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Enterobacter spp.: A new evidence causing bacterial wilt on mulberry

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Thirty-six pathogenetic bacterial strains were isolated from wilted mulberry plants in Hangzhou, Zhejiang province of China. The six representative strains were confirmed to be involved in more than one *Enterobacter* species by common bacteriological test, electron microscope observation, hypersensitive reaction, Koch's postulates, physiological and biochemical test, biolog, fatty acid methyl esters analysis (FAMEs), enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR), 16s rRNA sequences analysis, and comparative analysis with 7 type strains and 3 reference strains. This is the first report on mulberry disease caused by *Enterobacter* spp. in the world providing new evidence on induction of the plant disease in this genus. The results are not only important in the mulberry disease management but also have significant scientific value for further studies of opportunistic human pathogens and environmental strains in *Enterobacter*.

mulberry enterobacter wilting, *Enterobacter*, phenotype identification, ERIC-PCR, 16S rRNA

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Twenty-one species and two subspecies have been reported in genus *Enterobacer* previously [1], which are less in numbers as compared to *Salmonella typhi* [2] causing typhoid in human and *Erwinia* [3] inducing severe losses in economic crops. In the past 10 years people started to pay more and more attention on *Enterobacter* species which are thought to be important new opportunistic human pathogens and potential dangerous food pathogens [4,5]. However, fewer studies have been done on plant pathogens of *Enterobacter,* among which five species were reported to cause plant diseases [6,7]. No record of *Enterobacter* spp. involved in mulberry disease in the world although more than 100 diseases were reported before.

China is the largest country in silkworm and mulberry production, gaining huge amounts of foreign exchange by exporting the silk annually. Mulberry trees are the nutrition source for silkworms producing silk, therefore epidemiology of some mulberry diseases was concerned in China. In the summer of 2006, a severe outbreak of a foliar decline disease was noted in mulberry orchards of Hangzhou city in Zhejiang, China. The disease caused severe wilt, especially on 1- or 2-year-old mulberry plants, that resulted in premature plant death. Leaf wilt symptoms generally started on older leaves at the bottom of the plant and spread to the younger leaves. The leaves of infected plants became withered and dry, turned dark brown, and eventually the plants became defoliated. The root xylem of infected plants was moist and discolored with brown stripes. The phloem was asymptomatic, but in severe infections the phloem was de-

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cayed. It was diagnosed as plant bacterial disease and found that a majority of the wilt plants surveyed were not caused by *R. solanacearum*. Eight bacterial diseases of mulberry have been reported to be caused by different species in six genuses (*Erwinia*, *Ralstonia*, *Pseudomonas, Phytoplasm*, *Xanthomonas* and *Xylella*) [8–10]. However, no mulberry bacterial disease associated with *Enterobacter* was reported so far. The observation of wilting proceeding from the bottom of the plant to the top distinguishes this disease from bacterial wilt caused by *Ralstonia solanacearum*.

The objective of this study is to determine causal agent of the mulberry wilt diseaseas (MWD) because an accurate identification is a key point for the disease management.

1 Materials and methods

1.1 Type strains

Seven type strains of *Enterobacter* associated with plant (*Enterobacter cloacae* subsp**.** *dissolvens* LMG2683^T isolated from poplar, *E. cancerogenus* LMG2693^T isolated from maize, *E. pyrinus* LMG22970^T isolated from pear, *E. amnigenus* LMG2784^T isolated from soil, *E. turicensis* LMG23730^T isolated from fruit powder, *E. helveticus* LMG23732^T isolated from fruit powder, *E. nimipressuralis* LMG10245^T isolated from elm) and two reference strains (*E. agglomerans* LMG2557 isolated from *Pyrus communis*, *R. solanacearum* LMG 2299^T isolated from *Lycopersicon esculentum*) were provided by Prof. J Swing from the Belgian Co-ordinated Collection of Microorganisms, LMG Bacteria Collection, University of Ghent, Belgium. The reference strain of mulberry wilt, *R. solanacearum* ZJUM 19981 was offered by the Institute of Biotechnology, Zhejiang University.

