Characteristics and Rapid Diagnosis of *Pectobacterium carotovorum* ssp. Associated With Bacterial Soft Rot of Vegetables in China

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Abstract

Pectobacterium carotovorum, a causal agent of vegetable soft rot, contains three valid subspecies: *P. carotovorum* subsp. *carotovorum* (*Pcc*), *P. carotovorum* subsp. *brasiliensis* (*Pcb*), and *P. carotovorum* subsp. *odoriferum* (*Pco*). Using 16S rDNA sequencing and genus-specific PCR, we identified 72 *P. carotovorum* strains from Chinese cabbage, bok choy, and celery and assessed their pathogenicity on Chinese cabbage petioles and potato tubers. Based on phylogenetic analysis of *pmrA* sequences and confirmation by subspecies-specific PCR, the strains were divided into 18 *Pcc*, 29 *Pco*, and 25 *Pcb*. Several characteristic features were also assessed and supported the distinctiveness of the *Pco* strains. All *P. carotovorum* strains caused soft rot symptoms on Chinese cabbage and potato, but the *Pco* strains exhibited the greatest severity. We developed a conventional PCR and a quantitative PCR

(qPCR) assay for the identification of *Pco* based on its specific *srI*E gene encoding sorbitol-specific phosphotransferase. These two methods could specifically amplify the expected products of 674 and 108 bp, respectively, from all of the *Pco* strains. The assays demonstrated high sensitivity and could detect as little as 1 and 100 pg/μl of bacterial genomic DNA, respectively. Both assays could also detect the pathogens directly from plant tissues infected with as little as 2.5×10^{-2} CFU/mg of *Pco*, even before external symptoms appeared. These assays constitute effective tools for disease diagnosis and the rapid identification of soft rot pathogens.

Keywords: identification, *Pectobacterium carotovorum* subsp. *odoriferum*, qPCR, subspecies-specific PCR, virulence

Pectobacterium spp. are necrotrophic pathogens responsible for a wide range of diseases of important crops and ornamental plants worldwide, causing considerable economic and yield losses in both field production and storage (Onkendi and Moleleki 2014). This genus has been subjected to extensive taxonomic research, which has resulted in a current division into 12 recognized species: *P. carotovorum*, *P. atrosepticum*, *P. aroidearum*, *P. betavascularum*, *P. cacticidum*, *P. wasabiae*, *P. zantedeschiae*, *P. parmentieri*, *P. peruvienne*, *P. polaris*, *P. punjabense*, and *P. aquaticum* (Dees et al. 2017; Khayti et al. 2016; Pédrón et al. 2019; Sarfraz et al. 2018; Waleron et al. 2018). In contrast with other species with a more restricted host range (Gardan et al. 2003), *P. carotovorum* is considered to be one of the most devastating plant pathogens that causes soft rot disease globally and possesses the widest host range of all of the soft rot bacteria (Davidsson et al. 2013; Mansfield et al. 2012).

P. carotovorum consists of three morphologically similar but phylogenetically distinct subspecies (Nabhan et al. 2012a), of which *P. carotovorum* subsp. *brasiliense* (*Pcb*) and *P. carotovorum* subsp. *carotovorum* (*Pcc*) have been studied extensively because of their damage to a variety of crops (Czajkowski et al. 2009; Waleron et al. 2015), including Chinese cabbage, celery, and potato (Pritchard et al. 2016). The other recognized subspecies is *P. carotovorum* subsp. *odoriferum* (*Pco*), which was originally considered to have

a narrow host range and was isolated mainly from chicory (*Cichorium intybus*) (Gallois et al. 1992). However, because increasing numbers of *Pco* strains have been isolated from various vegetables, including leek, celery, cabbage, Chinese cabbage, artichoke, carrot, parsley, onion, sugar beet, and sweet potato, in many countries (Gao et al. 2016; Oskiera et al. 2017; Waleron et al. 2014), *Pco* is now recognized as an important causal pathogen of bacterial soft rot disease on vegetables. Our previous comparative genomics study indicated that the *Pco* strains possess several subspecific genes/clusters related to virulence, such as an additional type six secretion system (T6SS), compared with the other closely subspecies *Pcb* and *Pcc* (Li et al. 2018); however, the virulence differences among the three subspecies remain unclear. Considering that all three of these subspecies could cause serious and extensive vegetable loss worldwide, it is important to evaluate their population structure and virulence. This would provide useful information in developing an integrated management strategy against these pathogens.

