curtobacterium* obtained from a rotting onion bulb in Texas, USA, and of closely related *Curtobacterium* type strains.

Fatty acid %	Strains of *Curtobacterium*†					
	1‡	2§	3§	4§	5§	6§
$C_{15:0}$ iso	3.96	2.8	2.8	1.6	7.9	0.1
$C_{15:0}$ anteiso	48.14	31.3	19.6	37.2	39.5	1.3
$C_{16:0}$ iso	2.63	12.3	3.9	11.8	24	0.4
$C_{17:0}$ anteiso	39.44	29.3	18.6	36.6	18.4	2.4
† Strains: 1, 20TX0166^T; 2, *C. flaccumfaciens* JCM 9670^T; 3, *C. pusillum* JCM 1350^T; 4, *C. citreum* JCM 1345^T; 5, *C. luteum* JCM 1480^T; 6, *C. ammoniigenes* B55^T.						
‡ Data are from this study						
§ Data are from (Aizawa et al. 2007).						

Conclusion

In brief, based on physiological, chemotaxonomic, genotypic, and phylogenetic characterizations of strain 20TX0166^T, it is found to be different from all the most closely related species of *Curtobacterium*. Thus, we suggest that strain 20TX0166^T should be assigned as a novel species of Curtobacterium, for which the name *Curtobacterium allii* is proposed.

Description of *Curtobacterium allii* sp. nov.

Curtobacterium allii (al'li.i. L. gen. n *allii* of *Alliium cepa* (onion), pertaining to the isolation made from onion bulb tissue).

The cells of 20TX0166^T are Gram positive, non-spore-forming, non-motile, obligately aerobic, oxidase negative, and catalase positive. They are rod-shaped, 1.5-2.0 µm long and 0.6-1.0 µm wide. Colonies are 0.5-1.0 mm wide, smooth, creamy coloured, round, and entire on NBYA medium after 24 h of incubation at 25°C. The colony size increases to 2–3 mm in 3 d, becoming somewhat mucoid, and develops a light-yellow pigmentation. The bacterium has no pectolytic activity on potato slices and does not produce a hypersensitive response on tobacco. It can grow from a range of 4–39°C with 25°C the optimal temperature for growth. The bacterium can grow at a pH range of 5 to 10 with optimum growth at pH 7 and tolerate up to 9% NaCl in a YPGA medium, with optimal growth at 0–1% NaCl. The major cellular fatty acids of this strain were $C_{15:0}$ anteiso and $C_{17:0}$ anteiso. Other fatty acids detected in lower amounts were $C_{15:0}$ iso and $C_{16:0}$ iso.

The type strain, 20TX0166^T (= LMG 32517^T, =CIP112023^T, =NCIMB 15427^T), was isolated from symptomatic onion bulb tissue collected from Texas, USA. The estimated size of the genome is 3,986,426 nt, with a G + C content of 70.8%. The GenBank accession numbers for the 16S rRNA gene sequence and draft genome are OK275102 and JAIWKR000000000, respectively.

Abbreviations

ANI, Average nucleotide identity; dDDH, digital DNA-DNA hybridization; orthoANIu, orthologous ANI; ANIb, ANI based on BLAST; NCBI, National Centre for Biotechnology Information; TEM, transmission electron microscope; OD, optical density; FAME, fatty acid methyl ester.

Declarations

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Authors' contributions

Conceptualisation: MK, SM, ST. Data curation: MK, ST. Formal Analysis: MK, ST, SG. Funding acquisition: SM. Investigation: MK, SM. Methodology: MK, BP. Supervision: ST, KC, SM. Writing - original draft: MK. Writing - review & editing: SG, ST, KC, BP, SM.

Data availability

Data are publicly available on the GeneBank (https://www.ncbi.nlm.nih.gov/genbank/). The GenBank accession number for the genome of strain 20TX0166^T is JAIWKR000000000. The GenBank accession number for the 16S rRNA sequence of strain 20TX0166^T is OK275102.

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Conflicts of interest

The author(s) declare that there are no conflicts of interest.

Ethical approval

The study did not require an ethical committee approval.