1.2 Bacterial isolation

Twenty-two MWD samples were collected from the mulberry orchards. The phloem of fine root of each sample was washed by tap water, and disinfected by dipping in 70% ethanol for one min. Brown xylem surfaces of MWD were washed 3 times and macerated in sterile distilled water (SDW) for about 10 min after stripping away the phloem of the root. One loop of each suspension was streaked on the surface of plates containing NA or KMB medium and incubated at 30°C for 48 h. After observation under 365 nm UV light, representative colonies on the medium were picked and prepared in TZC (triphenyl tetrazolium chloride) medium for further differentiation [11].

1.3 Common bacteriological, pathogenecity and hypersensitive reaction tests

Colony morphology, cultural characteristics and hypersensitive reaction tests followed Klement's description [12]. The procedures of physiological and biochemical tests, and pathogenecity tests *in vitro* were referred to Schaad *et al*. [13] and Xu Lihui *et al*. [14], respectively. The cultivar of mulberry seedlings used for pathogenecity is the local variety Husang. After adjusting the concentration to 10^8 CFU/mL, 0.02 mL of inoculums were injected into the axils of the plant stem 10 cm above the soil. The development of the symptoms was recorded 10 days after inoculation. The same bacterial strains were re-isolated following Koch's postulation.

1.4 Electron microscopic observation

The pathogenic bacteria cultured for 48 h were made into a bacterial suspension with sterile water and filter filtrated, a few drops of fixative (pH 7.2, 0.15% glutaraldehyde in phosphate buffer) were added, and then centrifuged to collect cells. A few drops of fresh glutaraldehyde were added, and fixed in the refrigerator at 4°C overnight, with centrifugation again to collect cells, and then the bacterium concentration was adjusted to 10^8 CFU/mL. The final suspension with an equivalent amount of 2% osmium acid was used as a staining solution. It was sucked with sterile capillary suction and dropped on a copper omentum. Free water was removed with the help of a filter paper 4 min later, and the sample was observed using electron microscopy (TEM, KYKY-1000B, Japan) after it dried [13].

1.5 Biolog identification

The representative strains selected were tested for biochemical utilization by Biolog GN microplates containing 95 carbon source. Bacterial suspension of 150 μL (A = 0.3) was added to each well. The reaction results were determined by a Biolog reader 24 h after incubation at 30°C and the Biolog specific identification procedures (version 4.1) directly entered. The operations, in detail, were referred to Xie *et al.* [15].

1.6 FAMEs identification

FAMEs test was followed according to instructions of the microbial identification system from MIDI Company USA (MIS operating manual Nov. 2005)with Agilent 6890 Gas Chromatography System. All of the purified strains tested were cultured at NA medium, at 30°C for 24 h, and transferred to TSBA solid medium to culture for 24 h again. One loop of the culture was harvested from each strain to extract fatty acids. The identification results were obtained through the Microbial Identification System software (MIS6.0) and LGS6. 0 (library generation software) and compared with fatty acid information of standard strains in the database (MIDI Inc., Newark, DE, USA).

1.7 16S rRNA sequence analysis

The bacterial DNA was extracted using a modified tech-

nique from Harold Hoffmann *et al*. [16]. Nearly complete 16S rRNA genes were amplified by PCR using a forward primer P0: 5′-AGA GTT TGA TCA TGG CTC AG-3′, and a reverse primer P6: 5′- ACG GTT ACC TTG TTA CGA CTT-3′ [17], amplification followed the Programmable Temperature Cycler (PTC-200, MJ Research). The primer synthesis and the product sequencing were completed by Shenergy-Biocolor, Shanghai, China. The results were deposited in Genbank and the accession numbers were EU430750 to EU430755. These six sequences were compared to those of known *Enterobacter* spp. which were downloaded from the ribosomal RNA database [18] (http://rdp.cme.msu.edu/).

