Curtobacterium flaccumfaciens pv. *beticola*, A New Pathovar of Pathogens in Sugar Beet

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ABSTRACT

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Bacterial leaf spot of sugar beet was first discovered in 1995 in Inner Mongolia of China. The pathogen was shown to be a bacterium with properties of gram-positive bacteria: small irregular rods, lateral flagella, aerobic, and catalase-positive. The colonies of sugar beet strains produced a pale-yellow pigment. The optimum temperature for the bacteria to grow was 24 to 27°C. The bacteria could utilize a wide range of organic compounds, including hydrolyzed casein, starch, esculin and Tween 80, and released H_2S from cysteine, cystine, and $Na_2S_2O_3{\cdot}5H_2O$, but could not produce urease, oxidase, or indole. The cell wall peptidoglycan was based on ornithine (type B2β). The predominant menaquinone was MK-9. Polar lipids contained several glycosyldiacylglycerols. The DNA G+C content of a type strain of the new pathovar, T30^T, was 67.5%. DNA–DNA homology between T30^T and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (International Collection of Micro-Organisms from Plants, New Zealand [ICMP] 2584) was 70.1%. The new pathovar and *C. flaccumfaciens* pv. *flaccumfaciens* had 99.9% identity in DNA sequence of 16S rRNA. Close genetic relatedness was observed for the representatives of the species *Curtobacterium flaccumfaciens*, but a low level of similarity between the different pathovars was found. Based on these physiological, biochemical, chemotaxonomic, phylogenetic, and genetic characteristics, we demonstrate that the pathogen represents a new pathovar of *C. flaccumfaciens*, for which we propose the name *Curtobacterium flaccumfaciens* pv. *beticola* pv. nov. The type strain is T30^T (=ATCC BAA-144^T).

Additional keywords: plant pathogenic coryneform bacteria (PPCB), polyphasic analysis

In 1995, 1996, and 1998, we isolated a new pathogen from the leaves of diseased sugar beet (*Beta vulgaris* var. *saccharifera*) in Linhe City, Inner Mongolia autonomous region, China. The bacteria only damaged the leaves of sugar beet, and invaded from wounds of the leaves. The disease symptoms occurred on the leaves of sugar beet from June to October. Initially, the leaf spots were yellow and very small. They became brown with a yellow halo in a few weeks. In 1 month, the spots combined into brown patches. Finally, the entire leaves dehydrated and died (Fig. 1A). The roots of diseased sugar beets were observed to be much smaller than the roots of healthy plants. The disease was found only in Linhe City, not other cities in Inner Mongolia autonomous region (S. P. Liu, *personal communication*). Preliminary tests suggested that the pathogen responsible for these symptoms belonged to the group of bacteria called plant pathogenic coryneform bacteria (PPCB).

PPCB are classified into six genera: *Curtobacterium* (5,49), *Clavibacter* (8), *Rhodococcus* (18), *Rathayibacter* (7,39,54), and *Leifsonia* (15). The genus *Curtobacterium* consists of four PPCB, of which *Curtobacterium flaccumfaciens* pv. *betae* mainly causes silvering disease in red beet (*Beta vulgaris* var. *rubra*) and may be pathogenic to feed-stuff beet (*Beta vulgaris* var. *lutea*), but seldom damages sugar beet (27). The characteristic symptoms are white veins (5,27); this is very different from the brown spots seen on sugar beet, as described above. In this paper, we report the results of a comparative taxonomic study of T30^T and known PPCB, including the morphological, physiological, biochemical, chemotaxonomic characteristics, 16S rRNA phylogenetical and random amplified polymorphic DNA (RAPD) analysis. Our data suggest that a new pathovar, *Curtobacterium flaccumfaciens* pv. *beticola* pv. nov., exists. The new pathogen in this study is not correlative with the strain *Corynebacterium beticola*, as described by Collins and Jones (4).

MATERIALS AND METHODS

Bacterial strains and their cultivation. The novel isolates were T30^T, T28, and T56, which were isolated from sugar beet leaves with small spots in a field near Linhe City in July 1995, 1996, and 1998, respectively. Freshly infected leaves were washed with tap water and rinsed twice with sterilized water (SW); then individual small spots were cut into small pieces and soaked in about 20 µl of SW. The resulting bacterial suspension was streaked onto a petri dish containing 20 ml of D2 medium (26). After the plates were incubated at 28°C for 3 days, representative colonies were selected from each plate and further purified on the same medium. The type

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Yong-Fang Chen and Yan-Ni Yin contributed equally to this study and are regarded as joint first authors.

The GenBank accession number of the 16S rRNA sequence of *Curtobacterium flaccumfaciens* pv. *beticola* is AY273208.

* The *e*-Xtra logo stands for "electronic extra" and indicates that Figures 1 and 3 appear in color in the online edition.

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Fig. 1. Symptoms of sugar beet bacterial leaf spot caused by the new pathovar, *Curtobacterium flaccumfaciens* pv. *beticola*. **A,** Field symptoms; **B,** symptoms on leaves.

strain T30^T was used for the following experiments.

The type and reference strains used in this comparative study are presented in Table 1. All strains were grown on D2 medium at 27°C.

