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Identification and characterization of the *Enterobacter* complex causing mulberry (*Morus alba*) wilt disease in China

Guo-Fen Wang · Guan-Lin Xie · Bo Zhu · Jun-Sheng Huang · Bo Liu · Praphat Kawicha · Lesley Benyon · Yong-Ping Duan

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Abstract Mulberry wilt disease (MWD) was recently identified in Hangzhou, Zhejiang province, China. Typical symptoms of the disease are browning of vascular tissues, leaf wilt, defoliation, and tree decline. Unlike the symptoms of bacterial wilt disease caused by *Ralstonia solanacearum*, symptoms of MWD generally started from the bottom of the plants and moved upward. In inoculation experiments, four selected MWD strains caused mulberry shoot leaf wilt, discoloration, and defoliation. They also induced whole plant leaf wilt, defoliation and dark brown discoloration of vascular tissue. Based on Biolog metabolic profiles, fatty acid methyl ester analysis (FAME) and sequence analysis of the partial 16S rDNA and *rpoB* genes four MWD strains were identified as members of the genus *Enterobacter*. The 16S rDNA and *rpoB* gene sequences revealed a close relationship among two isolates, R2-2 and R6-2, and the *E. asburiae* type strain JCM6051. The isolates showed >98% similarity to *E. asburiae* JCM6051 in their *rpoB* gene. These results indicated that isolates R2-2 and R6-2 belonged to *E. asburiae*. No similarity in 16S rDNA sequences above 97% was found between either of the remaining isolates, R11-2 or R18-2, and any recognized *Enterobacter* species, suggesting that the two isolates may represent novel *Enterobacter* species. *rpoB* gene similarity values between the isolates and *Enterobacter* spp. type strains were <98%, providing further evidence that the two isolates may represent a novel species within the *Enterobacter*. The causal agent for MWD was previously reported to be *E. cloacae*, however, this study found that other *Enterobacter* spp. (*E. asburiae* and *Enterobacter* sp.) also cause MWD.

Keywords Pathogenicity · Phylogenetic analysis · FAME · BIOLOG

G.-F. Wang · G.-L. Xie (✉) · B. Zhu
State Key Laboratory of Rice Biology,
Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture,
Institute of Biotechnology, Zhejiang University,
P. O. Box 3-398, Hangzhou 310029, Zhejiang province,
People's Republic of China
e-mail: glxie@zju.edu.cn

G.-F. Wang · J.-S. Huang
Institute of Environmental and Plant Protection,
Chinese Academy of Tropical Agricultural Sciences,
Danzhou 571737, Hainan province,
People's Republic of China

B. Liu
Agricultural Bioresource Research Institute,
Fujian Academy of Agricultural Science,
Fuzhou 350003, Fujian province,
People's Republic of China

P. Kawicha
Faculty of Natural Resources and Agro-Industry
Kasetsart University,
Sakonnakhon Province 47000, Thailand

L. Benyon · Y.-P. Duan
USDA-ARS-USHRL,
Fort Pierce, FL 34945, USA

Introduction

Plant pathogenic bacteria that were Gram-negative, rod-shaped, peritrichously-flagellated, and facultatively anaerobic have previously been placed in the genus *Erwinia*. However, after taxonomic revisions several species were transferred to the genus *Enterobacter*. Currently, there are twenty-two species in the genus *Enterobacter* (Euzéby 2009), of which *E. cancerogenus* (Dickey and Zumoff 1988), *E. nimipressuralis* (Brenner et al. 1986), *E. cloacae* subsp. *dissolvens* (Rosen 1922), *E. pyrinus* (Chung et al. 1993) and *E. cloacae* complex (Neto et al. 2003; Hoffmann et al. 2005) are plant pathogens. Two of these pathogens cause diseases in woody plants: *E. cancerogenus* causes poplar canker (Dickey and Zumoff 1988) and *E. nimipressuralis* causes elm wetwood disease (Carter 1964; Murdoch and Campana 1983). Variants of *E. cloacae* have been associated with internal yellowing disease of papaya (Nishijima et al. 1987), internal decay of onion (Bishop and Davis 1990) and rhizome rot of ginger (Nishijima et al. 2004). Recently it was reported that *Enterobacter sakazakii* (renamed *Cronobacter sakazakii*) (Iversen et al. 2007) induces an atypical internal yellowing of papaya fruit in Hawaii (Keith et al. 2008).

Mulberry (*Morus alba* L.) is an important sericulture plant widely grown in China, Japan, Egypt and southeast Asian countries. It has been reported that *Pseudomonas syringae* pv. *mori* (Sato and Takahashi 1972), *Pseudomonas solanacearum* (He et al. 1983) renamed *Ralstonia solanacearum* (Taghavi et al. 1996), and *Erwinia carotovora* (Takahashi and Sato 1978) are the three most important bacterial pathogens of mulberry. A severe outbreak of a foliar decline disease was observed in the mulberry orchards in Hangzhou, Zhejiang province, China in summer, 2006. Leaf wilt symptoms began on older leaves at the bottom of the plants and then spread to the younger leaves. Leaves of infected plants became withered and dry, turned dark brown and eventually dropped. Xylem tissue in these plants was moist and discolored with brown stripe. Spring pruning of mulberry (April) induced slight field symptoms and formed the disease centre. The disease would then be aggravated by the summer pruning (July and August). This pruning, coupled with seasonal high temperatures and humidity, led to rapid disease progression and the eventual death of most infected plants. The causal agent has been presumed to be *R. solanacearum*. However, a facultatively-anaerobic, gram negative, rod-shaped bacterium was repeatedly isolated. Inoculations of the isolates produced the symptoms of mulberry wilt seen in the orchards, which upon inspection can be distinguished from mulberry bacterial wilt caused by *R. solanacearum*. The latter is characterized by flaccid wilted leaves without discoloration and defoliation (Lai et al. 1979). Five isolates of the bacteria were identified as *E. cloacae* and first reported in March, 2008 (Wang et al. 2008).

In this study, we selected four additional bacterial strains to confirm the initial identification and conduct further characterization of the causal agents of this new mulberry wilt disease (MWD) which included pathogenicity tests, biochemical and physiological features, carbon source oxidation (Biolog), and fatty acid methylesters (FAME) analyses. Additionally, the relationship of these four selected MWD strains to other *Enterobacter* spp. were evaluated using 16S rDNA and *rpoB* gene sequence analyses.

