Enterobacter mori sp. nov., associated with bacterial wilt on Morus alba L.

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Two isolates of mulberry-pathogenic bacteria isolated from diseased mulberry roots were investigated in a polyphasic taxonomic study. Comparative 16S rRNA gene sequence analysis combined with rpoB gene sequence analysis allocated strains R18-2T and R3-3 to the genus Enterobacter, with Enterobacter asburiae, E. amnigenus, E. cancerogenus, E. cloacae subsp. cloacae, E. cloacae subsp. dissolvens and E. nimipressuralis as their closest relatives. Cells of the isolates were Gram-negative, facultatively anaerobic rods, 0.3–1.0 μm wide and 0.8–2.0 μm long, with peritrichous flagella, showing a DNA G+C content of 55.1 ± 0.5 mol%. Calculation of a similarity index based on phenotypic features and fatty acid methyl ester (FAME) analysis suggested that these isolates are members of E. cancerogenus or E. asburiae or a closely related species. Biochemical data revealed that the isolates could be differentiated from their nearest neighbours by the presence of lysine decarboxylase activity and their ability to utilize D-arabitol. DNA–DNA relatedness also distinguished the two isolates from phylogenetically closely related Enterobacter strains. Based on these data, it is proposed that the isolates represent a novel species of the genus Enterobacter, named Enterobacter mori sp. nov. The type strain is R18-2T (=CGMCC 1.10322T =LMG 25706T).

White mulberry (Morus alba L.) is an important sericulture plant grown widely in Asia, Africa and Europe. White mulberry leaves are ecologically important as the sole food source of the silkworm (Bombyx mori), the pupa/cocoon of which is used to make silk. China produces about 75% of the world’s raw silk, which is valued at nearly $2 billion (Li et al., 2010). Therefore, epidemics of mulberry disease are of great concern in China. In the summer of 2006, a severe outbreak of a foliar decline disease was noted in mulberry orchards of Hangzhou, Zhejiang province, 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 and then turned dark brown and the plants eventually became defoliated. The root xylem of infected plants was moist and discoloured with brown stripes. The phloem was mostly asymptomatic; however, in severe infections, the phloem decayed.

Eight bacterial diseases of mulberry have been reported, caused by species of six genera (Erwinia,Ralstonia, Pseudomonas, Phytoplasma, Xanthomonas and Xylella) (Banerjee et al., 2009; Genin & Boucher, 2002; Gupta et al., 1995; Hernandez-Martinez et al., 2006; Kawakita et al., 2000; Seo et al., 2003). However, no mulberry bacterial disease associated with Enterobacter has so far been reported. Twenty-three species have previously been recorded in the genus Enterobacter (http://www.bacterio.cict.fr/e/enterobacter.html). Enterobacter species are both foes and friends; some are notorious plant pathogens or human opportunistic pathogens (Chow et al., 1991; Chung et al., 1993; Nishijima et al., 2007), while others are important engineering and plant growth-promoting bacteria (French et al., 1998; Nie et al., 2002). Moreover, others play important roles in biocontrol (Chernin et al., 1996).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and rpoB gene sequences of strains R18-2T and R3-3 are EU721605 and EU579858 and GO406571 (rpoB), respectively.

A supplementary figure and two supplementary tables are available with the online version of this paper.
Strain R18-2T and R3-3 were isolated from wilted mulberry plants. Colonies on nutrient agar medium (BD Difco) were light yellow, smooth, circular, entire and convex with no green fluorescent diffusible pigment on King’s medium B (King et al., 1954). The colonies were cream in colour on YDC agar (yeast extract, 10.0 g; glucose, 20.0 g; CaCO3, 20.0 g; Bacto agar, 15.0 g; distilled water, 1 l) and dark pink with translucent margins on TZC agar (peptone, 10.0 g; casein hydrolysate, 1.0 g; glucose, 5.0 g; Bacto agar, 12.0 g; distilled water, 1 l).

