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SHORT COMMUNICATION

# Characterization of *Pectobacterium carotovorum* subsp. *carotovorum* causing soft-rot disease on *Pinellia ternata* in China

**Xiu-Fang Hu · Fei-Xiang Ying · Yu-Bo He · Yuan-Yuan Gao · Hai-Min Chen · Ji-Shuang Chen**

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**Abstract** *Pinellia ternata* is a traditional Chinese herb which has been used in China for over 1,000 years. A soft-rot disease characterized by water-soaked lesions and soft-rot symptoms with a stinking odour was commonly observed in cultivated fields of this plant, and *Pectobacterium*-like bacteria were consistently isolated from the infected tissues. Two typical strains (SXR1 and ZJR1), isolated from Shanxi and Zhejiang, respectively, were identified. Pathogenicity tests revealed that these strains were virulent to *P. ternata* and induced the same symptoms as observed in the field. Characterization involving fatty acid profile, metabolic and physiological properties, 16S rDNA sequence and PCR-RFLP identified both isolates as *P. carotovorum* subsp. *carotovorum* (Pcc). The 16S rDNA of both isolates shared 97–99% sequence similarity with that of Pcc strains. The phylogenetic trees showed that both isolates were clustered in the group of Pcc and *P. carotovorum* subsp. *odorifera* and both PCR-RFLP profiles were consistent with the pattern E produced by the minority of Pcc strains. Thus, isolates SXR1 and ZJR1 were characterized as Pcc in spite of some differences. This is the first report that Pcc has been proven as a causal agent of soft-rot disease on *P. ternata*.

**Keywords** *Pinellia ternata* · Soft-rot disease · Characterization · Pathogen · *Pectobacterium carotovorum* subsp. *carotovorum*

**Abbreviations**
Pcc *Pectobacterium carotovorum* subsp. *carotovorum*

*Pinellia ternata (Araceae)* is a perennial medicinal herb that grows in the eastern part of Asia, mainly in China. As a natural medicine documented in the Chinese Pharmacopoeia, it has been used for more than 1,000 years in anti-vomiting, anti-coughing, analgesic and sedative efficacy (Luo et al. 2000), and novel functions of resisting viruses (Nagai et al. 2002) and terminating early pregnancy (Mao and Peng 2002) have been found recently. To meet increasing high demand and to protect the wild resource, *P. ternata* has been cultivated in China since the 1970s (Mao and Peng 2002).

A soft-rot disease causing major loss of yield has been noticed in cultivated *P. ternata*; however, the pathogen has not been isolated and characterized (Tang et al. 2005). According to our survey from 2004 to 2005, the disease occurs generally in summer

X.-F. Hu · F.-X. Ying · Y.-Y. Gao · H.-M. Chen · J.-S. Chen (✉)
Institute of Bioengineering, Zhejiang Sci-Tech University, Road 2, Xiasha,
Hangzhou 310018, People's Republic of China
e-mail: chenjs@zstu.edu.cn

Y.-B. He
College of Life Science, Dalian Nationalities University, Dalian 116600, People's Republic of China

and spreads widely in the Provinces Shanxi and Zhejiang, where *P. ternata* has been cultivated for a long time. *Pectobacterium*-like isolates were found to be associated with this soft-rot disease in our preliminary experiments. The objective of this study was to identify the causal agent through pathogenicity, phenotypical and phylogenetical characterization.

In July 2006, 50 infected samples of *P. ternata* were collected from two fields located in Shanxi and Zhejiang, respectively. Two types of bacteria, *Pectobacterium*-like and *Pseudomonas*-like, were isolated from the symptomatic tuber tissues on nutrient agar (NA) as described by Wright (1998). However, only *Pectobacterium*-like bacteria were proven to be correlated with the soft-rot disease of *P. ternata*, and were isolated from all the diseased samples consistently. Two typical *Pectobacterium*-like isolates, SXR1 and ZJR1, isolated from Shanxi and Zhejiang, respectively, were selected for further study. Reference strains Ecc01 (isolated from calla; Zhao et al. 2001) and ATCC15713, were ordered from the Chinese General Microbiological Culture Collection Centre (Beijing, China) and American Type Culture Collection (USA), respectively.