1.8 ERIC-PCR identification

The ERIC-PCR procedures were referred to Hoffmann and Fernandez-Baca's description [16,19] with the primers ERIC-1: 5′-ATG TAAGCTCCTGGGGATTCAC-3′ and ERIC-2: 5′- AAGTAAGTG ACTGGGGTGAGCG-3′. Each reaction included 30 ng of template DNA in a volume of 30 μL containing 500 pM primers, 200 $\mu molL^{-1}$ dNTP mixture, 10 $mmolL^{-1}$ Tris-Cl [pH 8.3], 5 $mmolL^{-1}$ KCl, 1.5 $mmolL^{-1}$ $MgCl_2$ and 2.5 U of *Taq* polymerase. The amplification program was performed with the following conditions: 10 min at 95°C , 35 cycles of 1 min at 95°C, 1 min at 45°C and 1 min at 72°C, and final extension at 72°C for 15 min. The PCR products were visualized in 3.0% agarose gels after electrophoresis and GoldenView staining.

1.9 Phylogenetic analysis

Nucleotide sequences were aligned by CLUSTAL X [20] and edited by BIOEDIT [21]. Phylogenetic trees were generated by MEGA4.0 [22], with Neighbor-joining [23] parameter estimation by 1000 bootstraps. Maximum composite likelihood method was used as the composition model [24].

2 Results

2.1 Cell morphology and cultural characteristics of the pathogenic bacteria

Six representatives were selected out of 36 pathogenic bacterial strains isolated from the wilted mulberry plants for cell morphology and cultural characterization. All the strains tested were gram negative, short-rod shaped, 0.3–1.0 μm wide and 1.0–3.0 μm long, with peritrichous flagella under the SEM (Figure 1).

All six representative strains showed no green fluorescent diffusible pigment on King's medium B. They were facultative anaerobic with no endospore production. On TZC media colonies were white pink at the initial stage and turned into translucent dark pink, non-mucoid colonies with regular edges and translucent margins later. They differed from those of *R. solanacearum* LMG 2299^T and ZJUM19981 which were strictly anaerobic, fluidial and white colonies with a slight pink color in the center and non-regular edges on TZC.

2.2 The key physiological and biochemical analysis of the pathogenic bacteria

The results of key physiological and biochemical tests of six representative MWD strains and three reference strains were shown in Table 1. The six strains were significantly-different from *R. solanacearum* $LMG2299^T$ in their utilization of the carbon source; however, they were similar to *Enterobacter* $LMG2693^T$ and $LMG2683^T$ except for D-melibiose, D-raffinose, D-sorbite, and thymidine. They were remarkably different from *R. solanacearum* LMG 2299^T in voges-proskauer, methylred, indole, gelatin liquefaction, arginine dextrose and ornithine decarboxylase reactions. The results of six MWD strains were identical to those of the two *Enterobacter* reference strains.

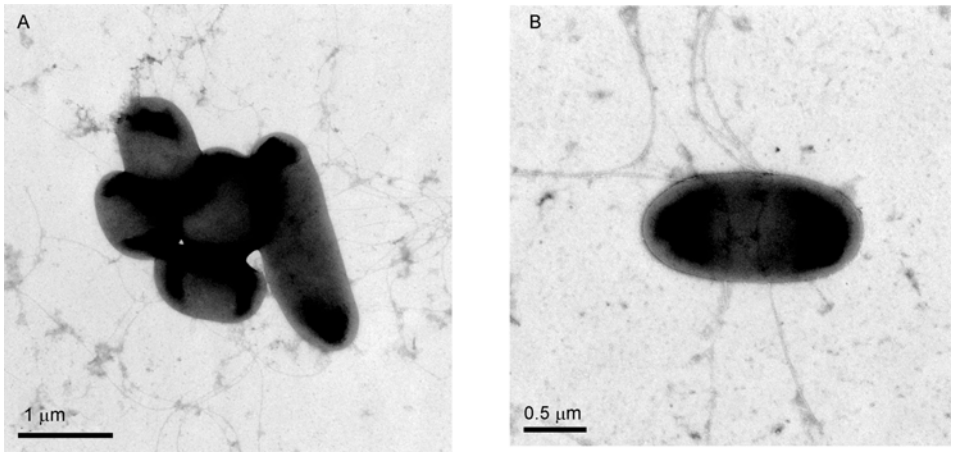

Figure 1 Electron microscopic photograph of *Enterobacter* sp. strain ZJUPD2 showing rod-shaped cell with peritrichous flagella. (A is the cellular individuals, B is the cell individual).