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Figures

Figure 1

Necrotic rotting observed on an onion bulb 12 d post inoculation with strain 20TX0166^{T} of *Curtobacterium* isolated from a rotten onion bulb in Texas, USA. A suspension of the bacterium (0.5 ml of 10^{8} CFU/ml) was injected into the upper shoulder of the bulb, and the bulb incubated at 25 °C.

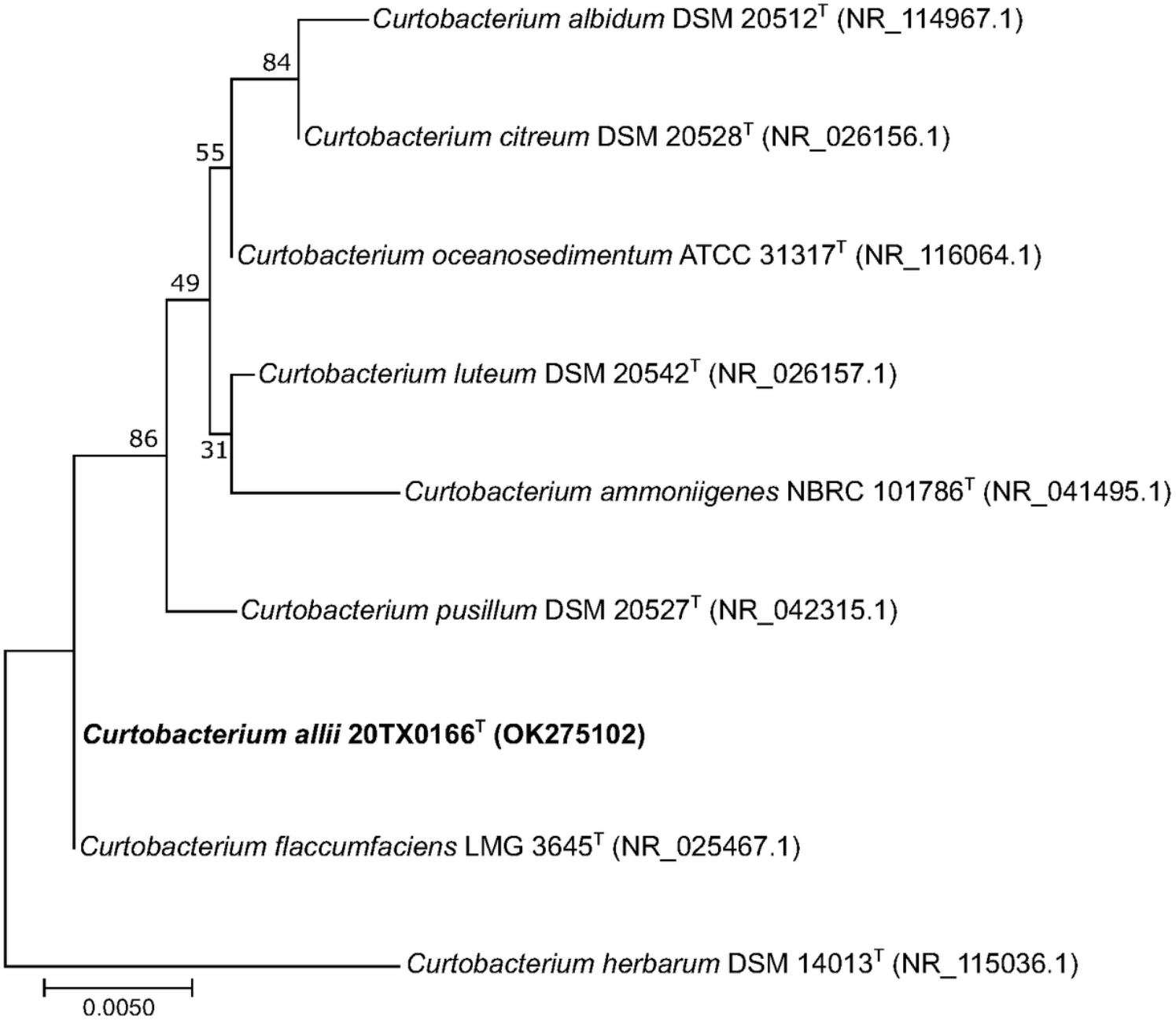

Figure 2

Maximum likelihood phylogenetic tree constructed using the 16S rRNA gene sequence of strain 20TX0166^T of *Curtobacterium* obtained from a rotting onion bulb in Texas, USA, and the sequences of closely related species of *Curtobacterium*. The gene sequences were aligned using MUSCLE v3.8.425, the tree was constructed using PhyML 3.0 and visualized using MEGA v10.2.6, and the text was reformatted using Inkscape v1.1. Bootstrap values at the branching nodes indicate the percentage of 1,000 replicates. The scale bar refers to the number of nucleotide substitutions per site. The superscript ^{T} denotes the type strain of the species used and the GenBank accession number of each strain is in parentheses.