Materials and methods

Bacterial collection and preliminary characterization

MWD bacteria initially were isolated from xylem tissue excised from the root and stem of infected mulberry plants (Fig. 2C) on TTC medium (1 gl^{-1} casamino acid, 10 gl^{-1} peptone, 5 gl^{-1} glucose, 17 gl^{-1} agar, 0.05 gl^{-1} triphenyltetrazolium chloride; pH 7.2). Pure cultures of each strain were maintained in frozen aliquots in 20% glycerol at −80°C and in 5 ml sterile distilled water (SDW) in screw-cap tubes (Nishijima et al. 1987). Two reference strains *E. cancerogenus* LMG2693 and *E. cloacae* subsp. *dissolvens* LMG2683 were acquired from BCCM/LMG Bacteria Collection at the University of Ghent, Ghent, Belgium (Table 1).

The cultures were recovered from storage and checked for purity by streaking on nutrient agar (NA: 3 gl^{-1} beef extract, 5 gl^{-1} peptone, 17 gl^{-1} agar; pH 7.2) after an aerobic incubation at 30°C for 48 h. The 3% KOH test was used as a presumptive indication of Gram reaction, and the ability to grow under anaerobic conditions was tested using Hugh and Leifson's medium sealed with mineral oil. The colony

Table 1 Origin and pathogenicity of the mulberry wilt disease-causing strains and reference strains of related *Enterobacter* spp

Strains	Host plant	Symptoms on mulberry observed in bioassay				
		Time (day)[a]	yellowing	wilting	necrosis	defoliation
R2-2	mulberry	14	+	+	+	v
R6-2	mulberry	14	+	+	−	v
R11-2	mulberry	12	+	+	+	+
R18-2	mulberry	12	+	+	(+)	(+)
LMG2683T	Corn	14	+	+	−	−
LMG2693T	Poplar	16	(+)	−	−	−

[a] the days after inoculation, first symptoms observed

Symbols: +, positive; −, negative; v, variable; (+), slightly positive

T type strain LMG2683 and LMG2693 are *E. cloacae* subsp. *dissolvens* and *E. cancerogenus*, respectively

appearance on TTC medium was recorded. Cell morphology of representative isolates R2-2 and R18-2 was observed by transmission electron microscope (TEM, KYKY-1000B, Japan). Measurements of 100 cells per isolate were made. Other basic identification tests, such as maximum and minimum growth temperature, NaCl tolerance, and catalase and oxidase activities, were conducted following the techniques described by Dickey and Zumoff (Dickey and Zumoff 1988) and Schaad (Schaad et al. 2001).

Pathogenicity tests

To test the pathogenicity of the four MWD strains, laboratory bioassays and whole plant inoculations in the greenhouse were performed. For the bioassay, healthy shoots of mulberry 'Nongsang', 15.0 cm long with five leaves, were cut and placed in flasks containing 50.0 ml SDW, and then 0.2 ml aliquots of bacterial cell suspension (1.0×10^8 CFU ml^{-1}) were injected into the axils of the plant stem. These were compared with injections with SDW or nutrient broth (NB) as controls. There were three replicates per treatment. The inoculated plants were incubated in a growth chamber with 30/25°C day/night temperatures, 95% relative humidity and a 12-h photoperiod. For the whole plant inoculations, 3-month old grafted mulberry seedlings (Nongsang × Heyebai) planted in individual pots were inoculated using the same method described above. After inoculation, the plants were covered with plastic bags and maintained in high humidity for 24 h, and then put in a greenhouse (25 to 30°C day and 23 to 25°C night with a 10 h photoperiod). The disease symptoms were observed 5 to 21 days after inoculation. At the end of the experiment, the internal symptoms were assessed, and leaves and discolored stem tissues were collected for isolation of the bacterial strains. The plant tissues were macerated in SDW for 15 min and the suspensions were streaked on TTC plates. The plates were monitored for reddish-purple nonmucoid colonies, with regular, translucent margins for 48 h. Selected bacterial colonies were further purified on NA and compared with the inoculum and reference strains using fatty acid analyses and carbon source oxidation tests. Each experiment was repeated three times.

Fatty acid analyses and carbon source oxidation

The total cellular fatty acid composition was extracted from 24-h-old bacterial lawns cultured at 30°C on Bacto™ Tryptic Soy Broth agar (Becton, Dickinson and Company Sparks, MD, USA). Gas chromatography using Agilent 6890 N (Agilent Chromatograph: Santa Clara, CA, USA) was conducted by Sherlock MIS4.5. Results were compared with the MIDI identification database TSBA50, version 5.00 (MIDI Inc., Newark, DE, USA). A species designation was assigned to each strain on the basis of similarity indexes. Oxidation of 95 different carbon sources was tested using the BIOLOG microtitre plate designed for Gram-negative bacteria (GN, BIOLOG, Inc., Hayward, CA). Testing was performed following manufacturers' instructions, and two microplates were inoculated per isolate. Colour development was recorded with a MAXLine microplate reader (Molec-

ular Devices) and the results checked by visual observation. Metabolic profiles were compared and identified after 24 and 48 h using the BIOLOG identification database GN4.01A. In addition, the arginine and lysine decarboxylase test was undertaken using Moeller medium (Difco) containing 1% (wt/vol) each of L-arginine and L-lysine according to manufacturer's instruction. MR/VP broth was used for the Voges-Proskauer test after 48 h and for the methyl red test after 4 days at 30°C. Indole, nitrate, malonate and starch reaction tests were conducted as described by Schaad (Schaad et al. 2001).

DNA isolation

DNA was extracted using a modified technique of the hexadecyl trimethyl ammonium bromide (CTAB) method (Rascoe et al. 2003). Two loops of 24-h-old bacterial lawn on NA medium were lysed in 570 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0), 30 μl 10% sodium dodecyl sulfate (SDS) and 60 μg of proteinase K. The mixture was incubated for 1 hour at 37°C, then 100 μl of 5 M NaCl and 80 μl of CTAB solution (10% CTAB in 0.7 M NaCl) were added and mixed, and the suspension was incubated at 65°C for 10 min. The solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1), followed by DNA-precipitation and recovered by isopropanol precipitation. The pellet was washed two times with 70% ethanol, redissolved in 50 μl TE, and quantified spectrophotometrically.

PCR amplification and sequencing

In order to exclude the possibility of *R. solanacearum* contamination, PCR analyses of the four testing strains was conducted using two sets of the *R. solanacearum*-specific primers *pehA*#3/*pehA*#6 (*pehA*#3: 5′ CAG CAG AAC CCG CGC CTG ATC CAG 3′, *pehA*#6: 5′ ATC GGA CTT GAT GCG CAG GCC GTT 3′) (Gillings et al. 1993) and 759/760 (759: 5′ GTC GCC GTC AAC TCA CTT TCC 3′, 760: 5′ GTC GCC GTC AGC AAT GCG GAA TCG 3′) (Ito et al. 1998). The PCR reactions were conducted as described by Gillings and Ito. Amplification was carried out with a Programmable Temperature Cycler (PTC-200, MJ Research). *R. solanacearum* GMI1000 was used as a positive control.