2.3 Hypersensitive reaction (HR) and pathogenicity tests of the pathogenic bacteria

Thirty-six strains out of 120 single bacterial colonies isolated from 22 MWD samples were obtained after HR and *in vitro* pathogenecity tests. Six representative strains selected and four reference strains (*E. cancerogenus* LMG2693^T, *E. cloacae* subsp**.** *dissolvens* LMG2683^T, *R. solanacearum* LMG 2299^T and ZJUM19981) were used for further tests. Bacterial suspensions were adjusted to 10^8 CFU/mL and injected into the tobacco leaves. The six strains and two *Enterobacter* reference strains showed weak HR with slight yellow zone appearing at the inoculation sites 48 h after injection, while *R.solanacearum* LMG 2299^T and ZJUM 19981 displayed normal HR with typical chlorotic halo 24 hours after injection. The results indicated that the strains of *Enterobacter* spp. were weak positive HR in non-host interaction while most of the plant pathogenic bacteria are positive. The field symptoms of MWD initiated at the margin of mulberry leaves from older ones at the bottom of the plant. The leaves curled inward from the edge like coke and quickly became withered (Figure 2). The six representative strains showed typical symptoms 12–13 days after inoculation similar to those in field. The initial symptoms of mulberry seedlings seemed to be wilt due to water deficiency, and recovered after watering. The defoliation started two to three days after wilt symptom appearance (Figure 3). The symptoms still continued to develop when the temperature was higher than 30°C. The symptoms initiated 7 to 10 days after inoculation of mul- berry cuttings *in vitro*. The leaves of the cutting turned yellow, or discolored, wilted leaves fell off and some shoot tops showed necrosis (Figure 4). Finally, all of the tested shoots died. When *R. solanacearum* ZJ M19981 and LMG 2299^T were inoculated onto the mulberry seedlings, the disease symptoms develop- ed slowly and the wilted leaves did not show defoliation. According to the symptoms described by Lai *et al*. [25], the field symptoms caused by *R. solanacearum* were wilting, leaves losing their shiny luster and no defoliation. The susceptible variety of older mulberry trees infected by *R. solanacearum* may have defoliation but the process was very slow.

2.4 Biolog identification of the pathogenic bacteria

Biolog results of the six representative MWD strains showed that they belong to *Enterobacter* genus (Table 2). ZJUPD1-5 were identified as *E. cloacae* with Biolog similarity index ranging from 0.555 to 0.844 and similar to that of *E. cloacae* subsp. *dissolvens* LMG2683^T.

ZJUPD6 was identified as *E. cancerogenus*. with the similarity index of 0.621 and similar to that of *E.cancerogenus* LMG2693^T. The Biolog results of two *R.Solanacearum* reference strains were identical to previous identification.

2.5 FAMEs identification of the pathogenic bacteria

The FAMEs identification analysis was referred to Buyer's description [26] : If the FAMEs similarity index <0.2, the identification was ignored. It could be identified to the genus level if the similarity ⩾0.5. FAMEs results of ZJUPD1-ZJUPD6 strains were also classified into the *Enterobacter* genus, similar to Biolog results. All of the 6 strains were identified as *E.cancerogenus* with FAME similarity index ranging from 0.593 to 0.839 (Table 3). Two *R.solanacearum* reference strains were identical to previous identification.

2.6 ERIC-PCR analysis of the pathogenic bacteria

ERIC-PCR is a powerful tool suitable for identification and classification of *Enterobacter* spp [19]. TREECON software [27] was used to analyze the fingerprinting results (Figure 5) in this study. The genetic distance matrix suggested that 5 MWD strains were obviously different from 7 *Enterobacter* type strains whereas MWD strain ZJUPD1 was similar to

Table 1 Comparison of the results of 6 MWD strains and three type strains for key physiological and biochemical tests.