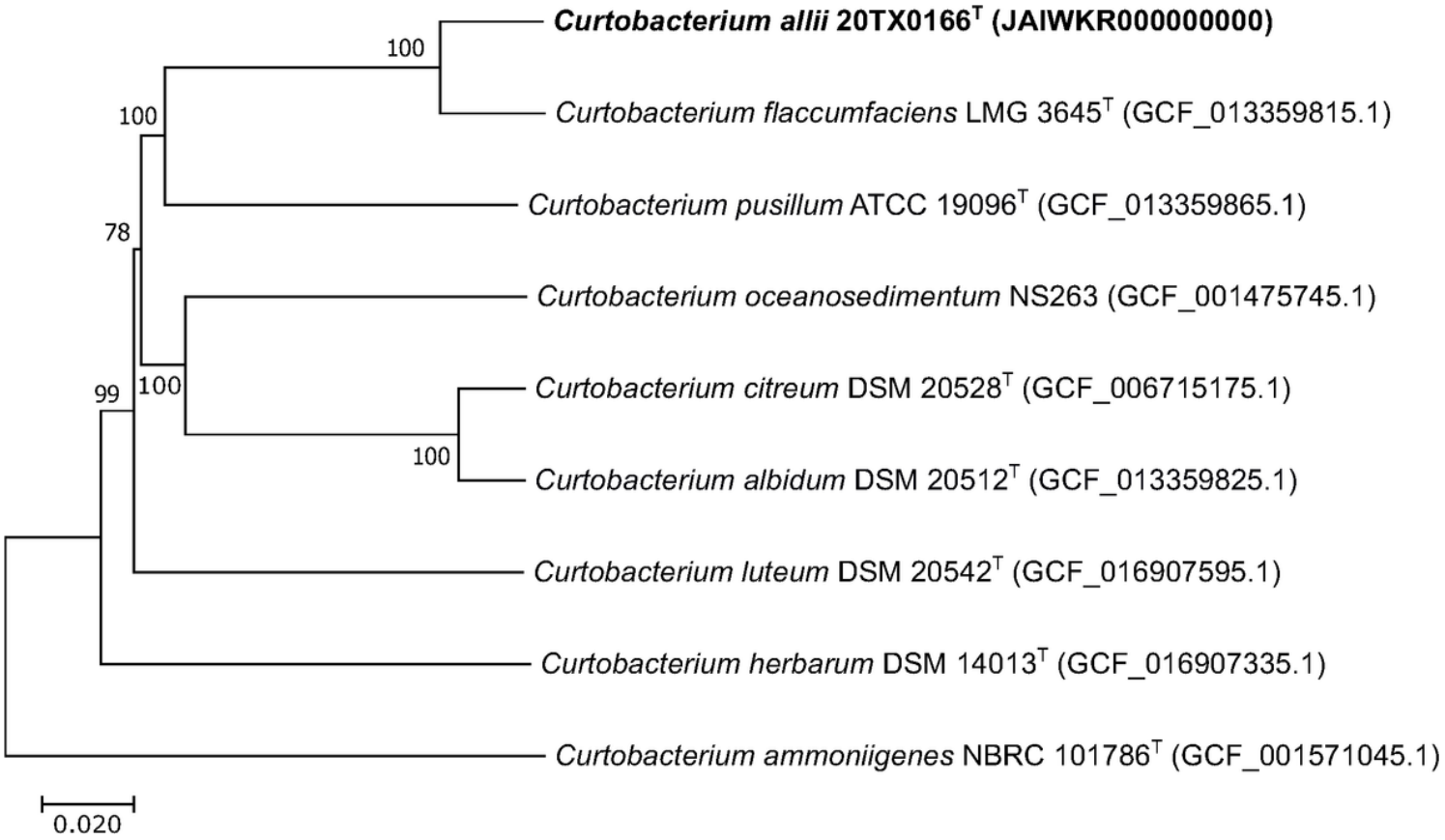

Figure 3

Phylogenetic tree based on the whole genome sequence of strain 20TX0166^T of *Curtobacterium* isolated from a rotting onion bulb in Texas, USA, and whole genome sequences of closely related species of *Curtobacterium*. The tree was constructed using the TYGS web server, and the text was reformatted using MEGA v10.2.6 and Inkscape v1.1. Bootstrap values at the branching nodes indicate the percentage of 1,000 replicates. The scale bar refers to the number