Using sterile water and strain Ecc01 as controls, pathogenicity was confirmed by pricking bacterial suspensions ($1\times10^8$ CFU $ml^{-1}$ ) into tubers of 6 week-old *P. ternata* plants of peach-leaved and willow-leaved cultivation groups (Azad et al. 2000). Inoculated plants were transferred to pots filled with sterile soil, and five pots were used for each treatment. These plants were kept in a growth chamber at 25°C with humidity ranging from 80 to 90%. Isolates SXR1 and ZJR1 both induced typical soft-rot symptoms on the two cultivation groups as observed in the field in May, July, September and November (Fig. 1; data on peach-leaved group not shown). Water-soaked lesions were first observed on the stem-base, and then the plant collapsed 36 h post-inoculation, although the upper portion remained asymptomatic. Subsequently, the lesions expanded rapidly over the entire plant. The macerated tuber was usually reduced to a whitish, mushy and foul-smelling pulp surrounded by undecayed periderm. The infected

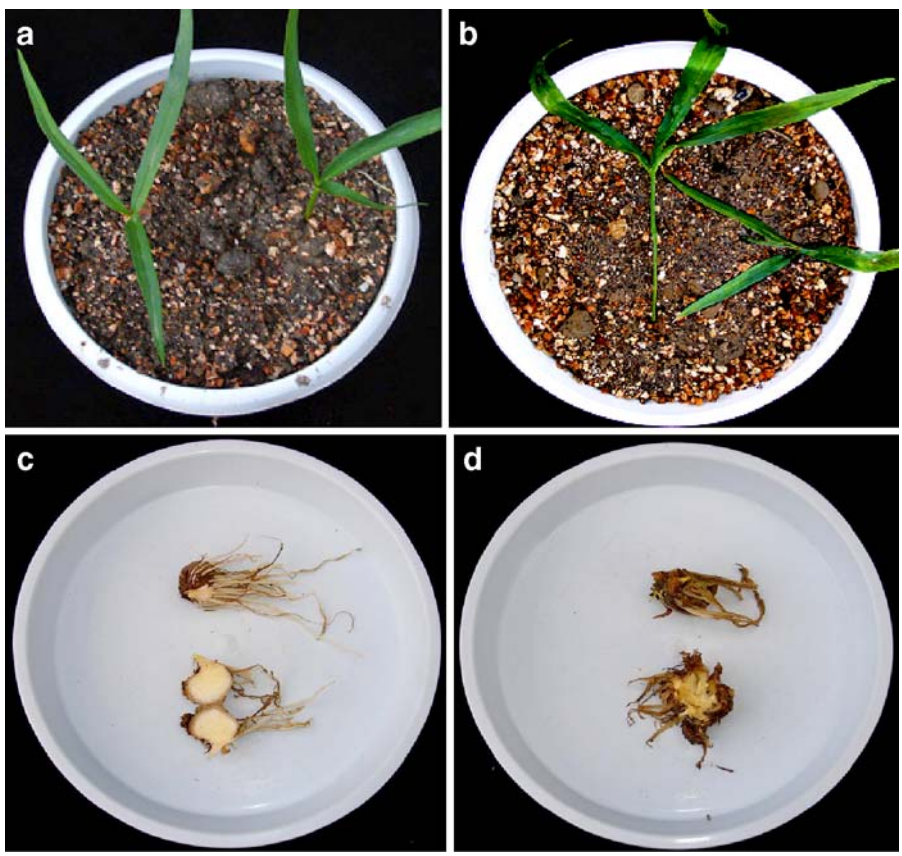


**Fig. 1** Disease symptoms of willow-leaved *P. ternata* associated with pathogenic isolates in the greenhouse at 48 h post-inoculation. **a** Control plants inoculated with sterile water. **b** Symptoms of *P. ternata* induced by pathogenic isolates. **c**–**d** Tubers and roots from control and soft-rotted plant, respectively

roots became transparent and slimy, until completely decayed (Fig. 1d). The disease with similar symptoms induced by strain Ecc01 progressed more slowly. Control plants treated with sterilized water showed no symptoms. The same bacteria as the original inocula were re-isolated from symptomatic tubers, demonstrating that isolates ZJR1 and SXR1 were the casual agents of the soft-rot disease.