Pathogenicity test. For the pathogenicity test of T30^T, T28, T56, and International Collection of Micro-Organisms from Plants, New Zealand (ICMP) 2594, sugar beet, red beet, tulip (*Tulipa gesneriana*), common poinsettia (*Euphorbia pulcherrima*), and bean (*Phaseolus vulgaris*) were used. Seedlings were transplanted into plastic pots (9 in.) containing a horticultural soil–vermiculite mixture and grown in a greenhouse at 25 to 30°C. To prepare inoculum, bacteria were grown on D2 agar for 2 to 3 days at 27°C, suspended in sterile distilled water, and adjusted to 10^8 CFU/ml. Sugar beet and red beet at the seventh to eighth leaf stage were inoculated by using scrape and infiltration methods. Quartz was applied to the backsides of expanded leaves, then the backsides of leaves were brushed with a brush pen dipped in 10^8 CFU/ml inoculum. A drop (~8 µl) of 10^8 CFU/ml inoculum was infiltrated under the epidermis of the leaves. For beans, at the fourth to fifth leaf stage, a drop (~8 µl) of 10^8 CFU/ml inoculum was infiltrated to the basal part of the stem. For common poinsettia at the fourth to fifth leaf stage, a drop (~8 µl) of 10^8 CFU/ml inoculum was infiltrated to the axil of the lowest leaf. For tulips, puncture the bulb with a needle dipped in 10^8 CFU/ml inoculum, then transplant into a pot and observe the onset of symptoms after 25 days. As for sugar beet, red beet, bean, and common poinsettia, observe the onset of symptoms at 7, 14, and 21 days after inoculation. Eight to 10 plants of each host were used for each strain. Three leaves were used for each plant. Inoculated plants were placed in the greenhouse at 25 to 30°C. After microscopic examination of the fragments of inoculated sugar beet leaves (taken from the border between healthy and infected tissues), the pathogen was reisolated. The hypersensitivity (HR) test was determined using the method described by Fang (16). Each bacterial suspension (≈10^8 CFU/ml) was infiltrated into fully expanded purple jasmine (*Mirabilis jalapa*) leaves, and the reaction was recorded at 6 to 24 h after infiltration.

Physiological and biochemical characterizations. The morphology and life cycle of sugar beet strains were studied in cultures grown on D2 agar by 3% KOH test (40), and observed under optical and electronic microscopy. Motility was also determined by the hanging drop method (33). The activities of catalase, gelatinase, urease, oxidase, and arginine dihydrolase were determined according to standard methods (24). Oxidase activity was determined using 1% (wt/vol) tetramethyl-*p*-phenylenediamine solution on filter paper disks, as reported by Groth et al. (19). The production of indole, the reduction of nitrate to nitrite, and the hydrolysis of starch, esculin, Tween 80, starch hydrolysis, methyl red, Voges–Proskauer, litmus, and milk tests, sucrose deoxidize, levan production, methods of acid production, H_2S production, casein hydrolysis, acid-fast staining, 5% NaCl tolerance, growth situation at 37°C, growth factor and NH_3 production were measured according to the procedures described in the literature (16,24,43,44). The utilization of 32 different sugars and alcohols, leading to the formation of acid with or without gas production, was monitored according to Hugh and Leifson (25). Eleven different organic acids were used to test the ability of the culture/strain to utilize a carbon compound that was provided as a sole carbon source (13). The basal medium used for carbohydrate utilization tests was used to check the strains for their tolerance to antibiotics and growth inhibitors. All these morphological, physical, and biochemical characterization experiments were repeated in triplicate.

Chemotaxonomic characterizations. Methods used for the analysis of menaquinones were described previously by Collins et al. (6). Elucidation of the peptidoglycan structure was accomplished as described previously (30). Analysis of enantiomeric diamino acid isomer was performed according to the method reported by Sasaki et al. (39). Isolation and analysis of polar lipids were performed according to the method of Minnikin et al. (34). Sugars in whole-cell hydrolysates (1 M HCl, 105°C, 30 min) were analyzed by thin-layer chromatography (22).

DNA base composition and DNA–DNA hybridization. We isolated genomic DNA from thawed T30^T cells resuspended in TE buffer, using the technique of Bradley et al. (2). Genomic DNA G+C base composition was determined by the method of Marmur and Doty (31), replicated twice. The DNA–DNA hybridization was examined for T30^T and *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (ICMP2584) according to the method described by Martin et al. (32).

16S rRNA sequencing and phylogenetic analysis. The 16S rRNA gene of T30^T was amplified by using universal rDNA primers R16F0 and CBR16R1 (28). The amplified 16S rRNA fragment was then isolated from a 1.4% agarose gel followed by purification using the crush-and-soak method, and sequenced with an ABI PRism 377XL DNA sequencer (23,38). The nearly complete 16S rRNA sequence was compared with sequences available from the Ribosomal Database Project and EMBL/GenBank. Similarity values were based on a pairwise comparison of sequences. For phylogenetic analysis, the tree was constructed using the neighbor-joining method (17,37) with DNAMAN (program version 6.0; 45,48). Distance matrices were processed using the same software. Tree topologies were evaluated by bootstrap analysis of the neighbor-joining tree using the 1,000 bootstrap datasets.