Nearly complete 16S rRNA genes were amplified by PCR using a forward primer fD2 (5`-AGA GTT TGA TCA TGG CTC AG-3`) and a reverse primer rP1 (5`-ACG GTT ACC TTG TTA CGA CTT-3`) (Weisburg et al. 1991). The amplification program was performed with the following conditions: 5 min at 94°C; 35 cycles of 30 s at 94°C, 60 s at 55°C, and 2 min at 72°; and final elongation at 72°C for 10 min. The PCR products were excised from the 1% agarose gels after electrophoresis, and purified using 3S EZ-Resin DNA gel purification Kit (Shenergy-Biocolor, Shanghai, China). Products were ligated into PMD18-T vector (Takara, Japan) according to the manufacturer's manual and transformed into *Escherichia coli* (DH5α) competent cells. White clones with the correct insert were bi-directional sequenced by an ABI 3730 automatic sequencer (Sangon Biological Engineering Technology Co. Ltd, Shanghai, China). Amplification of the *rpoB* genes was performed by PCR as described by Mollet et al. (1997) using specific primers CM7 (5`-AAC CAG TTC CGC GTT GGC CTG G-3`) and CM31b (5`-CCT GAA CAA CAC GCT CGG A). Both strands of the amplicons were sequenced using the primers CM81, CM81b and CM32b and the reaction condition as described by Mollet et al. (1997).

16S rRNA and *rpoB* genes sequence comparison and phylogenetic analysis

Resulting sequences were aligned and edited using DNAMAN software (Lynnon BioSoft, USA). Analyses of sequences were performed with the basic sequence alignment BLAST program run against the nucleotide database (http://www.ncbi.nlm.nih.gov/blast). 16S rRNA and *rpoB* gene sequences were aligned using MEGA (version 3.1, Kumar et al. 2004) by the Clustal W program and the phylogenetic tree was constructed by the neighbor-joining method. Other member sequences of the family *Enterobacteriaceae* were selected from GenBank. Sequence similarities were calculated by the MegAlign program in the DNAstar package 5.00 (DNASTAR Inc. Steve ShearDown R.P.M.S). The significance of branching was evaluated by bootstrap analysis of 1000 replicates. The bootstrap values were placed at the internal branches of the distance trees. Branches with bootstrap values less than 50 were collapsed.

Results

Isolation and general characteristics of bacteria

All four MWD bacterial strains formed white to light cream, circular, opaque, convex colonies on NA. They also grew on NA containing up to 5% NaCl and had a maximum growth temperature of 45°C. Strains R2-2 and R6-2 showed growth at the 5°C minimum temperature after a 72 h incubation, but R11-2 and R18-2 did not. On TTC media, after 24 h incubation at 30°C, dark pink, non-mucoid colonies with regular edges and translucent margins were formed. The strains were facultatively anaerobic, catalase positive, oxidase negative, Gram negative, short-rod shaped, 0.3–1.0 μm wide and 1.0–3.0 μm long, with peritrichous flagella (Fig. 1).

Pathogenicity tests

MWD show leaves discoloration and defoliation characteristic in the field (Fig. 2A). Symptoms were unlike the wilt disease caused by *R. solanacearum* which is characterized by flaccid, wilted leaves with no discolouration and defoliation (Lai et al. 1979). However, in both diseases, vascular tissues are dark brown (Fig. 2C), and neither disease shows soft rot.

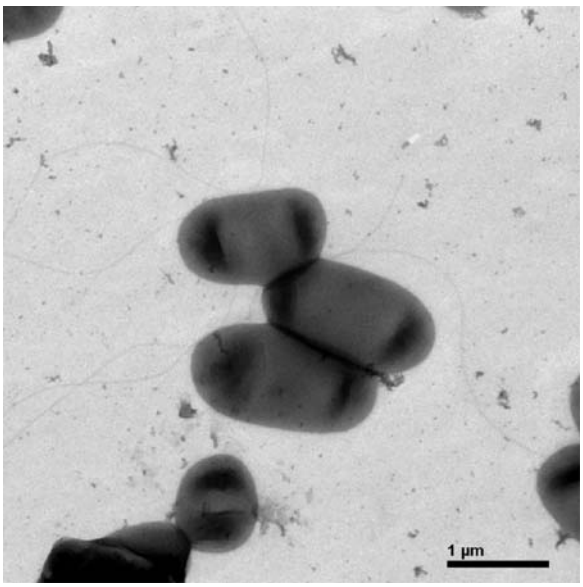


Fig. 1 Electron microscopic photograph of *Enterobacter* sp. strain R6-2 showing rod-shaped cell and peritrichous flagella. Black bar, 1 μm