Both isolates were further characterized in morphology and physiology through standard methods, with Ecc01 and ATCC15713 as reference strains. They were also identified for production of different enzymes on crystal violet pectate (CVP; Perombelon et al. 1987) and carboxyl methyl cellulose (CMC; Cantwell and McConnell 1983). Both isolates were morphologically similar to the reference strains when incubated at 30°C for 48 h. They formed circular, translucent, slightly convex and entire colonies with regular edges, and did not produce any pigment. The cells were Gram-negative and rod-shaped, with a peritrichous flagella arrangement. On the CVP medium, both isolates had circular, smooth and concave colonies. On the CMC medium, both isolates induced clear zones larger than those produced by Ecc01, indicating a comparatively higher activity of cellulase. The two isolates had similar physiological and biochemical characteristics to strain ATCC15713 (Table 1) except that they could grow at 39°C and utilize tartrate.

The metabolic profiles were determined using the Microlog GN2 microplates system, and the data were analyzed with MicroLog™ version 3.5 database

**Table 1** Selected biochemical and physiological characteristics of the reference strains and bacterial isolates causing soft-rot of *P. ternata*

| Characteristics | SXR1 | ZJR1 | Ecc01 | ATCC15713 |
|---|---|---|---|---|
| Oxidative glucose fermentation | + | + | + | + |
| Pectate hydrolysis | + | + | + | + |
| Catalase | + | + | + | + |
| Oxidase | − | − | − | − |
| Production of reducing substances from sucrose | − | − | − | − |
| Growth at 39°C | + | + | + | − |
| Growth in 7% NaCl | + | + | + | + |
| Urease | + | + | + | + |
| Gas production from D-Glucose | + | + | + | + |
| Gelatin liquefaction | + | + | + | + |
| Nitrate reduction | + | + | + | + |
| Lecithin hydrolysis | − | − | − | − |
| Cellulase | + | + | + | + |
| Acid production from | | | | |
| D-Arabinose | − | − | − | − |
| Lactose | + | + | + | + |
| Melibiose | + | + | + | + |
| a-Methyl glucoside | − | − | − | − |
| Raffinose | + | + | + | + |
| D-Glucose | + | + | + | + |
| Utillization of | | | | |
| Citrate | + | + | + | + |
| Malonate | − | − | + | − |
| Tartrate | + | + | + | − |
| D Arabinose | + | + | + | − |
| D-Cellobiose | + | + | + | + |
| Trehalose | + | + | + | + |
| D-Galacturonate | + | + | + | + |
| Sorbitol | − | − | − | − |
| Arabitol | − | − | − | − |

+ Positive, − negative.

software. The Microlog system identified the two isolates as *P. carotovorum* subsp. *carotovorum* (Pcc), based on a similarity index of more than 50% (66.9% for SXR1, and 61.6% for ZJR1). Although both isolates were identified as the same subspecies, they had different biochemical profiles. Compared with strain ATCC15713, isolate ZJR1 could not oxidize Tween 40, Lactulose, D-Melibiose, D-Glucuronic Acid, Succinamic Acid, Inosine, Uridine and Thymidine, and isolate SXR1 could not oxidize L-Lactic Acid and Succinamic Acid. Thus, they were regarded as metabolically dissimilar.

Gas chromatography of fatty acid methyl esters (FAME) was analyzed using the MIS (Sherlock Microbial Identification System, Newark, DE, USA) standard method (Sasser 1990). The major fatty acid composition of isolates SXR1 and ZJR1 was similar to that of strain ATCC 15713 (Table 2). The MIS identification programme judged SXR1 and ZJR1 as Pcc, with maximum similarities of 78.2 and 72.9%, respectively. However, fatty acids ratios 12:0/14:0 and 16:0/12:0 demonstrated that both isolates were, to some degree, different from the typical strains of Pcc, even though the isolate SXR1 matched the ratio 16:0/12:0 and the isolate ZJR1 matched the ratio 12: 0/14: 0.

The G+C content was determined from thermal denaturation temperature as described by Marmur and Doty (1962). The G+C mol% contents of isolates SXR1 and ZJR1 were 51.0 and 51.3 mol%, respectively. The values were consistent with 50.5 to 53.1 mol% reported for Pcc (Hauben et al. 1998).