Table 1. A list of reference strains and the new strains isolated from sugar beet used in this study

Bacterial names	Strains	Host	Origin
Curtobacterium flaccumfaciens pv. *flaccumfaciens* pv. nov.	2584	Leguminous plants	ICMP[a]
C. flaccumfaciens pv. *poinsettiae*	2566	Common poinsettia	ICMP
C. flaccumfaciens pv. *betae*	2594	Beet (mainly red beet)	ICMP
C. flaccumfaciens pv. *oortii*	2632	Tulip	ICMP
C. flaccumfaciens pv. *beticola*	T30^T	Beet (mainly sugar beet)	Linhe City, Inner Mongolia of China, 1995
C. flaccumfaciens f. pv. *beticola*	T28	Beet (mainly sugar beet)	Linhe City, Inner Mongolia of China, 1996
C. flaccumfaciens pv. *beticola*	T56	Beet (mainly sugar beet)	Linhe City, Inner Mongolia of China, 1998
Clavibacter michiganensis subsp. *michiganensis*	2550	Solanaceous plants	ICMP
C. michiganensis subsp. *insidiosum*	2621	Clover	ICMP
C. michiganensis subsp. *tesselarius*	7221	Wheat	ICMP
C. michiganensis subsp. *sepedonicus*	MH5	Potato	Jiangsu, China
C. michiganensis subsp. *nebraskense*	3298	Maize	ICMP
Leifsonia xyli subsp. *cynodontis*	8790	Bermuda grass	ICMP
Rathayibacter rathayi	2574	Cereal plants	ICMP
R. iranicum	3496	Wheat (Iran)	ICMP
R. tritici	2626	Wheat	ICMP
Rhodococcus fascians	5833	Sweet pea	ICMP
Arthrobacter ilicis	2607	American holly	ICMP

[a] International Collection of Micro-Organisms from Plants, New Zealand.

RAPD analysis. Bacterial DNA was extracted by following a version of the hexadecyltrimethylammonium bromide (CTAB) method (11). Random primers (10 bases of oligonucleotides with 60 or 70% G+C content and random sequence; Table 2), dNTP, *Taq* DNA polymerase, and agarose (Spanish) were purchased from Takara Company. RAPD–polymerase chain reaction (PCR) was carried out in an MJ Research thermocycler (PTC 100). The reaction mixture (25 µl) contained 50 ng of template DNA, 1 U of *Taq* DNA polymerase, 200 µM of each dNTP, 0.64 mM of a single primer, and 2 mM $MgCl_2$ in 10× buffer (10 mM Tris-HCl, pH 8.3; 50 mM KCl). The reaction mixture was heated for 1 min to 94°C and submitted to 30 reaction cycles of 94°C for 30 s, 37°C for 90 s, and 72°C for 90 s. After the final reaction cycle, the mixture was held at 72°C for 5 min and stored at 4°C. Amplified PCR products (4 to 8 µl) were separated by 1.5% agarose gel electrophoresis in 1× Tris-acetate–EDTA buffer (40 mM Tris-acetate, pH 8.0, and 1 mM EDTA) at 4 V/cm for 5 h. Gels were stained with ethidium bromide and photographed under UV light. DNA Ladder Plus (MBI) (100 bp) was used as marker (M) in all gels. RAPD-PCR experiments were repeated at least twice with independently prepared DNA stocks to confirm the banding patterns.

For data analysis, the genetic similarity among strains was measured according to the profiles. Each band with a different electrophoretic mobility was assigned a position number and scored as either 1 or 0 based on the presence or absence of the band, respectively, for this position. Dendrograms were constructed from the similarity coefficient data by the unweighted pair group, with the arithmetic averages clustering (UPGMA) method. The similarity value (*Sxy*) was calculated according to the following equation: [$Sxy = 2Nxy/(Nx + Ny)$], where *Nxy* is the number of bands common to *X* and *Y*, *Nx* is the total number of bands in *X,* and *Ny* is the total number of bands in *Y* (9,41).

RESULTS

Pathogen isolation and pathogenicity test. Strains $T30^T$, T28, and T56 formed yellow, entire margin, convex, butyrous colonies on D2 medium. Cells formed from each of the three strains with clubbed ends were frequently seen. In cultures of more than 5 days, irregular rods predominated and occurred singly, in pairs, or in 'V' form, pleomorphic rod or cococci, without a rod-cococci growth cycle. This observation matches that of previous reports (7,40). Cells of the bacteria were gram positive, motile, with one to three lateral flagella (Fig. 2), non-acid-fast, nonendospore-forming, and noncapsulated.

Sugar beet scraped with $T30^T$, T28, and T56 showed the same symptoms as field observations (Fig. 1B). Red beet scraped with the three strains presented the same symptoms as with sugar beet. One week after infiltration with the three strains, yellow spots occurred on the leaves of sugar and red beets, then became brown and expanded with a chlorotic halo, but there were no silvering symptoms on red beet. Bacterial ooze was observed through microscopic examination, then isolates from the lesions were cultured on D2 medium and identified to the inoculum based on morphological characteristics previously described. Tulip, common poinsettia, and bean displayed no symptoms after inoculation with the three strains. Red beet scraped with ICMP 2594 developed typical silvery lesions, sometimes discrete and sometimes coalescent, the silvering often being most apparent at first along the veins. Three weeks after infiltration with ICMP 2594, veinal silvering was seen on the inoculated red beet, and after a further week this symptom became more intense and identical with those seen in the field (27). Sugar beet, tulip, common poinsettia, and bean displayed no symptoms after inoculation with strain ICMP 2594. HR on purple jasmine leaves occurred for all tested pathogenic *Curtobacterium* strains (HR for $T30^T$ in Figure 3).