Items for test	Reference strain [a)]			Six representative MWD strains					
	1	2	3	4	5	6	7	8	9
Voges-proskauer	+[b)]	+	~	+	+	+	+	+	+
Methylred	–	–	–	–	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–
Gelatin liquefaction	+	+	–	+	+	+	+	+	+
Arginine dihydrolase	+	+	–	+	+	+	+	+	+
Urease	w	w	+	w	w	w	w	w	w
Catalase	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	–	+	+	+	+	+	+
Deoxyribonuclease	+	+	+	+	+	+	+	+	+

a) 1-3 : LMG2693^T, LMG2683^T and LMG2299^T; 4-6: ZJUPD1-6. b) +: positive; – : negative; W: weak positive.

Figure 2 The wilt symptoms in the field

Figure 3 The symptoms on the seeding plant by inoculation of *Enterobacter* sp. ZJUPD2 (The left plant is control).

E. agglomerans LMG2557 (Table 4). The closest genetic distance also had a value of 35.29 between ZJUPD4 and *E. helveticus* LMG23732^T while further studies comfirmed that they were never reported as plant pathogenic bacteria on *Enterobacter*.

2.7 16S rRNA sequence and phylogenetic analysis of the pathogenic bacteria

The partial 16s rRNA gene sequences of MWD strains ZJUPD1-ZJUPD6 were obtained and submitted to GenBank. The sequences from different *Enterobacter* species were downloaded from RDP standard database, and BLAST [28] procedure was used for the local search of the 6 strains. Based on Carol Iversen's criteria for species classification of *Enterobacter* unless the sequences are >1300 bp and homology between their 16S rRNA sequences is >98.7%, the two strains could be classified into the same species [17]. BLAST analyses showed that 16S rDNA sequences of these 6 MWD strains yielded 97% to 98% similarity with 16 enterobacter species associated with plant-environment which indicates the possibility of new species. Phylogenetic trees (Figure 6) revealed that ZJUPD6 with *E. asburiae*, ZJUPD1, ZJUPD3 with *E. agglomerans* and ZJUPD2, 4, 5 with *E. cancerogenus* were located into the same group, respectively. It suggested that there might be more than one new enterobacter species involved in these strains.

3 Discussion

Six representative pathogenetic strains isolated from wilted mulberry plants were confirmed as bacterial pathogens of *Enterobacter* species in our present study by common bacteriological, physiological and biochemical tests, electron microscope observation, pathogenicity, Koch's postulate test, Biolog, FAMEs analysis, ERIC-PCR, 16S rRNA sequence analysis and comparative analysis with 10 standard reference strains. This is not only the first report of

Figure 4 The symptoms on mulberry cuttings by *in vitro* inoculation of *Enterobacter* sp. ZJUPD2 (Left 1 was inoculated by sterile water).

Table 2 Biolog identity of the 6 MWD strains and 3 type strains

Strain	Genbank No.	Host	Former identification	Similarity	Biolog result
ZJUPD1	EU430750	Mulberry	–	0.555	*E.cloacae*
ZJUPD2	EU430751	Mulberry	–	0.536	*E.cloacae*
ZJUPD3	EU430752	Mulberry	–	0.620	*E.cloacae*
ZJUPD4	EU430753	Mulberry	–	0.574	*E.cloacae*
ZJUPD5	EU430754	Mulberry	–	0.844	*E.cloacae*
ZJUPD6	EU430755	Mulberry	–	0.621	*E.cancerogenus*
LMG2693		Poplar	*E.cancerogenus*	0.673	*E.cancerogenus*
LMG2683		Corn	*E. dissolvens*	0.662	*E.cloacae*
LMG2299		Mulberry	*R. solanacearum*	0.682	*R. solanacearum*

Table 3 The FAME identity of the 6 MWD strains and three type strains

Strain code	Genbank No.	Host	Original identification	Similarity	FEMEs results
ZJUPD1	EU430750	Mulberry	–	0.593	*E.cancerogenus*
ZJUPD2	EU430751	Mulberry	–	0.692	*E.cancerogenus*
ZJUPD3	EU430752	Mulberry	–	0.644	*E.cancerogenus*
ZJUPD4	EU430753	Mulberry	–	0.639	*E.cancerogenus*
ZJUPD5	EU430754	Mulberry	–	0.710	*E.cancerogenus*
ZJUPD6	EU430755	Mulberry	–	0.839	*E.cancerogenus*
LMG2693	–	Poplar	*E. cancerogenus*	0.740	*E.cancerogenus*
LMG2683	–	Corn	*E. dissolvens*	0.644	*E.cloacae*
LMG22299	–	Mulberry	*R. solanacearum*	0.702	*R. solanacearum*