Some progress has already been made toward the rapid detection and identification of *P. carotovorum* ssp. Although various phenotypic features that differentiate the subspecies have been reported, most of them vary depending on the isolate and growth conditions (Czajkowski et al. 2015). Sequencing of the 16S rDNA gene is an accurate method for identifying soft rot bacteria at the genus or species level, but this method lacks resolution at the subspecies level (Naum et al. 2008; Staley 2006). Many other molecular methods, such as amplified fragment length polymorphism and multilocus sequence analysis, have been successfully used for differentiating *Pcc*, *Pcb*, and *Pco* strains but typically involve repetitive, time-consuming cloning processes and procedural difficulties (Nabhan et al. 2012b). Phylogenetic analysis based on the single gene *pmrA*, which is required for resistance to plant-derived antimicrobial peptides in *Pectobacterium* spp., has become an efficient and rapid tool for subspecies identification (Kettani-Halabi et al. 2013).

Specific PCR constitutes a rapid and inexpensive method for the taxonomic classification of soft rot pathogens. Genus-specific PCR primers, such as Y1/Y2 for *Pectobacterium* spp. (Darrasse et al. 1994), and species-specific primers, such as Eca1f/Eca2 for *P. atrosepticum* (De Boer and Ward 1995) and PhF/PhR for *P. wasabiae* (De Boer et al. 2012), have been successfully used for the

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identification of *Pectobacterium* strains. In addition, two subspecific PCR primers, namely BR1f/L1r (Duarte et al. 2004) and EXPCCR/EXPCCF (Kang et al. 2003), for the identification of *Pcb* and *Pcc* strains, respectively, have been published. However, a later study found that EXPCCR/EXPCCF is not universal for all genotypes of *Pcc* subspecies (De Boer et al. 2012). To our knowledge, no *Pco*-specific primer has been reported. Thus, the currently available primers do not allow for the clear separation of all *Pectobacterium* subspecies from one another. This study developed two *Pco*-subspecific primers for conventional PCR and quantitative PCR (qPCR) based on six genes (i.e., *srlAEBDMR*) of a *Pco*-subspecific *srl* operon related to sorbitol metabolism (Li et al. 2018), providing rapid PCR/qPCR-based identification tools for *Pco*. Using these, we identified newly isolated *P. carotovorum* strains to assess the current distribution of these *P. carotovorum* ssp. in economically important vegetables that are severely impacted by bacterial soft rot disease in China. We also compared the ability of these subspecies to induce disease symptoms on both Chinese cabbage petioles and potato tubers.

Materials and Methods

Bacterial strains and culture conditions. A total of 72 strains from the petioles of 72 different plant samples of three economically important Chinese vegetables, namely Chinese cabbage (*Brassica rapa* subsp. *pekinensis*), bok choy (*B. rapa* subsp. *chinensis*), and celery (*Apium graveolens*), growing in different fields and exhibiting soft rot symptoms were collected from seven districts of Beijing, China (Supplementary Table S1). These strains were preserved at the Beijing Key Laboratory of Agricultural Genetic Resources and Biotechnology, Beijing Academy of Agriculture and Forestry Science. Isolation of bacteria from diseased samples was carried out according to Perombelon and van der Wolf (2002). Single colonies obtained from crystal violet pectate medium were purified at 28°C on Luria-Bertani (LB) agar plates, after which they were stored in 30% glycerol and LB broth medium (vol/vol, 1:1) at -80°C (Czajkowski et al. 2015).

DNA isolation, gene sequencing, and phylogenetic analysis. Total DNA was extracted from each strain using an EasyPure Bacteria Genomic DNA Kit (TransGen Biotech). DNA quantity and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The 16S rDNA and *pmrA* genes were amplified with the primers described in Table 1. The purified *pmrA* products with a length of 666 bp were cloned into the pMD19-T vector (Takara) for sequencing. The *pmrA* sequences of 72 strains, together with the other published *Pectobacterium pmrA* sequences in the NCBI database, were aligned using the ClustalW program of MEGA X software (version 10.0.4) (Kumar et al. 2018), and a phylogenetic tree based on these sequences was constructed using the maximum likelihood (ML) method with 1,000 bootstrap replications

(Kettani-Halabi et al. 2013). All of the 16S rDNA and *pmrA* gene sequences generated in this study were deposited in the GenBank database under the accession numbers shown in Supplementary Table S1.