A weak hypersensitive reaction was observed on tobacco plants 3 days after inoculation. Results of pathogenicity tests and cell morphology analysis have been reported previously (Wang et al., 2008, 2010), in an initial characterization of this pathogen. Here, we further compare the physical and biochemical properties of the strains with those of reported Enterobacter type strains and use phylogenetic analysis and DNA–DNA relatedness tests to distinguish the two isolates from closely related Enterobacter type strains. Based on the results of our polyphasic taxonomic study, it can be concluded that the two isolates belong to the same species and represent a novel species in the genus Enterobacter.

Bacterial DNA was extracted using a modification of a previously described procedure (Hoffmann & Roggenkamp, 2003) and amplified using primers targeting the 16S rRNA gene (P0, 5’-AGAGTTTGATCCTGGCTCAG-3’; P6, 5’-GTACGCGTACCTTGTAGCA-3’) and the rpoB gene (CM7, 5’-AACCAGTTCCGCGTTGGCCTGG-3’; CM31bp, 5’-CCTGGAACAAACGCCTCGGA-3’) (Iversen et al., 2007; Mollet et al., 1997). PCR procedures followed Iversen et al. (2007) and Mollet et al. (1997). Primer synthesis and sequencing of products were completed by Shanghai Shenergy-Biocolour Company, Shanghai, China. The resultant sequences were compared to sequences from representative species from GenBank and were aligned with CLUSTAL_X (Jeanmougin et al., 1998). Phylogenetic relationships were determined by the neighbour-joining method (Saitou & Nei, 1987) with the program package MEGA 4.0 (Tamura et al., 2007). Bootstrap confidence values were obtained using 1000 resamplings.

The 16S rRNA gene sequences of isolates R18-2T and R3-3 showed 99.4% similarity to each other and formed a monophyletic group within the genus Enterobacter (Fig. 1). The 16S rRNA gene sequence similarity to the type strain of the type species of the genus, Enterobacter cloacae, was 98.08%. Strains R18-2T and R3-3 grouped most closely with a cluster containing the type strains of Enterobacter nimpresuralis, E. amnigenus, E. cancerogenus and E. asburiae, with 97.95–98.89% sequence similarity (Fig. 1). 16S rRNA gene sequence analysis therefore indicated that both strains belong to the genus Enterobacter; however, based on the 16S rRNA gene sequence alone, the novel strains could not be allocated unequivocally to a narrower taxonomic level. Since rpoB sequence analysis has been used successfully for species discrimination within the family Enterobacteriaceae (Drancourt et al., 2001; Kämper et al., 2005; Li et al., 2004; Madhaiyan et al., 2010; Mollet et al., 1997), this approach was used to determine the taxonomic position of the two strains. The rpoB sequences of the two isolates showed 99.19% sequence similarity to each other, indicating that they probably belong to the same species. The highest rpoB sequence similarities were obtained with the type strains of E. cancerogenus (95.99%), E. cloacae subsp. dissolvens (97.49%), E. cloacae subsp. cloacae (97.70%) and E. asburiae (97.83%).

The phylogenetic branch formed by E. cancerogenus, E. asburiae and the novel strains was supported by a high bootstrap value (85% in neighbour-joining analysis) (Supplementary Fig. S1, available in IJSEM Online), confirming that the novel strains belong to the genus Enterobacter. The similarity with their nearest neighbours was low (95.99–97.83%) compared with the intraspecies similarity range of 98–100% found within the family Enterobacteriaceae (Mollet et al., 1997). The result confirmed that the two isolates represented a novel species within this family. The phylogenetic results were also consistent with our previous report that R18-2T was close to E. cancerogenus (Wang et al., 2010).

The phenotypic features described previously (Peng et al., 2009) were used to group the isolates. Phenotypic analyses covered utilization of sugars, amino acids, alcohols and organic acids as sole carbon sources, determined using Biolog GN2 MicroPlates (Madhaiyan et al., 2010). Tests for motility and indole production were performed in SIM medium (Amresco) at 37 °C. A similarity index based on phenotypic features was analysed in our previous paper (Wang et al., 2010); the results suggested that these isolates are members of either E. cancerogenus or E. asburiae or a closely related species.

Biochemical characteristics were tested by using the API 20E kit (bioMérieux). The data revealed that the isolates could be differentiated from their nearest neighbours by the presence of lysine decarboxylase activity and their ability to utilize d-arabitol (Table 1).