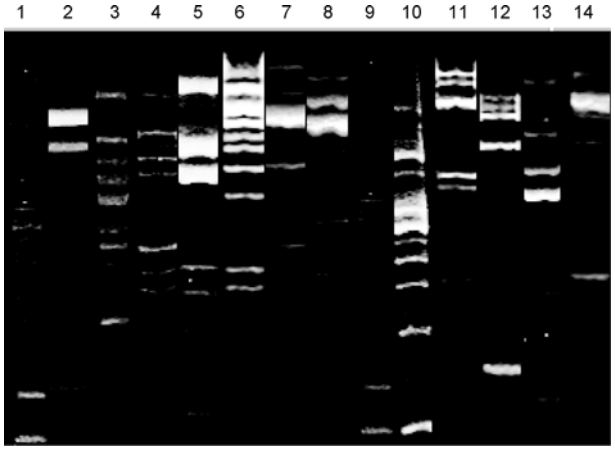

Figure 5 ERIC-PCR fingerprinting results of 14 *Enterobacter* Strains (1-8: *E. agglomerans* LMG2557, *E. amnigenus* LMG2784^T, *E. cancerogenus* LMG2693^T, *E.cloacae* subsp. *dissolvens* LMG2683^T, *E. helveticus* LMG23732^T, *E. nimipressuralis* LMG10245^T, *E. pyrinus* LMG22970^T, *E. turicensis* LMG23730^T; 9-14: ZJUPD1-ZJUPD6).

mulberry (*Morus alba*) bacterial wilt caused by *Enterobacter* species in China but also the first incidence to isolate and validate a new bacterial disease of mulberry associated with *Enterobacter* spp. in the world. ZJUPD6 was identified as *E. cancerogenus* by Biolog and FAMEs analysis. However, by comparing to the type strain *E. cancerogenus* we noted that LMG2693^T could infect poplar only and not mulberry while ZJUPD6 caused severe infection on mulberry plants. The homology of 16S rRNA sequences between ZJUPD6 and *E. cancerogenus* in RDP standard database was below 98%. The other 5 MWD strains were also significantly different from ZJUPD6 in Biolog, FAMEs, ERIC-PCR and 16s rRNA analysis. It indicated that the causative organism of mulberry wilt might involve more than one species in *Enterobacter*. A few reports about *Enterobacter* spp. causing plant disease were available. Until now, only five pathogenetic species associated with plants were reported (*E. cancerogenus*, *E. cloacae* subsp. *Dissolvens*, *E. nimipressuralis*, *E. pyrinus* and *E. cloacae* which infect poplar, maize, elm, pear tree and stone fruit, respectively). In this study we found that the bacteria of *Enterobacter* were more easily adapted to the niche of woody plants. When the two reference *Enterobacter* strains *E. cancerogenus* LMG2693^T and *E. cloacae* subsp**.** *dissolvens* LMG2683^T which caused disease on poplar and maize, respectively, were inoculated on mulberry seedlings they did not induce any disease. It indicated the narrow host range of these bacteria as Saddler reported [6,29]. *Enterobacter* spp. are both our foes and friends like *Burkholderia cepacia* complex [30]. As foes they are both notorious plant patho-

gens and human opportunistic pathogens [31]. As friends they are important plant engineering and plant growth-promoting bacteria, and serve as biopesticides which play an important role in biocontrol [32–34]. In the present study six MWD strains were identified as *Enterobacter* spp. by phenotypic and genotypic determination. As mentioned above 21 species and two subspecies have been reported in Enterobacer genus, most of which are related to animals and environments. We collected the 8 species related to plants and confirmed the genetic distances with the six MWD strains, but it is still hard to define the accurate scientific name of species causing mulberry wilt because of difficult access for hybridization with other type strains.