Physiological and biochemical characteristics. All of the strains—$T30^T$, T28, and T56—were metabolically versatile, and all could utilize a variety of organic compounds, including sugars, alcohol, organic acid, amino acids, and nucleotides. They differed in the utilization of sorbose, sorbitol, and rhamnose. Only strain T56 could utilize sorbose, strain T28 could utilize sorbitol, while strain $T30^T$ could utilize rhamnose. Levan was produced from sucrose. H_2S could be produced from cystine, cysteine, and $Na_2S_2O_3$. Both potato and soluble starch were decomposed, but not gelatin. Methyl red and Voges–

Table 2. Codes and sequences of the primers

Primer	Sequence	%G+C	Primer	Sequence	%G+C
S301	CTGGGCACGA	70	S366	CACCTTTCCC	60
S302	TTCCGCCACC	70	S367	AGCGAGCAAG	60
S303	TGGCGCAGTG	70	S368	GAACACTGGG	60
S304	CCGCTACCGA	70	S369	CCCTACCGAC	70
S305	CCTTTCCCTC	60	S370	GTGCAACGTG	60
S306	ACGCCAGAGG	70	S371	AATGCCCCAG	60
S307	GAGCGAGGCT	70	S372	TGGCCCTCAC	70
S308	CAGGGGTGGA	70	S373	GGTTGTACCC	60
S309	GGTCTGGTTG	60	S374	CCCGCTACAC	70
S310	CCCTAGACTG	60	S375	CTCCTGCCAA	60
S311	GGAGCCTCAG	70	S376	GAGCGTCGAA	60
S312	TCGCCAGCCA	70	S377	CCCAGCTGTG	70
S313	ACGGGAGCAA	60	S378	CCTAGTCGAG	60
S314	ACAGGTGCTG	60	S379	CACAGGCGGA	70
S315	CAGACAAGCC	60	S380	GTGTCGCCAG	70
S316	CTCTGTTCGG	60	S381	GGCATGACCT	60
S317	GACACGGACC	70	S382	TGGGCGTCAA	60
S318	GACTAGGTGG	60	S383	CCAGCAGCTT	60
S319	TGGCAAGGCA	60	S384	GACTGCACAC	60
S320	CCCAGCTAGA	60	S385	ACGCAGGCAC	70
S361	CATTCGAGCC	60	S386	GAGGGAAGAG	60
S362	GTCTCCGCAA	60	S387	AGGCGGGAAC	70
S363	CCAGCTTAGG	60	S388	AGCAGGTGGA	60
S364	CCGCCCAAAC	70	S389	TGCGAGAGTC	60
S365	TCTGTCGAGG	60	S390	TGGGAGATGG	60

Fig. 2. Scanning electron micrograph of cells from an 18-h cultured strain $T30^T$ grown at 28°C on solid D2 medium (×20,000).

Fig. 3. Hypersensitive reaction of the strain $T30^T$ (BV30 = T30) of *Curtobacterium flaccumfaciens* pv. *beticola* on purple jasmine (*Mirabilis jalapa*).

Proskauer tests were negative. Sucrose was not deoxidized. Nitrate was not reduced. Esculin, Tween 80, and Casein (not T56) were hydrolyzed. Oxidase and urease were negative, whereas catalase was positive. Acid fast staining was negative. Acid could be produced by both oxidation and fermentation. The optimal growth temperature is between 24 and 27°C. In contrast, the bacteria did not grow at 4 or 37°C. The strains could not grow in D2 medium that was amended with NaCl at 10%, but could grow at 5%. Indole production and NH_3 production were negative. In litmus and milk tests, acid and medium peptonization occurred.

Chemotaxonomic characterization. Cell-wall analysis revealed that strain T30^T has a B2β-type peptidoglycan based on D-ornithine, the major isoprenoid quinone MK-9, with MK-8 (H_2) and MK-8 as minor components. The polar lipid pattern of T30^T is composed of PG (phosphatidyl glycerol), DPG (diphosphatidyl glycerol) and several unknown glycolipids. PI (phosphatidylinositol) and PIM (phosphatidylinositol mannose) were not detectable. T30^T contains galactose, glucose, rhamnose, and xylose, but no arabinose, mannose, and ribose in their whole cells.

DNA base composition and DNA–DNA hybridization. The G+C content of T30^T is 67.5%. The above characteristics are typical for the genus *Curtobacterium* (3,6). DNA–DNA homology between strain T30^T and *C. flaccumfaciens* pv. *flaccumfaciens* (ICMP 2584) is 70.1%, which is high enough to reach the threshold value of 70% that is considered for species delineation (17,42,47).