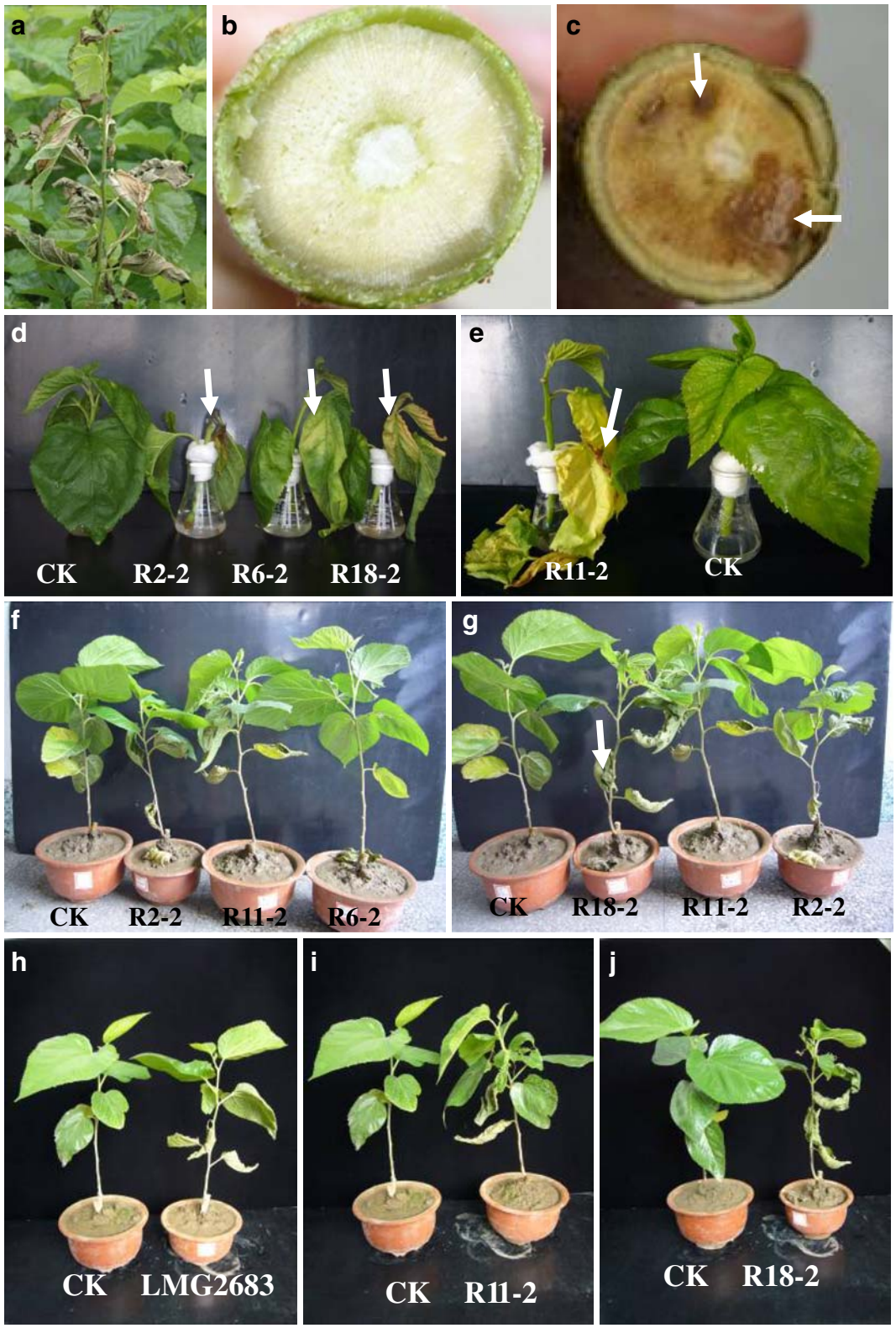
In the bioassay, in which mulberry cuttings were inoculated, typical wilt symptoms first appeared at 12 days, in the case of R11-2 and R18-2, and at 14 days, in the case of strains R2-2 and R6-2, post inoculation. Two days later the leaves on the same sides as the inoculation point showed browning and crinkling. At 17 days post inoculation, the wilted leaves fell off and the other parts of the cutting turned yellow, or discoloured, and wilted. At 19 days after inoculation, the tested plants were withered, and some shoot tops showed necrosis. Finally, all of the tested shoots died at 21 days after inoculation, but the controls were still fresh with a small amount of leaf yellowing due to lack of nutrients (Table 1, Fig. 2D, E).

In the whole plant inoculation test, the initial disease symptom appeared 10 days after inoculation, two days earlier than in the cutting bioassay test. The lower, older leaves showed wilting symptoms first. In the following 2 days, the middle and upper leaves also withered and showed crinkling from the edges in toward the vein. The affected leaves dried and defoliated 16 days after inoculation (Fig. 2F–J). Control plants remained symptomless. One plant inoculated with the type strain LMG2683 showed slight wilting and defoliation (Fig. 2H). Strains R2-2 and R6-2 had milder symptoms than the other strains, with only one leaf yellowing at 16 days after inoculation than other strains (Fig. 2F–J).

All the diseased seedlings showed dark brown vascular bundles when dissected, and some bacterial exudation was seen from the cross section of the stem. Colonies with the same Biolog and FAME profiles were re-isolated into pure cultures. The mulberry plants inoculated with SDW and NB as controls remained symptomless.

Fatty acid analysis, carbohydrate oxidation profiles and other biological features

Fatty acid similarity index analyses matched the four MWD isolates to *E. cancerogenus*. Comparisons of the fatty acid profiles of the four MWD strains with those of known bacteria in the MIDI database were matched to *E. cancerogenus* with similarity index of 0.59 to 0.84 (Table 2). Dendrogram analyses of fatty acid profiles were represented as Euclidean distances between the four MWD isolates. MIDI instructions suggest that a Euclidean distance ≥10 is indicative of separate species, while values of ≤6


a
b
c
d
CK
R2-2
R6-2
R18-2
e
R11-2
CK
f
CK
R2-2
R11-2
R6-2
g
CK
R18-2
R11-2
R2-2
h
CK
LMG2683
i
CK
R11-2
j
CK
R18-2

◀ **Fig. 2** Symptoms of mulberry wilt disease in the field (**A**-**C**), bioassay (**D**, **E**) and the whole plant inoculation (F–J). **A**. field shoot wilt symptoms; **B**. mulberry healthy stem; **C**. dark brown lesions on the stem xylem; **D**, **E**. three symptoms of the bioassay; the arrows indicate top necrosis (R2-2, R11-2), discoloration (R18-2, R11-2) and wilt symptoms (R6-2, R18-2) on the mulberry shoots 19 days post inoculation with four MWD isolates. **F**, **G**. Leaves with wilt and difoliation symptoms on mulberry seedling 15 days post artificial inoculation with *Enterobacter* sp. isolates R2-2, R6-2, R11-2 and R18-2 (typical symptoms marked by arrows) ; **H**, **I**, **J**. Seedling symptoms in type strain LMG2683 (H), R11-2 (I) and R18-2 (J) inoculation; CK indicates control

suggest that two strains are in the same subspecies or biotype (Sherlock 2002). Bacteria with Euclidean distances ≤2.5 are recommended for assignment to the same species. The four MWD isolates were in the same cluster with a Euclidean distance of ≤10, along with *E. cancerogenus* and *E. asburiae* (Euclidean distances of 3.7 and 6.7, respectively) (Fig. 3). These results show that the MWD isolates are either members of *E. cancerogenus* and/or *E. asburiae* or are closely related.

The metabolic profiles of the four MWD strains identified them all in the genus *Enterobacter*. Three of the strains were identified as *E. cloacae* with similarity indices ranging from 0.56 to 0.65 (Table 2). R11-2 had a high similarity index (0.73) to *E. aerogenes*, which is the homotypic synonym of *Klebsiella mobilisa* (Skerman et al. 1980). All four MWD strains oxidized α-hydroxybutyric acid and producted phenylalanine ammonium lyase, while reference strains of *E. asburiae*, *E. cancerogenus* and *E. cloacae* subsp. *dissolvens* were negative. The MWD strains differed from the reference strains and among each other in a number of other traits as listed in Table 3.