A phylogenesis analysis was performed on the basis of 16S rDNA sequence. Polymerase chain reaction (PCR) and sequencing of the 16S rDNA were carried out as described by Hu et al. (2006). DNA sequence similarity was analyzed by NCBI BLAST server (http://www.ncbi.nlm.nih.gov), and phylogenetic trees were constructed using three methods: neighbour-joining (bioNJ), maximum parsimony (MP) and maximum likelihood (ML). The 16S rRNA gene sequences of isolates SXR1 and ZJR1 have been submitted into GenBank Data under the accession numbers of DQ785511 and DQ785510, respectively.

Phylogenetic analysis revealed that both isolates shared 99.9% homology, and they had a 97–99% maximum similarity with Pcc strains. The phylogenetic trees constructed by the above methods (data of MP and ML not shown) showed that isolates SXR1 and ZJR1 were grouped together with *Pectobacterium* species, especially with Pcc and *P. carotovorum* subsp. *odorifera* in one cluster. However, they formed a unique cluster independent of the two subspecies together with Pcc strains Ecc01, M1 and M3 from China (Fig. 2).

To clarify the relationship of both isolates with the two subspecies, PCR-based specific detection and RFLP analysis were performed as described by Kang

**Table 2** Relative fatty acid compositions of the pathogenic isolates and strain ATCC15713

| Feature[a] | Percentage of total ($n$=3) | | |
|---|---|---|---|
| | SXR1 | ZJR1 | ATCC15713 |
| 12:0 | 8.34±0.425 | 8.90±0.379 | 6.76±0.413 |
| 13:0 | 0.16±0.007 | 0.16±0.005 | 0.88±0.025 |
| 14:0 | 2.37±0.107 | 2.17±0.114 | 1.60±0.102 |
| Unknown 14.502 | 0.51±0.022 | 0.54±0.019 | 1.17±0.109 |
| 15:0 | 0.30±0.013 | 0.43±0.016 | 1.75±0.107 |
| Summed feature 2 | 8.12±0.415 | 8.90±0.565 | – |
| Summed feature 3 | 33.69±1.894 | 32.27±2.135 | – |
| 16:0 | 27.33±1.425 | 25.68±1.241 | 27.72±1.388 |
| 17:1 w8c | 0.22±0.006 | 0.31±0.008 | 0.89±0.014 |
| 17:0 | 0.29±0.013 | 0.53±0.014 | 1.45±0.112 |
| 18:1 w7c | 18.20±0.751 | 17.90±0.832 | 20.35±0.926 |
| 18:0 | 0.25±0.015 | 0.25±0.009 | 0.32±0.017 |
| 12:0/14:0 | 3.52 | 4.10 | 4.21 |
| 16:0/12:0 | 3.28 | 2.89 | 4.10 |

[a] Summed feature 2 comprises any combination of 14:0 3OH and 16:1 iso I. Summed feature 3 comprises 15:0 iso 2OH, 16:1 w7c, or both.