Phylogenetic characterization. Nearly the complete nucleotide sequences of the 16S rRNA of strain T30^T (1,410 nt) were obtained. T30^T had a similarity of 99.9% to *C. flaccumfaciens* pv. *flaccumfaciens*, *C. flaccumfaciens* pv. *betae*, *C. flaccumfaciens* pv. *oortii,* and *C. flaccumfaciens* pv. *poinsettiae* in the DNA sequences of 16S rRNA. T30^T and *C. flaccumfaciens* pv. *flaccumfaciens*, *C. flaccumfaciens* pv. *betae*, *C. flaccumfaciens* pv. *oortii*, and *C. flaccumfaciens* pv. *poinsettiae* were clustered into one group, with a 92% bootstrap confidence level (Fig. 4).

RAPD fingerprinting. To identify primers that generate RAPD patterns characteristic of PPCB, we tested 50 different primers with DNA extracted from five strains (Table 2). Twenty primers (S304, S307, S309, S311, S312, S314, S315, S316, S317, S362, S367, S370, S371, S372, S373, S375, S376, S377, S380, S381) gave a total of 225 RAPD fragments, and the number of fragments scored per primer varied from 6 to 15 (Fig. 5). The sizes of the products ranged from about 0.2 to 3.0 kb. A total of 181 of the total fragments (80.4%) were polymorphic. The primers S316, S370, S371, and S377 amplified two common DNA fragments from *Curtobacterium*. The RAPD profiles that were amplified with primer S315 revealed pathovar-specific DNA fragments, permitting the identification and differentiation of the *C. flaccumfaciens* pathovars. UPGMA analysis revealed genetic diversity among all the strains, and they were divided into seven distinct groups (maximum similarity: approx. 60%; Fig. 6). T30^T, T28, T56, and *C. flaccumfaciens* pv. *flaccumfaciens*, *C. flaccumfaciens* pv. *betae*, *C. flaccumfaciens* pv. *oortii,* and *C. flaccumfaciens* pv. *poinsettiae* were the largest group (minimum similarity value: 0.65). T30^T, T28, and T56 displayed a higher similarity to *C. flaccumfaciens* pv. *flaccumfaciens* and *C. flaccumfaciens* pv. *oortii* (0.70), but a lower similarity to *C. flaccumfaciens* pv. *betae* (0.66). The three strains revealed the highest similarity (0.89). These results suggested that T30^T should be assigned to a novel pathovar below species *C. flaccumfaciens*.

DISCUSSION

This is the first report of bacterial leaf spot disease on sugar beet in Linhe City, Inner Mongolia autonomous region, China. Sugar beet is an important sugar plant, as well as an oil plant crop, in China and has economic importance. The disease, which is caused by *C. flaccumfaciens* pv. *beticola* pv. nov., has been a serious obstacle to the culture of sugar beet. In fields that are widely affected by the disease, there are sometimes total losses in sugar beet. In pathogenicity tests, the pathogen is also pathogenic to red beet, but in the field we don't find the disease (S. P. Liu, *personal communication*).

Genus *Curtobacterium* embraces six species (1), but only the species *C. flaccumfaciens* includes plant-pathogenic strains that are allocated to various pathovars (3,5,10). The results of G+C content of T30^T (67.5%) and DNA–DNA homology between T30^T and *C. flaccumfaciens* pv. *flaccumfaciens* (70.1%), as well as the results of comparing the 16S rRNA gene sequences, are powerful evidence that T30^T should be classified with species *C. flaccumfaciens*. The 16S rRNA gene sequence of T30^T showed a high similarity (99.9%) to four existing pathovars. This result also indicates high similarity among these pathovars.

Pathovar classification and nomenclature is based on the capacity of plant pathogenic bacteria to cause distinctive symptoms or by reference to their proved host range (12,14,50,52). Compared with silvering disease of red beet, the symptoms of brown leaf spot without representative white veins caused by T30^T are conspicuously different. Moreover, T30^T mainly damages sugar beet, whereas *C. flaccumfaciens* pv. *betae* often infects red and feeding-stuff beets (27; S. P. Liu, *personal communication*). In applying the RAPD method, we distinguished each of five independent pathovars using just a single 10-nt primer (S315), and found that strains of T30^T, T28, and T56 exhibited 70% homology with the *C. flaccumfaciens* pv. *oortii* strain. Based on the differences in pathogenicity and their genetic relationships, it is necessary to allocate T30^T to a novel pathovar, *C. flaccumfaciens* pv. *beticola*. Additionally, morphological and physiological properties are both appropriate for this classification. Colonies of T30^T are more yellow and viscous on D2 medium than those of *C. flaccumfaciens* pv. *betae* ICMP 2594. T30^T can also be differentiated from *C. flaccumfaciens* pv. *betae* ICMP 2594 by acid formation from starch, utilization of acetate, H_2S production, casein hydrolysis, acid production, and litmus deoxidation. The host plant of *C. flaccumfaciens* pv. *poinsettiae* is common poinsettia (36), whereas that of T30^T is sugar beet, so we did not regard this strain as pv. *poinsettiae*, although physiological analysis of phenotypes significantly demonstrates that T30^T was highly similar to *C. flaccumfaciens* pv. *poinsettiae*. Recently, Young et al. (53) proposed that the authentic pathogen of American holly became a novel pathovar, *Curtobacterium flaccumfaciens* pv. *ilicis*, and *Arthrobacter ilicis* ICMP 2608 proved pathogenic to *Ilex opaca*, which is designated as the pathotype strain. In our research, *A. ilicis* ICMP 2607 is in a phenon that is separate from all species that all other PPCB belonged to, agreeing with the results of Young and Fletcher (51) that ICMP 2607 is not a typical strain of *C. flaccumfaciens* pv. *ilicis*. This result is in accordance with that of Dye et al. (12).