PCR amplification and sequence analysis

Using *R. solanacearum*-specific primers *pehA*#3/*pehA*#6 and 759/760, the PCR amplification yielded the expected amplicons from the *R. solanacearum* GMI1000 control but not from the four MWD strains (Fig. 4), indicating that the MWD strains were not contaminated with *R. solanacearum*.

The partial 16S rDNA and *rpoB* gene sequences of the four isolates were obtained and submitted to GenBank (Table 2). BLASTn analyses showed that the 16S rDNA sequences of these four MWD strains yielded 96% to 99% similarity with *Enterobacter* spp. strains that did not have species identifications in Genbank. Based on this result, the 16S rDNA sequences of 15 phytopathogens belonging to the *Enterobacteriaceae* (Hauben et al. 1998), and the *rpoB* gene sequences of 28 strains of the genus

Table 2 Fatty acid, BIOLOG identification, and GenBank accession numbers for 16S r RNA and ribosomal RNA-encoding gene (*rpoB*) sequences of mulberry wilt disease strains and *Enterobacter* spp. used in this study

Strains	FAME SI[a]	BIOLOG SI[b]	GenBank assession number	
			16S rDNA	*ropB*
R2-2	0.59	0.56	EU078565	EU579855
R6-2	0.64	0.62	EU078566	EU579856
R11-2	0.84	0.73[c]	EU078564	EU579857
R18-2	0.73	0.65	EU721605	EU579858
LMG2683T	0.47[d]	0.64	Z96079	AJ543725
LMG2693T	0.53	0.74[e]	Z96078	AJ566943

[a] MIDI identification database TSBA50, version 5.00 (MIDI Inc., Newark, DE, USA) total cellular fatty acid composition similarity index: identified as *Enterobacter cancerogenus* unless noted otherwise

[b] BIOLOG Similarity Index: identified as *Enterobacter cloacae* unless noted otherwise

[c] *Enterobacter aerogenes*

[d] *Enterobacter cloacae*

[e] *Enterobacter cancerogenus*

T type strain LMG2683 and LMG2693 are *E. cloacae* subsp. *dissolvens* and *E. cancerogenus*, respectively

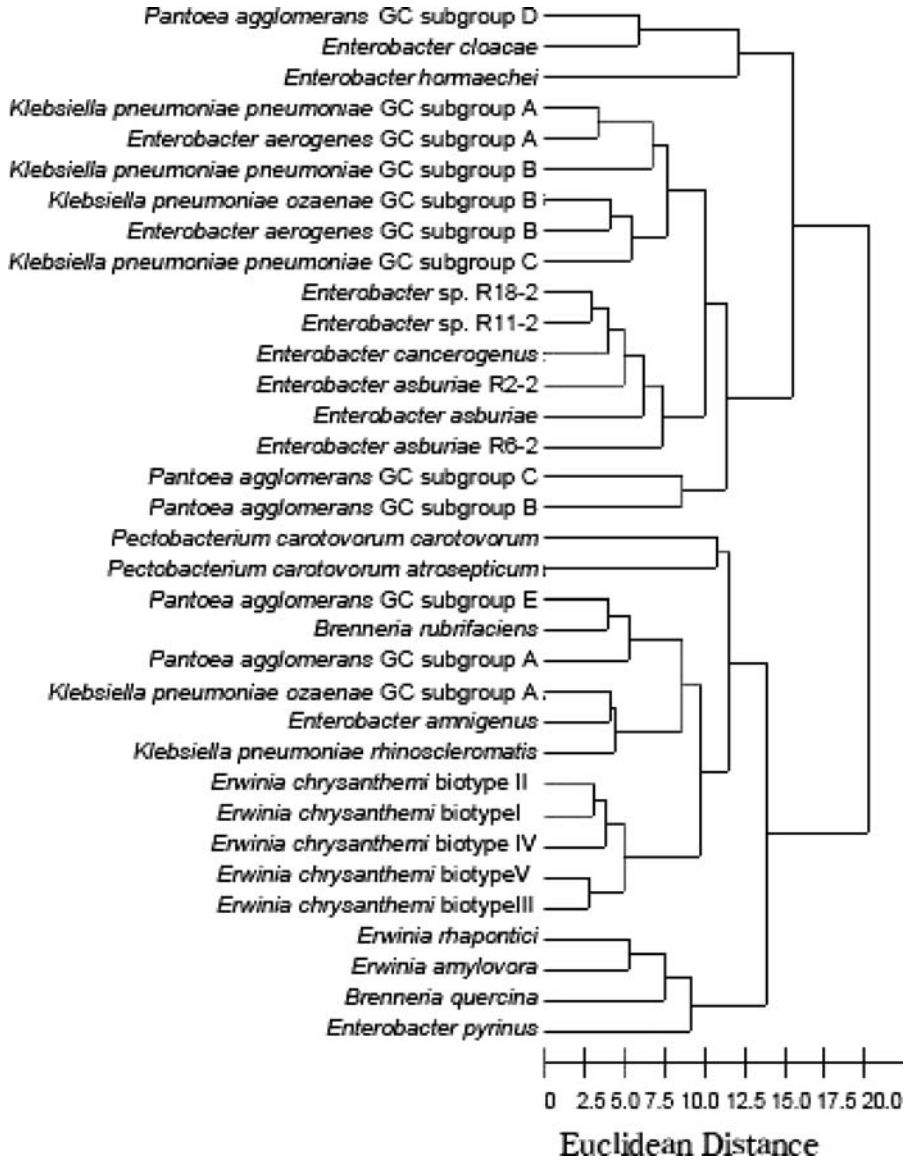


Fig. 3 Dendrogram cluster analysis of unweighted pair matchings based on fatty acid compositions

Enterobacter were obtained from GenBank (Stephan et al. 2007; Stephan et al. 2008). R3-3 and R8-2 are the *E. cloacae* strains isolated previously (Wang et al. 2008). Neighbour-joining phylogenetic distance trees were constructed based on the 16S rDNA sequence data (Fig. 5) and the *rpoB* gene sequence data (Fig. 6) using MEGA software.

The 16S rDNA sequences of the four MWD strains formed a separate clade with two type strains, *E. cancerogenus* LMG2693 and *E. asburiae* JCM6051 (Fig. 5). Strains R2-2 and R6-2 shared 98.1% and 97.7% identity to *E. cancerogenus* LMG2693, respectively, and 98.3% and 97.2% identity to *E. asburiae* JCM6051, respectively. Strains R11-2 and R18-2 were 94.8% and 96.8% identical to *E. cancerogenus* LMG2693, respectively, and 94.3% and 95.9% identical to *E. asburiae* JCM6051, respectively. Two other type strains also had high sequence similarities with the four new strains. The first, *E. cloacae* subsp. *cloacae* LMG2783 shared similarities ranging from 97.8% to 94.6%, and the second, *E. cloacae* subsp. *dissolvens* LMG2683, shared similarities ranging from 97.7% to 94.4% (data not shown).