In recent bacteriological studies people are more focused on bacteria in the family Enterobacteriaceae which includes the first bacterium whose genome was sequenced. By now, the genomes of many strains of *Enterobacteriaceae* have been sequenced or are being sequenced, including the model bacterium, *Escherichia coli*, animal-pathogen interaction bacteria (*Salmonella* spp., *Yersinia* spp., *Shigella* spp.) and plant-pathogen bacteria (*Erwinia* spp.) [35]. With the development of the sequencing technology and increase of the genome data, comparative genome analysis becomes more popular. More and more microbiologists are focusing on the bacteria that are serious pathogens and can also survive in different environments. Most bacteria in *Enterobacteriaceae* have unique hosts (*Salmonella* spp., *Yersinia* spp., *Shigella* spp. and *Escherichia coli* can only cause disease in animals, while *Erwinia* spp. can only cause severe disease in plants), a fact which limits the use of their functions as a tool in comparative genome research. However, the characteristics of *Enterobacter* spp. make it possible to be an excellent material in such research. Nowadays, only two species (*Enterobacter sakazakii* ATCC BAA-894 (syn *Cronobacter sakazakii*) and *Enterobacter* sp. 638) [17] have been sequenced in genus *Enterobacter*. No experimental evidence on pathogenicity mechanisms and virulence genes of *Enterobacter* species causing plant diseases is presently available. The only information is that *E. cloacae* could secrete some toxic biochemical substances to induce the

Table 4 Genetic distance matrix results of 14 *Enteronacter* strains[a)]

0.00	75.00	45.18	72.22	76.47	66.67	89.47	78.95	0.00	36.84	62.50	89.47	64.71	89.47
75.00	0.00	70.59	87.50	93.33	94.44	76.92	71.43	75.00	63.16	86.67	76.92	80.00	93.33
41.18	70.59	0.00	75.00	87.50	76.47	87.50	66.67	41.18	35.29	73.33	94.12	66.67	87.50
72.22	87.50	75.00	0.00	60.00	45.46	60.00	66.67	72.22	80.00	75.00	60.00	85.71	72.73
76.47	93.33	87.50	60.00	0.00	50.00	100.00	83.33	76.47	90.00	70.00	80.00	83.33	80.00
66.67	94.44	76.47	45.46	50.00	0.00	84.62	78.57	66.67	80.95	54.55	75.00	78.57	75.00
89.47	76.92	87.50	60.00	100.00	84.66	0.00	83.33	89.47	90.00	91.67	66.67	83.33	66.67
78.95	71.43	66.67	66.67	83.33	78.57	83.33	0.00	78.98	73.68	84.62	83.33	85.71	72.73
0.00	75.00	41.18	72.22	76.47	66.67	89.47	78.95	0.00	36.84	62.50	89.47	64.71	89.47
36.84	63.16	35.29	80.00	90.00	80.95	90.00	73.68	36.84	0.00	72.22	84.21	66.67	95.24
62.50	86.67	73.33	75.00	70.00	54.55	91.67	84.62	62.50	72.22	0.00	91.67	63.64	81.82
89.47	76.92	94.12	60.00	80.00	75.00	66.67	83.33	89.47	84.21	91.67	0.00	72.73	80.00
64.71	80.00	66.67	85.71	83.33	78.57	83.33	85.71	64.71	66.67	63.64	72.73	0.00	72.73
89.47	93.33	87.50	72.73	80.00	75.00	66.67	72.73	89.47	95.24	81.82	80.00	72.73	0.00
14[a)]	13	12	11	10	9	8	7	6	5	4	3	2	1

a) Strain names are the sameas in Figure 5.