In many approaches to the taxonomy of plant pathogenic bacteria, analyses of chemotaxonomic and 16S rRNA sequence are a good choice (21,29). Chemotaxonomic methods, such as the determination of major isoprenoid quinones, fatty-acid analysis and polar lipid patterns, play an important role in the taxonomy of a genus and species. In our study, the chemotaxonomic properties of strain T30^T correspond with those of the genus *Curtobacterium*. The diamino acid ornithine (Orn) is found in the peptidoglycan, which was of the B2β type with acetyl residues, and the major respiratory menaquinone is MK-9. 16S rRNA similarity reveals phylogenetic and evolutionary relationships among bacteria. More available data and development of software accelerate the application of the method. Meanwhile, this is necessary to respect the need for a reasonable degree of genetic stability. High 16S rRNA conformity cannot differentiate pathovars or strains (1). Comparison of FT-IR spectra (35), Biolog identification system, and methods based on genome fingerprinting (20) all prove to be useful for distinguishing pathovars. In conclusion, there is no phylogenetic standard for species, genus,

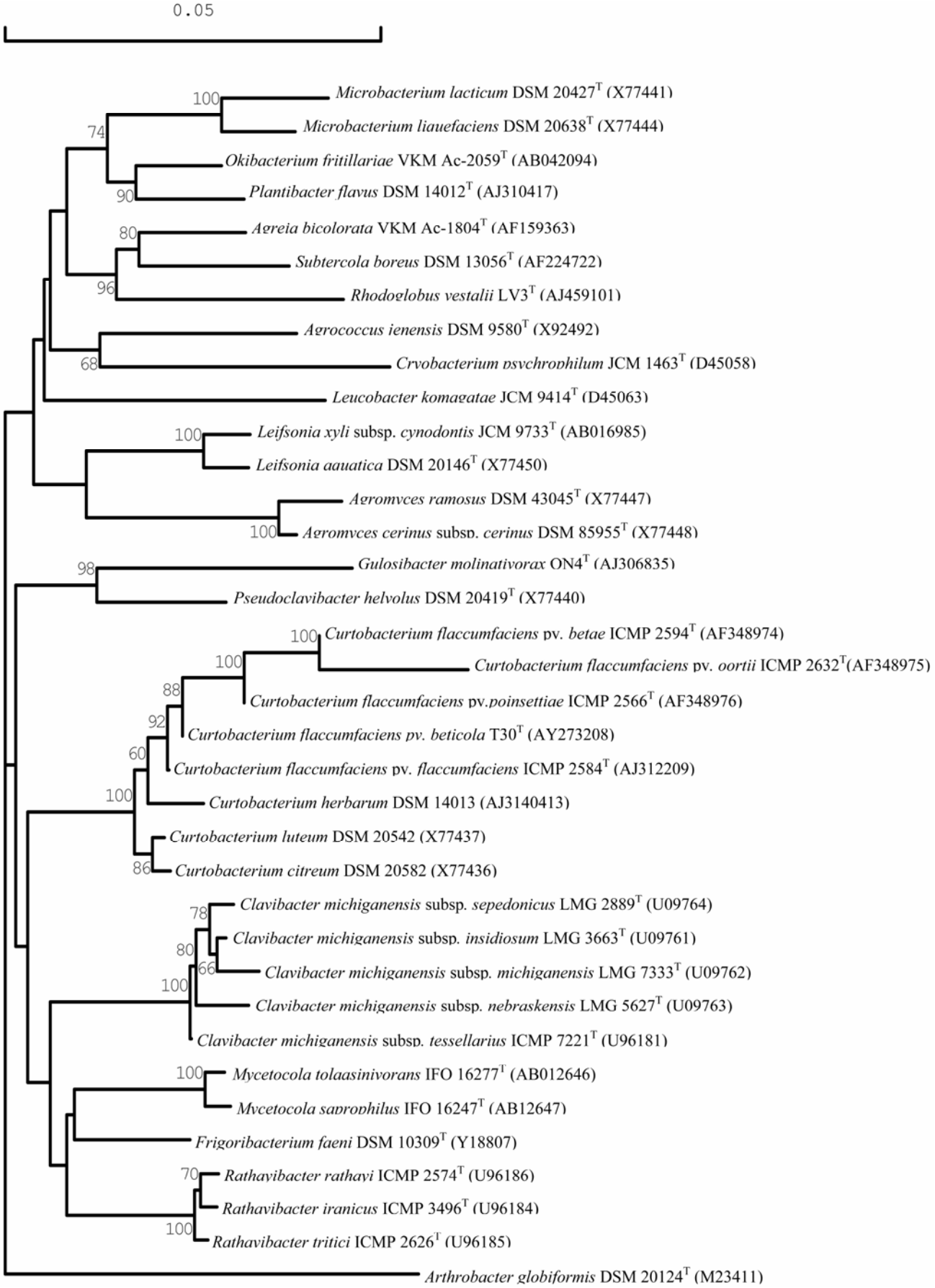

Fig. 4. A phylogenetic tree, based on the neighbor-joining method, derived from an alignment comprising 16S rRNA sequences from phylogenetically related *Curtobacterium* spp., and genera within the family *Microbacteriaceae*. The 16S rRNA sequence of *Arthrobacter globiformis* DSM 20124^T was used as an outgroup.