Subspecific PCR assays. Specific PCR assays for all strains were performed using three frequently used pairs of primers (Table 1), including primers Y1/Y2 to amplify a 434-bp *pelY* fragment for *Pectobacterium* spp. (Darrasse et al. 1994), EXPCCR/EXPCCF to amplify a 550-bp fragment in *Pcc* or *P. wasabiae* (Kang et al. 2003), and BR1f/L1r to amplify a 322-bp fragment only in *Pcb* (Duarte et al. 2004). Here, a conventional PCR primer (srIE-F1: 5'-TGACTGTGGTGGAACTTCG-3'; srIE-R1: 5'-CAGCCAGTGATAACCAACCG-3') was developed for *Pco* based on the gene *srlE* of the *srl* operon to produce a specific 674-bp fragment. PCR amplification for this locus was performed in a total volume of 20 µl containing 1× Taq PCR Master Mix, 10 pM of primers, and 10 ng of DNA. The PCR program was as follows: 95°C for 5 min; 35 cycles at 95°C for 30 s, 62°C for 25 s, and 72°C for 1 min; and 72°C for 5 min. The amplified products were detected by agarose gel electrophoresis. We further developed *Pco*-specific qPCR primers (srIE-qF1: 5'-CGCAGGCAGCATGTGATTTT-3'; srIE-qR1: 5'-CTGTAAGGAAGCGGCCTACC-3') based on the *srlE* sequence, and the qPCR assay of these 72 strains was conducted using TB Green Premix Ex Taq II (Tli RNaseH Plus; Takara). The thermal cycling program was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 68°C for 30 s. The amplified 108-bp fragments were further tested by 3% agarose gel electrophoresis.

Moreover, to check the sensitivity of primers srIE-F1/srIE-R1 and srIE-qF1/srIE-qR1, a test was performed using 10-fold gradient dilutions of purified genomic DNA (100, 10, and 1 ng/µl; 100, 10, and 1 pg/µl; and 100, 10, 1, and 0.1 fg/µl) from the *Pco* BCS7 strain (genome accession number CP009678) (Li et al. 2018). The conventional PCR and qPCR were completed as described above (ddH₂O as the negative control).

To test the applicability of the conventional PCR and qPCR assays on field-plant samples, 1-month-old celery seedlings were transplanted to the field, and plants at leaf stage 7 to 8 were inoculated with bacterial suspensions (5 µl/inoculation site, 2×10^8 CFU/ml) of *Pcc* B5, *Pcb* BC1, *Pco* BCS7 (Li et al. 2018), as well as sterilized water, using a sterilized needle. The infected plants were covered with plastic film to maintain high humidity (Ren et al. 2001b). The tissues of both the local sites with soft rot symptoms and the distant sites (asymptomatic tissue close to the lesions) were collected at 24 h after incubation, and 200 mg per sample was used for total DNA extraction (TransGen Biotech). The experiment was repeated three times. The specific PCR and qPCR assays were used to detect these DNA samples from different plants inoculated with different strains and sterilized water, with the genomic DNA of *Pcc* B5, *Pcb* BC1, and *Pco* BCS7 and the control ddH₂O.

Table 1. Primers used in this study^a

Primer	Primer sequence (5' to 3')	Target organism	Target gene	Tm (°C)	Expected size (bp)	Reference
27f	AGAGTTTGATCCTGGCTCAG	Most bacteria	16S rDNA	50	1,500	Osborne et al. (2005)
1492r	AGGTGATCCAACCGCAGGTT					
F0145	TACCCTGCAGATGAAATTATTGATTGTTGAAGAC	Most Enterobacteriaceae	<i>pmrA</i>	55	666	Kettani-Halabi et al. (2013)
E2477	TACCAAGCTTTGGTTGTTCCTCTTGGTCA					
Y1	TTACCGGACGCCGAGCTGTGGCGT	<i>Pectobacterium</i> spp.	<i>Pel</i>	60	434	Darrasse et al. (1994)
Y2	CAGGAAGATGTCGTTATCGCGAGT					
EXPCCF	GAAGCTTCGACCCGACCTTCTA	<i>Pcc</i> and <i>Pw</i>	Clone pECC2F	60	550	Kang et al. (2003)
EXPCCR	GCGTAATTGCTACCTGCTTAAG					
BR1f	GCGTGCCGGTTTATGACCT	<i>Pcb</i>	Intergenic spacer of rRNA operon	62	322	Duarte et al. (2004)
L1r	CAAGGCATCCACCGT					
srIE-F1	TGACTGTGGTGGAACTTCG	<i>Pco</i>	<i>srlE</i>	62	674	This study
srIE-R1	CAGCCAGTGATAACCAACCG					
srIE-qF1	CGCAGGCAGCATGTGATTTT	<i>Pco</i>	<i>srlE</i>	68	108	This study
srIE-qR1	CTGTAAGGAAGCGGCCTACC					