The DNA G+C content of strains R18-2T and R3-3 was determined according to the HPLC method (Mesbah et al., 1989). The values (means of three independent analyses of the same DNA sample) for R18-2T and R3-3 were 55.1 ± 0.5 mol%. These values are consistent with the DNA G+C contents of other members of the genus Enterobacter (Stephan et al., 2007, 2008).

Total cellular fatty acids were extracted from 48-h-old bacterial cells cultured at 28 °C on tryptic soy broth agar (BD Difco). Gas chromatography using an Agilent 6890 gas chromatography system was conducted by using the Microbial Identification System software (MISE6.0). Results were compared with the MIDI identification database TBSA50, version 5.00 (MIDI Inc.). The fatty acid profile of strain R18-2T contained summed feature 2 (iso-C16:1 1 and/or C14:0 3-OH), C14:0, summed feature 3 (C16:1 iso and/or iso-C15:0 2-OH), C18:1 iso and C17:0 cyclo and C16:0 as major constituents (Supplementary Table S1).

Finally, to confirm whether the novel strains represent an independent genospecies within the genus Enterobacter,
DNA–DNA hybridizations were performed. DNA–DNA relatedness was determined by the fluorimetric method (Gonzalez & Saiz-Jimenez, 2005). Thermal denaturation was quantified from SYBR green fluorescence measurements, using an ABI Prism 7500 Realtime PCR System. The accompanying software was used to generate melting curves. The temperature at which 50 % of the initial double-stranded molecules have denatured (50 % fluorescence) is called the thermal denaturation midpoint ($T_m$), and $\Delta T_m$ is the difference between the $T_m$ of the reference strain and the $T_m$ of the hybrid DNA. Its value is used to measure the DNA–DNA relatedness; if the $\Delta T_m$ between homologous and hybrid DNA is greater than 5 °C, the DNAs are considered to originate from members of distinct species. Thermal denaturation curves are shown in Fig. 2 and $T_m$ and $\Delta T_m$ values are given in Supplementary Table S2. This experiment was repeated five times. The DNA–DNA hybridization results revealed that strains R18-2 and R3-3 showed very high DNA–DNA relatedness ($\Delta T_m > 5 ^\circ C$), and they therefore belong to the same genomospecies. Strain R18-2 showed low levels of DNA–DNA relatedness ($\Delta T_m < 5 ^\circ C$) with its close relatives Enterobacter amnigenus LMG 2784T ($\Delta T_m 19.2 ± 1.6 ^\circ C$), Enterobacter cloacae subsp. dissolvens LMG 2683T ($\Delta T_m 14.8 ± 1.8 ^\circ C$), Enterobacter cloacae subsp. cloacae ATCC 13047T ($\Delta T_m 5.7 ± 1.2 ^\circ C$) and Enterobacter hormaechei ATCC 10341 ($\Delta T_m 5.9 ± 1.0 ^\circ C$). Strain R18-2 showed low levels of DNA–DNA relatedness ($\Delta T_m < 5 ^\circ C$) with its close relatives Enterobacter amnigenus LMG 2784T ($\Delta T_m 19.2 ± 1.6 ^\circ C$), Enterobacter cloacae subsp. dissolvens LMG 2683T ($\Delta T_m 14.8 ± 1.8 ^\circ C$), Enterobacter cloacae subsp. cloacae ATCC 13047T ($\Delta T_m 5.7 ± 1.2 ^\circ C$) and Enterobacter hormaechei ATCC 10341 ($\Delta T_m 5.9 ± 1.0 ^\circ C$). Strain R18-2 showed low levels of DNA–DNA relatedness ($\Delta T_m < 5 ^\circ C$) with its close relatives Enterobacter amnigenus LMG 2784T ($\Delta T_m 19.2 ± 1.6 ^\circ C$), Enterobacter cloacae subsp. dissolvens LMG 2683T ($\Delta T_m 14.8 ± 1.8 ^\circ C$), Enterobacter hormaechei ATCC 10341 ($\Delta T_m 5.9 ± 1.0 ^\circ C$).
**Table 1.** Phenotypic characteristics that differentiate the novel strains R18-2$^T$ and R3-3 from related species of the genus *Enterobacter*