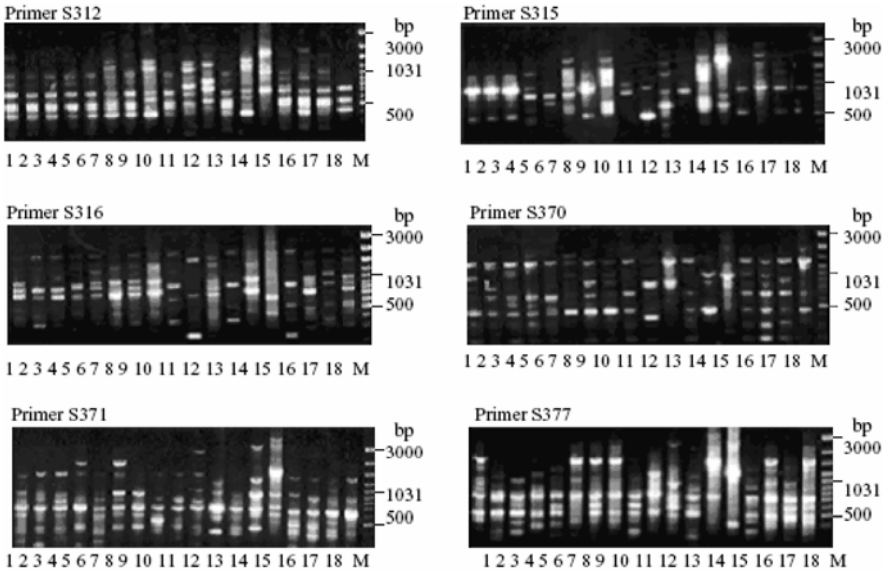

Fig. 5. Random amplified polymorphic DNA (RAPD) patterns of plant pathogenic coryneform bacteria (PPCB) strains amplified with several representative primers. Strains 1, *Curtobacterium flaccumfaciens* pv. *beticola* T28; 2, *C. flaccumfaciens* pv. *beticola* T30^T; 3, *C. flaccumfaciens* pv. *beticola* T56; 4, *C. flaccumfaciens* pv. *poinsettiae* ICMP 2566; 5, *C. flaccumfaciens* pv. *flaccumfaciens* ICMP 2584; 6, *C. flaccumfaciens* pv. *betae* ICMP 2594; 7, *C. flaccumfaciens* pv. *oortii* ICMP 2632; 8, *Clavibacter michiganense* subsp. *michiganense* ICMP 2550; 9, *C. michiganense* subsp. *tessellarius* ICMP 7221; 10, *C. michiganense* subsp. *insidiosus* ICMP 2621; 11, *C. michiganense* subsp. *sepedonicus* MH5; 12, *C. michiganense* subsp. *nebraskensis* ICMP 3298; 13, *Rathybacter rathayi* ICMP2574; 14, *R. tritici* ICMP 2626; 15, *R. iranicum* ICMP 3496; 16, *Leifsonia xyli* subsp. *cynodontis* ICMP 8790; 17, *Rhodococcus fascians* ICMP 5833; 18, *Arthrobacter ilicis* ICMP 2607.