The 16S rDNA sequence similarity between strains R2-2 and R6-2 was 98.3%. Their highly similarity (> 97%) to the reference strains indicated that they

Table 3 General characteristics, partial substrate utilization profiles and other biological features of mulberry wilt disease-causing strains (R2-2, R6-2, R11-2, R18-2) and reference strains of related *Enterobacter* spp.[a]

Test	*E. asburiae*[b]	R2-2	R6-2	R11-2	R18-2	LMG2683T	LMG2693T
General characteristics							
Catalase	+	+	+	+	+	+	+
Oxidase	−	−	−	−	−	−	−
5% NaCl	ND	+	+	+	+	±	−
Growth at 5°C	ND	+	+	−	−	−	±
Growth at 45°C	ND	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+
BIOLOG							
Glycogen	±	±	±	+	+	±	−
Tween 40	−	+	+	+	+	±	−
Tween 80	−	+	+	+	+	±	±
N-Acetyl-D-Galactosamine	−	−	+	+	+	+	+
Adonitol	−	−	−	±	−	−	−
Itaconic acid	−	−	−	±	−	−	−
D-Arabitol	−	−	−	±	±	−	−
L-Fucose	−	−	−	+	±	−	+
Meso-Inositol	±	+	+	+	+	+	−
α-D-Lactose	+	±	±	+	±	−	−
Lactulose	±	±	−	+	±	−	−
D-Melibiose	−	+	+	−	+	+	−
D-Raffinose	±	+	+	+	+	+	−
Turanose	±	+	+	±	+	+	−
Xylitol	−	−	−	+	±	−	−
Monomethyl-Succinate	+	+	±	+	±	±	±
Acetic acid	+	±	±	−	±	±	±
D-Galactonic acid lactone	+	−	+	+	+	+	+
D-Glucosaminic acid	−	±	−	+	±	−	−
α-Hydroxybutyric acid	−	+	+	+	+	−	−
β-Hydroxybutyric acid	−	+	+	±	±	±	−
ρ-Hydroxyphenylacetic acid	+	+	+	+	+	+	+
α-Keto Butyric acid	−	−	−	±	±	−	−
α-Keto Glutaric acid	±	−	−	±	±	−	±
α-Keto Valeric acid	−	−	−	±	−	−	−
Propionic acid	−	−	−	±	±	−	−
Quinic acid	−	±	−	±	±	−	−
D-Saccharic acid	−	+	+	+	+	+	+
Sebacic acid	−	−	−	±	−	−	−
Succinamic acid	±	−	−	−	−	−	−
Glucuronamide	±	±	±	+	+	±	±
Alaninamide	±	+	±	+	+	±	−
L-Histidine	−	−	−	±	±	±	−
Hydroxyl-L-proline	−	−	−	±	−	−	−
L-Leucine	−	−	−	±	−	−	−
L-Phenylalanine	−	+	±	+	+	−	−

Table 3 (continued)

Test	*E. asburiae*[b]	R2-2	R6-2	R11-2	R18-2	LMG2683T	LMG2693T
DL-Carnitine	−	−	±	−	−	−	−
Y-Aminobutyric	−	−	±	−	−	−	−
Phenylethylamine	−	−	±	−	−	−	−
2-Aminoethanol	−	−	±	−	−	−	−
L-Proline	±	+	±	+	+	±	−
Additional biological features							
Arginine decarboxylase	ND	+	+	+	+	+	−
L-Lysine decarboxylase	−	−	−	−	−	−	−
L-Arginine dihydrolase	±	−	−	−	−	−	−
Ornithine dihydrolase	−	+	+	+	+	±	−
Voges-Proskauer	−	+	+	+	+	+	+
Methyl Red	+	−	−	−	−	−	−
Indole	−	+	+	+	+	−	−
Nitrate	+	+	+	+	+	+	−
Malonate	−	−	−	+	+	+	−
Starch reaction	−	−	−	−	−	−	−

[a] BIOLOG metabolic profiles are presented for only 41 of 95 substrates. These are the reactions in which MWD isolates differed unequivocally from those of reference strains. Symbols: −, no activity; +, activity; ±, questionable; ND, no data.

[b] BIOLOG information for *E. asburiae* is from the GN 4.01 database. General and other biological features from Brenner et al. 1986 and Hoffmann et al. 2005.

LMG2683T and LMG2693T are the type strain *E. cloacae* subsp. *dissolvens* and *E. cancerogenus*, respectively

belonged to either *E. cancerogenus* or *E. asburiae* (Stackebrandt and Goebel 1994). The 16S rDNA sequence similarity between strains R11-2 and R18-2 was 97.7%. No 16S rDNA sequence similarity above 97% was found between either of these isolates and any recognized species, suggesting that the two isolates might represent a novel *Enterobacter* species (Stephan et al. 2007).

In the phylogenetic tree created using the *rpoB* gene sequences, the four MWD strains formed two distinct clades. The larger clade contains *E. asburiae* JCM6051, *E. cloacae* subsp. *cloacae* LMG2783, *E. cloacae* subsp. *dissolvens* LMG2683 and *E. cancerogenus* LMG2693 (Fig. 6). The relationship between R2-2 and R6-2 and the type strain *E. asburiae* JCM6051 was confirmed by the *rpoB* gene sequence. These strains showed a similarity of 98.4% and 99.0%, respectively, to *E. asburiae* JCM6051. The intraspecies similarity range in the family *Enterobacteriaceae* is 98–100% (Mollet et al. 1997). It was therefore concluded that R2-2 and R6-2 should be identified as plant pathogenic strains of *E. asburiae*. The other two strains, R11-2 and R18-2 had *rpoB* gene similarity values lower than 98%, suggesting that the two isolates represent novel species within this family.