of nucleotide substitutions per site. The superscript ^{T} denotes the type strain of the species used and the GenBank accession number of each strain is in parentheses.

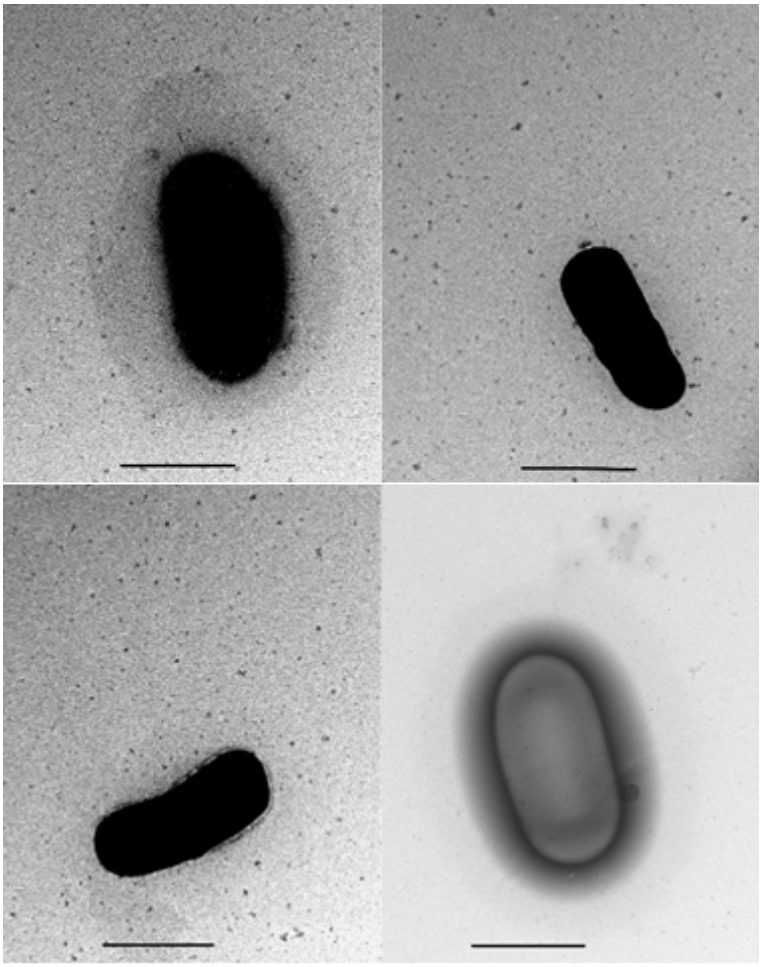

Figure 4

Cells of the novel strain 20TX0166^{T} of *Curtobacterium* isolated from a rotting onion bulb in Texas, USA, observed with a transmission electron microscope. The scale bar in each of the four images measures 1 µm.

Supplementary Files

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