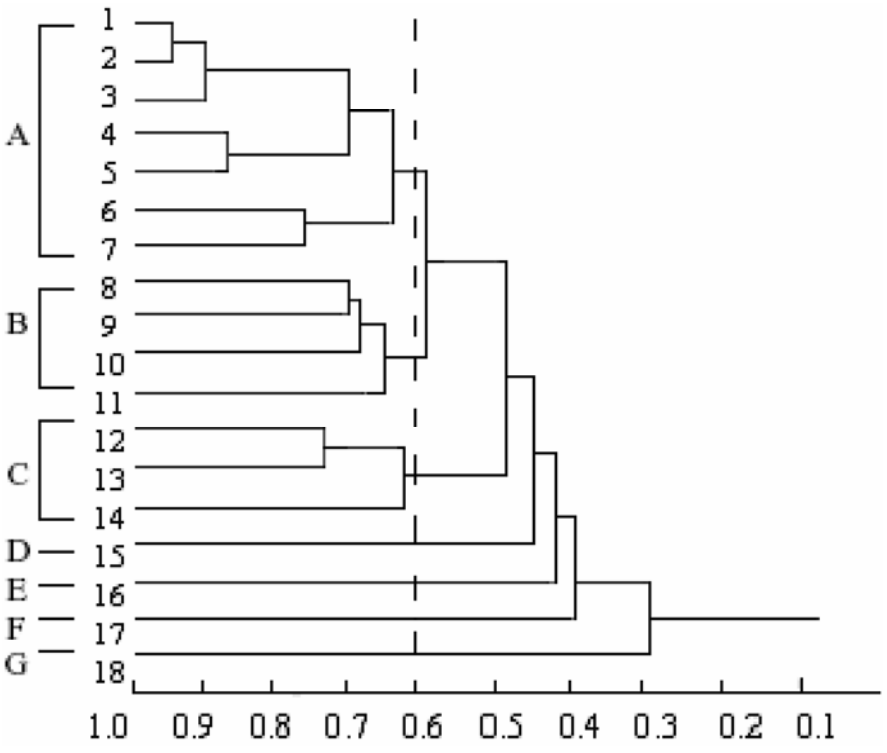

Fig. 6. Cluster dendrogram of random amplified polymorphic DNA (RAPD) in 18 plant pathogenic coryneform bacteria (PPCB) strains. Strain 1, *Curtobacterium flaccumfaciens* pv. *beticola* T56; 2, *C. flaccumfaciens* pv. *beticola* T30^T; 3, *C. flaccumfaciens* pv. *beticola* T28; 4, *C. flaccumfaciens* pv. *flaccumfaciens* ICMP 2584; 5, *C. flaccumfaciens* pv. *oortii* ICMP 2632; 6, *C. flaccumfaciens* pv. *poinsettiae* ICMP 2566; 7, *C. flaccumfaciens* pv. *betae* ICMP 2594; 8, *Clavibacter michiganense* subsp. *tessellarius* ICMP 7221; 9, *Rathybacter iranicum* ICMP 3496; 10, *C. michiganense* subsp. *nebraskensis* ICMP 3298; 11, *Rhodococcus fascians* ICMP 5833; 12, *Clavibacter michiganense* subsp. *michiganense* ICMP 2550; 13, *Rathybacter rathayi* ICMP2574; 14, *Leifsonia xyli* subsp. *cynodontis* ICMP 8790; 15, *Arthrobacter ilicis* ICMP 2607; 16, *C. michiganense* subsp. *insidiosus* ICMP 2621; 17, *C. michiganense* subsp. *sepedonicus* MH5; 18, *Rathybacter tritici* ICMP 2626.

or family delineation. Thus, a polyphasic approach is valuable (46).

Summarizing the results of this study and previous characterizations of the genus *Curtobacterium*, the bacterium isolated from leaves of diseased sugar beet reveals a high similarity to *C. flaccumfaciens* and obvious differences from other pathovars in pathogenicity. A distinct pathovar, *C. flaccumfaciens* pv. *beticola* pv. nov., is proposed to accommodate the new pathogen.

DESCRIPTION

Curtobacterium flaccumfaciens pv. *beticola* (be.ti´co.la. L. n. *beta* beet; L. subsp. *cola* dweller; M.L. n. *beticola* beetdweller) causes bacterial leaf spot of sugar beet and was first found in 1995 in Inner Mongolia of China.

A morphological and physiological description of the pathovar is based on type strain T30^T. Cells are gram positive, strictly aerobic, non-spore-forming, noncapsulated, motile, irregularly shaped rods that sometimes form V-shapes. Colonies are pale-yellow, entire margin, convex, and butyrous. Oxidase, urease, Methyl red, and Voges–Proskauer reactions are negative, whereas catalase is positive. Aesculin, casein, Tween 80, and starch are hydrolyzed, but gelatin is not. Levan is produced from sucrose. H_2S can be produced from cystine, cysteine, and $Na_2S_2O_3$. Sucrose is not deoxidized. Nitrate is not reduced. Acid fast staining is negative. Strains produce acid from the oxidative fermentation of adonitol, cellobiose, dextrin, erythritol, fructose, galactose, glucose, glycerol, inositol, inulin, maltose, mannitol, mannose, melibiose, salicin, sucrose, rhamnose, trehalose, xylose, and α-methyl-D-glucoside. Acid is not produced from dulcitol, ethanol, methanol, ribose, sorbitol, sorbose, or starch. Strains produce acid weakly from raffinose, lactose, arabinose, arbitol, and hepatin. Acetate, barbiturate, benzoate, butyrate, citrate, formate, glutamate, malonate, pectate, succinate, and tartrate are utilized. The optimum growth temperature is 24 to 27°C. No growth was observed at 4°C, 37°C, or in the presence of 10% NaCl, although there is some growth in the presence of 5% NaCl. Growth factor is needed. Indole production and NH_3 production were negative. In litmus and milk tests, acid and medium peptonization occurred. The diagnostic diamino acid of the peptidoglycan is D-Orn; peptidoglycan is of the B2β type with acetyl residues as described for the genus. The major menaquinone is MK-9. The predominant polar lipids are PG (phosphatidyl glycerol), DPG (diphosphatidyl glycerol), and several unknown glycolipids. DNA G+C composition for the type strain is 67.5 mol %. Type strain T30^T has been deposited in ATTC, USA (ATTC- BAA-144^T). The EMBL accession number for the 16S rRNA gene sequence is AY273208.

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