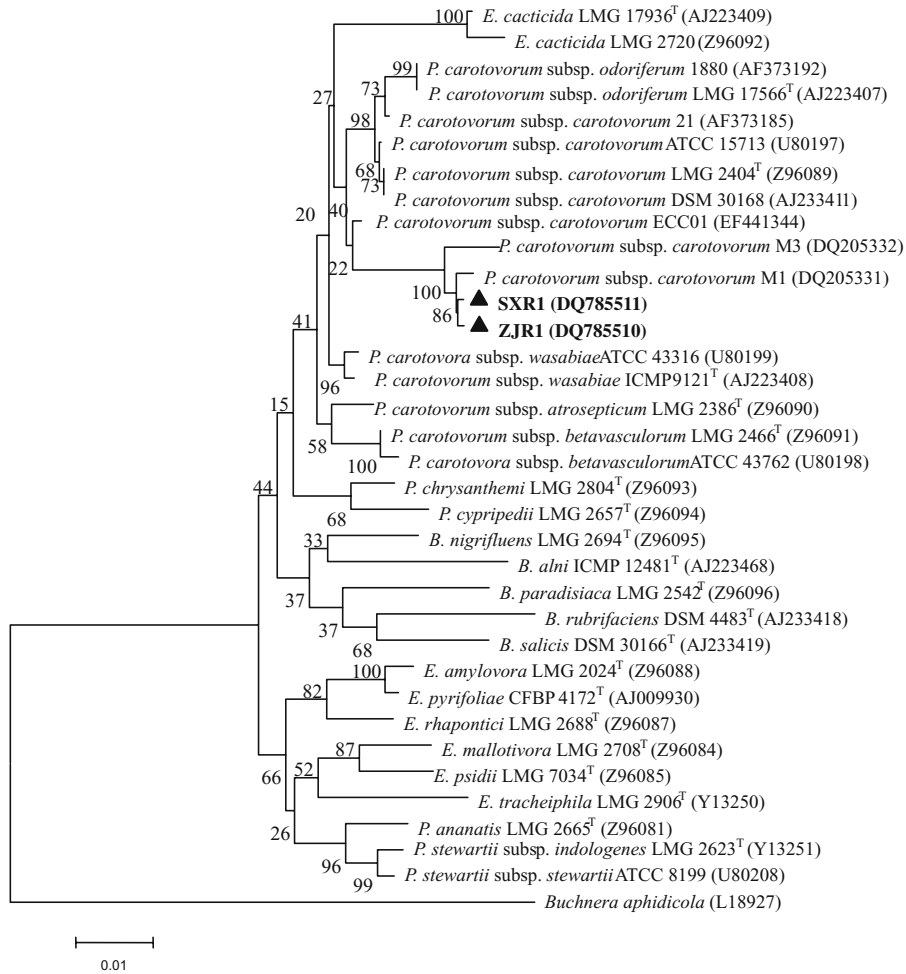


**Fig. 2** Phylogenetic tree showing the relationship of the pathogenic isolates and the closely related strains of plant-pathogenic *Enterobacteriaceae*. On the basis of the alignment of 16S rDNA sequences, a phylogenetic tree was constructed using the neighbour-joining method. Stability of the tree was assessed by 1,000 bootstrap replications with Felsenstein confidence limits. The sequence of *Buchnera aphidicola* was used as an outgroup

et al. (2003). Banding patterns were observed on 3% NuSieve GTG agarose gel (Flowgen, Ashiby de la Zouch, UK) in TBE. A 550 bp DNA fragment was amplified from each of SXR1, ZJR1 and Ecc01, consistent with Pcc as reported by Kang et al. (2003; Fig. 3a). Two bands of 350 bp and 200 bp were observed from both isolates and strain Ecc01 after digestion with restriction endonuclease *Rsa* I. The pattern was consistent with profile E which was one of the five polymorphic Pcc patterns.

Both phenotypic and phylogenetic analyses identified isolates ZJR1 and SXR1 as Pcc yet with some differences, and PCR-RFLP analysis confirmed that they belonged to the minority of Pcc. Soft-rot diseases are usually associated with *P. carotovorum* (Kotoujansky 1987), of which Pcc is known to have a broad host range and is variable in pathogenicity and genetic diversity (Smith and Bartz 1990). The discovered Pcc strains are reported as pathogens infecting plants in family *Solanaceae*, *Apiaceae*, *Brassicaceae*, *Cucurbitaceae* and *Liliaceae* (Toth et al. 2003). Seldomly reported to be associated with the plants in family *Araceae,* Pcc was first found to be

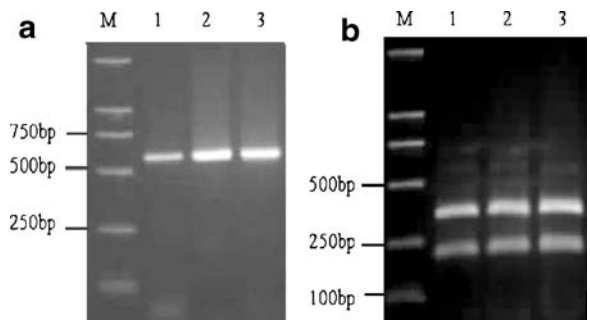


**Fig. 3** Subspecies-specific PCR amplification and restriction enzyme-digested profiles of the pathogenic isolates and strain Ecc01. Amplified products using primers EXPCCF/EXPCCR (**a**) were digested with restriction endonuclease *Rsa*I (**b**). Lane M, 100 bp ladder; lanes 1–3, isolates SXR1, ZJR1 and strain Ecc01, respectively

correlated with the soft-rot disease of *P. ternata*. Differences existed between the two isolates and other Pcc strains from Europe and America, which might be caused by geological distribution or diversities of host adaptation; further evidence is needed to confirm their origins.

Taken together, isolates SXR1 and ZJR1 were identified as Pcc, and they were the causal agents inducing soft-rot disease of *P. ternata*.

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