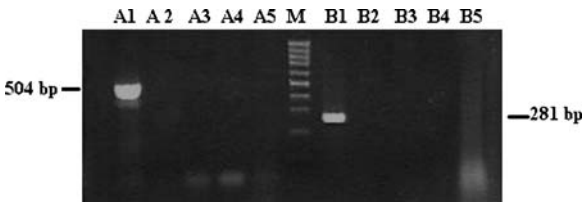


Fig. 4 PCR detection from the DNA samples of *R. solanacearum* and *Enterobacter* spp. using *R. solanacearum*-specific primers. A indicates primer set *pehA*#3/*pehA*#6 and B indicates primer set 759/760. Numbers 1 through 5: *Ralstonia solanacearum* GMI1000, R2-2, R6-2, R11-2 and R18-2, respectively

Discussion

Mulberry wilt disease was first reported in Hangzhou suburb of Zhejiang province, China in 2006. The disease infected areas from a few hectares in 2004 to over 200 hectares in 2007. Based on the field surveys

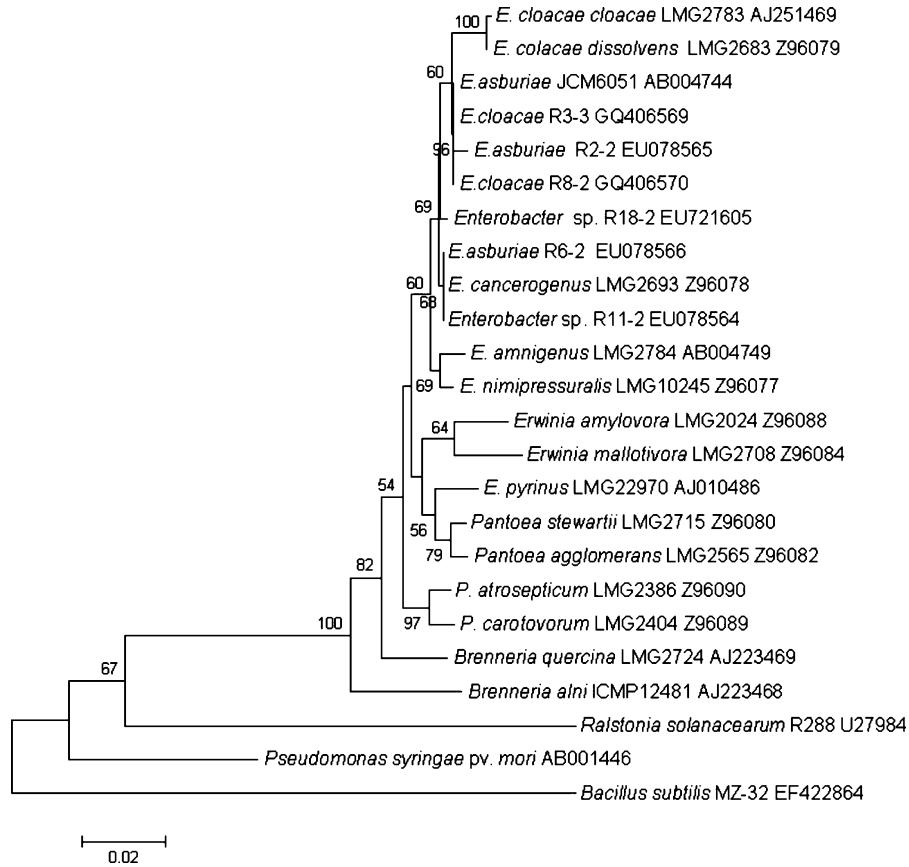


Fig. 5 Bootstrap Neighbor-Joining Phylogenetic distance tree compiled from 16S rDNA sequence data using Clustal W program in the MEGA software package version 3.1, generated by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. Booststrap analyses (1,000 replicates) for node values from 50% are indicated. *P. atrosepticum* is *Pectobacterium atrosepticum*; *P. carotovorum* is *Pectobacterium carotovorum*. R3-3 and R8-2 are the *E. cloacae* strains isolated previously

conducted, none of the popular mulberry cultivars, including Nongsang, Husang and Heyebai, are resistant to this disease. Strains of the MWD pathogen were initially reported as *E. cloacae* (Wang et al. 2008), a common species surviving in diverse habitats and ecological niches, and existing widely in soil, water, plants, insects and animals (Nishijima et al. 2004). Characterization of the four additional MWD strains in this study revealed they belong to *Enterobacter* but are not *E. cloacae.* Two of the isolates were identified as *E. asburiae* and other two may belong to a new *Enterobacter* species. All four strains were able to cause the disease individually, but two caused a more severe phenotype. Further analyses, such as DNA-DNA hybridization of the species in *Enterobacter* and G + C content of the DNA, and more MWD strains need to be characterized before the new species can be nominated.

E. asburiae is comprised of insignificant clinical strains which were formerly placed in Enteric Group 17 (Brenner et al. 1986). Other environmental and plant strains which belong to the *E. asburiae* group have been identified, and one strain has been associated with the ginger soft rot symptoms. They are also considered a companion bacterium to *R. solanacearum* which induces ginger wilt, but not as independent plant pathogens (Jones et al. 1993; Hoffmann and Roggenkamp 2003; Tsang and Shintaku 1998). This research identified two *E. asburiae* strians, R2-2 and R6-2, and other two *Enterobacter* sp. strains, R11-2 and R18-2, which caused mulberry shoot and leaf yellowing, wilting, necrosis and defoliation in the bioassay experiments. They also induced leaf wilt, defoliation and vascular tissue browning symptoms in the mulberry whole plant inoculation tests. PCR analyses using *R. solanacearum*-specific primers and 16S rDNA universal primers indicated that the four strains were purely *Enterobacter* spp. without any contamination of *R. solanacearum* in the cultures. According to the pathogenicity tests, four tested strains all independently cause mulberry wilt symptoms. It will be important to investigate if there is a synergistic effect when two or more strains are co-inoculated, or when the *R. solanacearum* is co-inoculated into host plants.