Table 5 16s rRNA results of the six isolates

Strain code	Genbank No.	Highest similarity	Type strain of the highest similarity
ZJUPD1	EU430750	97%	*E. agglomerans*
ZJUPD2	EU430751	98%	*E. cancerogenus*
ZJUPD3	EU430752	97%	*E. agglomerans*
ZJUPD4	EU430753	98%	*E. cancerogenus*
ZJUPD5	EU430754	98%	*E. cancerogenus*
ZJUPD6	EU430755	98%	*E. asburiae*

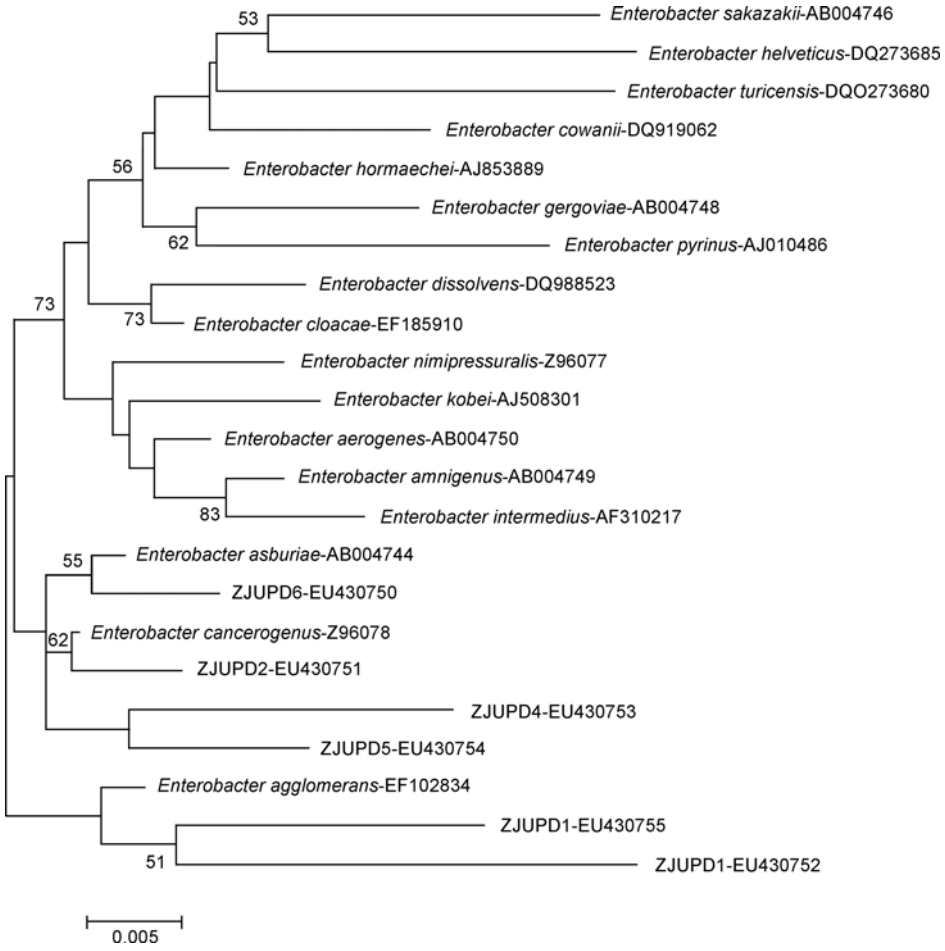

Figure 6 16s rRNA phylogenetic tree generated by Neighbor-Joining. Numbers indicate branch support from 1000 bootstrap analyses. Only those with value above 50% are shown.

diseases of woody plants. Based on pathogenicty and weak HR of the MWD strains we deduced that these bacteria might not impose Type III secretion system which plays an important role in virulence determination in many gram-negative phytobacteria to infect hosts. With the constant in-depth study of these bacteria, important information about the evolution of phytobacteria, their differentiation, origin and transfer of virulent genes, and their pathogenic mechanisms will be achieved in the future.

4 Conclusions

(1) Six representative MWD strains were identified as new bacterial pathogens of mulberry by common bacteriology, electron microscope observation, pahogenicity and Koch's postulates, physiological and biochemical analysis, Biolog, FAMEs analysis and comparative analysis against four type strains. They belong to *Enterobacter* which has never been reported before as a mulberry pathogen in the world.

(2) After phenotypic and pathogenicity tests the six MWD strains (Genbank accession number. EU430750-EU430755) were further studied by ERIC-PCR, partial 16s rRNA sequences compared with 10 standard reference strains and comparative analysis of sequences with 16 *Enterobacter* species associated with plants and environments. It was confirmed that the causal organisms of mulberry wilt involved more than one new species in *Enterobacter.*

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