| Characteristic                                        | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
|------------------------------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Voges–Proskauer reaction                             | +  | +  | +  | +  | +  | +  | +  | +  | +  | –  | –  | +  | –  | –  | –  | +  | +  | –  | +  | –  | +  | +  | +  | +  | +  | +  | +  |
| Methyl red test                                       | –  | +  | –  | –  | –  | –  | –  | –  | –  | +  | +  | –  | –  | –  | –  | +  | +  | –  | +  | –  | +  | +  | +  | +  | +  | +  | +  |
| Ornithine decarboxylase                               | +  | +  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Arginine dihydrolase                                  | +  | +  | +  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Motility                                             | +  | +  | –  | +  | +  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Aesculin hydrolysis                                   | +  | +  | +  | +  | +  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Lysine decarboxylase                                  | +  | +  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Carbon source utilization                             | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Sucrose                                              | +  | +  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Melibiose                                            | +  | –  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| D-Arabinol                                           | +  | +  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| D-Sorbitol                                           | +  | +  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| L-Fucose                                             | +  | –  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Putrescine                                           | +  | +  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| α-L-Rhamnose                                         | +  | +  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Citrate                                              | +  | +  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| Mucate                                               | +  | +  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |
| 1-O-Methyl α-D-galactopyranoside                      | +  | –  | –  | –  | –  | –  | –  | –  | –  | –  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  | +  |

$\text{cloacae ATCC 130477}^T$ (14.5 ± 1.5 °C), *E. asburiae* ATCC 35953$^T$ (9.1 ± 0.6 °C) and *E. cancerogenus* LMG 2693$^T$ (7.5 ± 1.1 °C), indicating that the new isolates are genotypically different from these type strains.

The 16S rRNA and rpoB gene sequence similarity data and DNA–DNA hybridization differentiated the two isolates from other members of the genus *Enterobacter*. Furthermore, these strains produced lysine decarboxylase and utilized D-arabitol, while their nearest neighbours did not. Based on these results and the current definition of bacterial species, we propose the classification of the novel pathogenic bacteria isolated from white mulberry (*Morus alba* L. 'HuSang') as members of the novel species *Enterobacter mori* sp. nov.

**Fig. 2.** Thermal denaturation of genomic DNA from R18-2$^T$ with itself and with DNA from strain R3-3 and other *Enterobacter* type strains phylogenetically related by 16S rRNA and rpoB gene sequence analysis.
**Description of Enterobacter mori sp. nov.**

*Enterobacter mori* (mo’ri L. gen. n. mori of a mulberry tree, of *Morus*, the generic name of the white mulberry, *Morus alba* L., from which the first strains were isolated).

Cells are Gram-negative, motile, short rods, 0.3–1.0 μm wide and 0.8–2.0 μm long, with peritrichous flagella, appearing singly or in pairs. Facultatively anaerobic. Growth occurs at 20–30 °C with an optimum at 28 °C. Grows at pH 4–10, with optimal growth at pH 7. Colonies are cream in colour on YDC agar and dark pink with translucent margins on TZC agar. In API 20E tests, positive for catalase, oxidase, ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, asacilin hydrolysis, Voges–Proskauer reaction and gelatin liquefaction; negative for methyl red test and indole production. Using Biolog GN2 microplates, positive for utilization of citrate, dextrin, glycerol, melibiose, Tween 40 and 80, N-acetyl-D-glucosamine, L-arabinose, cellobiose, D-arabitol, D-fructose, D-galactose, gentiobiose, D-D-glucose, myo-inositol, maltose, D-mannitol, D-mannose, methyl β-D-glucoside, D-psicose, raffinose, L-β-thalianose, D-sorbitol, sucrose, trehalose, turanose, methyl pyruvate, citric acid, cis-aconitic acid, formic acid, D-galactonic acid-γ-lactone, D-galacturonic acid, D-glucuronic acid, D-glucuronic acid, 2,4,6-trinitrotoluene. This work was supported by the National Natural Science Foundation of China (30871655), the Agriculture Ministry of China (201003029; nhyzx072056), the Zhejiang Agriculture Department (J-fa 2008–97) and the 863 project (2006AA10211). We thank the BCCM/LMG Bacteria Collection, University of Ghent, Belgium, for providing most of the type strains used in this study.

**References**


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