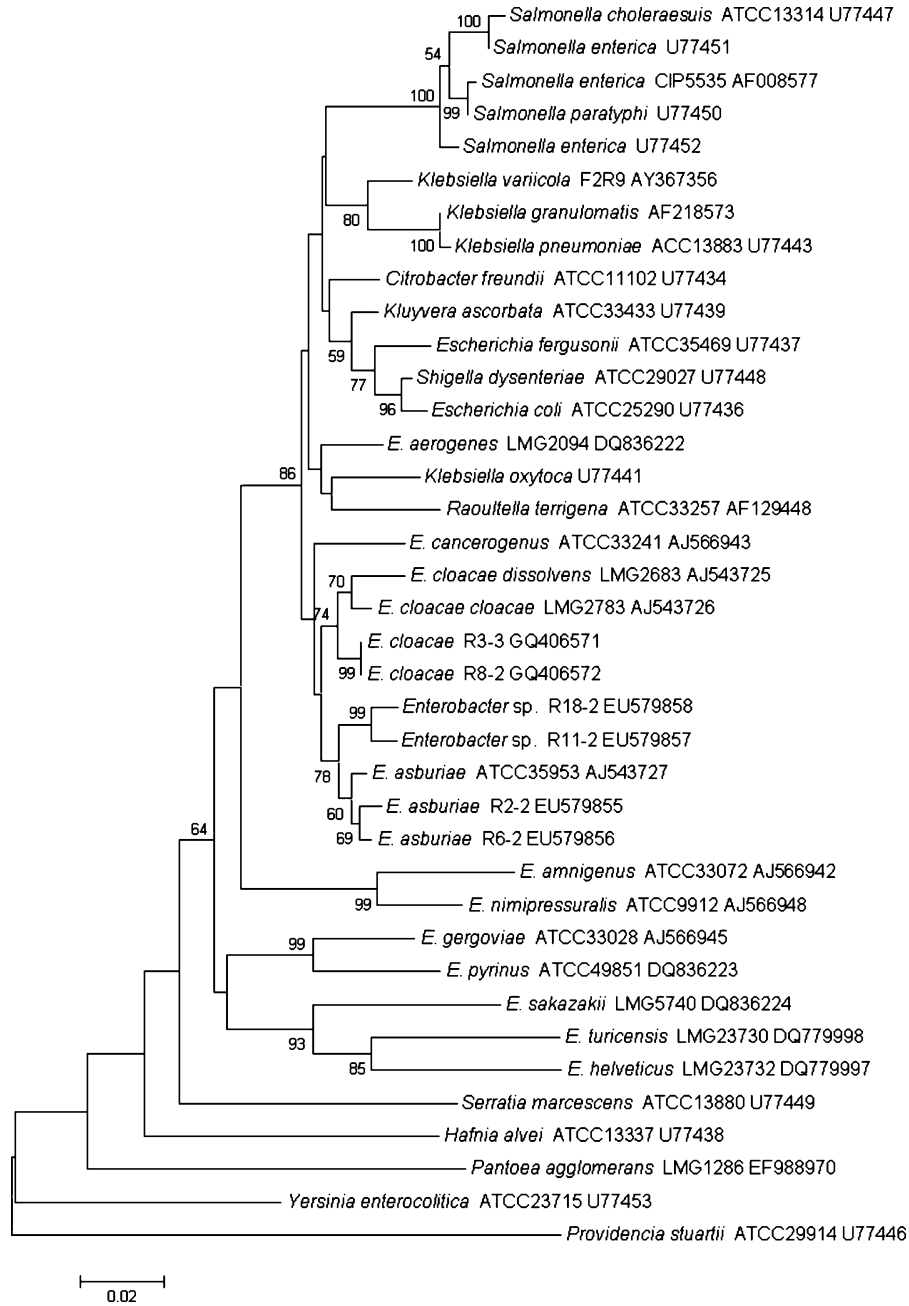


Fig. 6 Bootstrap Neighbor-Joining Phylogenetic distance tree compiled from *rpoB* gene sequence data using Clustal W program in the MEGA software package version 3.1, generated by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. Bootstrap analyses (1,000 replicates) for node values from 50% are indicated. R3-3 and R8-2 are the *E. cloacae* strains isolated previously

Result from this study indicated that *Enterobacter* spp. MWD could be distinguished from *R. solanacearum* mulberry bacterial wilt based on the symptoms. In both diseases, vascular tissues are dark brown, and neither disease shows soft rot. However, leaves turning dull green and the whole plant wilt are the typical symptoms of the *R. solanacearum* mulberry bacterial wilt (Lai et al. 1979), while the leaf discoloration and defoliation are unique to the *Enterobacter* spp. MWD. Besides, a recovering of plant flaccidity in the morning and night is seen in the early stage of the *R. solanacearum* mulberry bacterial wilt, whereas the MWD generally started leaf yellowing and defoliation symptoms from the bottom of the plants and moved upward.

Pathogenicity, biochemical and physiological features, oxidation of carbon sources (Biolog), fatty acid methylester (FAME) analysis and protein profiling aid

in the identification of novel plant bacterial pathogens (Jones et al. 1993; Janse et al. 2001; Schaad et al. 2001). In this study, the metabolic profiles firmly placed all of the isolates within the genus *Enterobacter* but did not clearly define the species. This may be due to the heterogeneity of metabolic functions within *Enterobacter* species in general. Only 14% of strains identified as *E. asburiae* displayed the original phenotypic characterizations (Hoffmann et al. 2005), and atypical test reactions were especially common for ornithine dihydrolase, VP/RM, D-melibiose, *D*-raffinose and malonate (Brenner et al. 1986). In the FAME analysis, all strains possessed a mixture of hydroxyl (14:0 3OH) and isobranch (16:1 ISO I) fatty acids. Only R6-2 had the hydroxy fatty acids 14:0 2OH, which occurs in some *Enterobacter* species (Schaad et al. 2001). However, the similarity indices did not show close matches with the two *Enterobacter* reference strains. One explanation for this result is that the 14:0 3-hydroxyl, which is the characteristic fatty acid for the *Enterobacteriaceae* family, cannot be accurately detected by gas chromatography due to the presence of the isobranch (16:1 ISO I) form. Another possibility is that fatty acid analysis has limited use for distinguishing species of some genera, such as *Xanthomon*as spp. (Li et al. 2006) and *Serratia* spp. (Rascoe et al. 2003).

Information regarding the ability of *E. asburiae* to function as a plant pathogen is limited, but other *Enterobacter* species are known to induce plant diseases. *E. cancerogenus* (Dickey and Zumoff 1988), *E. nimipressuralis* (Brenner et al. 1986), *E. cloacae* subsp. *dissolvens* (Rosen 1922) and *E. pyrinus* (Chung et al. 1993) were the first four assigned *Enterobacter* species, and their phylogenetic relationship was analyzed by 16S rDNA and *atpD*, *carA*, *recA* genes, but the sequences showed such a diversity that a definite conclusion for this genusspecific placement could not be reached (Hauben et al. 1998; Young and Park 2007). In 2007, two new *Enterobacter* species (*E. turicensis* sp. nov., *E. helveticus* sp. nov.) were identified using 16S rDNA and *rpoB* gene sequence analyses, followed in 2008 by yet another novel species (*E. pulveris* sp. nov.) (Stephan et al. 2007; Stephan et al. 2008). In our work here, two identification systems (FAME and BIOLOG) revealed that four MWD isolates were members of the genus *Enterobacter*. Fatty acid dendrogram analyses indicated that these isolates have a close relationship with *E. cancerogenus* and *E. asburiae*. Sequence analyses of 16S rDNA and *rpoB* genes confirmed that two isolates, R2-2 and R6-2, belonged to species *E. asburiae*, and indicated that the remaining two isolates, R11-2 and R18-2, might be classified in a new species of the genus *Enterobacter*.

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