^a Tm = melting temperature, *Pcc* = *P. carotovorum* subsp. *carotovorum*, *Pw* = *P. wasabiae*, *Pcb* = *P. carotovorum* subsp. *brasiliense*, and *Pco* = *Pectobacterium carotovorum* subsp. *odoriferum*.

To determine the limit of detection of *Pco* in plants using this protocol, the celery plants were inoculated with 10-fold gradient dilutions of *Pco* BCS7 from 10 to 1×10^9 CFU/ml (5 μ l/inoculation site). Total genomic DNA was then extracted from 200 mg of celery stem sample, and the specific PCR and qPCR assays were performed as per the above procedure. Two microliters of DNA was used as the template for detection in different plants inoculated with different dilutions of *Pco*. The experiment was repeated three times.

Pathogenicity testing. To comprehensively evaluate the maceration ability of these *P. carotovorum* strains, Chinese cabbage petioles and potato tubers were selected for pathogenicity tests. Chinese cabbage and potato from a local grocer were surface sterilized with 75% ethanol for 1 min and thoroughly rinsed with sterile distilled water. The Chinese cabbage petioles were inoculated with each strain by injecting a bacterial suspension (5 μ l/inoculation site, 2×10^8 CFU/ml) into the petiole surface, with 5 μ l of sterile

Fig. 1. Maximum likelihood tree based on the entire *pmrA* gene sequences of the 72 soft rot strains. The tree indicates phylogenetic relationships between the strains isolated during this study and other published strains as well as the model strains of species/subspecies. Sequences obtained from GenBank are indicated by accession numbers followed by strain numbers. Bootstrap values (expressed as percentages based on 1,000 replications) are given at the nodes. Bootstrap values < 50% are excluded.

distilled water as a control (Xie et al. 2018). The petioles were placed in separate Petri dishes (9 cm in diameter) containing filter paper moistened with sterile distilled water and sealed with sealing membrane. The diameter of decay surrounding the inoculation point was measured at 6, 10, 24, and 30 h, respectively, at 28°C and >95% RH, as described previously (Łojkowska and Kelman 1994; Waleron et al. 2013). Each strain was represented by three replicate petioles, and the experiment was performed three times.

Sterilized potato tubers were cut into 7- to 8-mm-thick slices. A 5-mm-deep slit was made in the slices and filled with 20 µl of 2×10^8 CFU/ml suspension of each strain (Terta et al. 2010). The control tubers were treated with 20 µl of sterile distilled water. After incubation at 28°C and >95% RH for 24 h, the decay diameter and weight (weight difference of the tubers before and after removing the decayed tissue) were measured. The assay was replicated six times for each strain. The data were subjected to one-way analysis of variance (ANOVA) using SPSS 10.0 software (SPSS Inc.) (Waleron et al. 2014). Means were compared using Duncan's tests, and statistical significance was determined at the 5% level.

Biochemical tests. Five biochemical traits that are known to be distinctive among the subspecies were tested, including acid production ability from α -methylglucoside, D-sorbitol, D-arabitol, palatinose, and inulin (Perombelon and van der Wolf 2002; Schaad et al. 2001). These tests were repeated at least three times for each strain.

Results

Classification of the bacterial strains. A total of 72 strains obtained from the different infected plants yielded 1,400 to 1,500-bp-long fragments from the 16S rDNA amplification. They were identified as *P. carotovorum* based on their high sequence identities (99.6 to 100%) compared with other published *P. carotovorum* strains. In addition, all strains generated a 434-bp PCR product with the *Pectobacterium*-specific primers Y1/Y2, which also supported their classification as *Pectobacterium* spp. (Darrasse et al. 1994) (Supplementary Fig. S1A).

These strains were further characterized by phylogenetic analysis based on the entire *pmrA* gene sequence. All strains yielded a 666-bp fragment with the specific primers for *pmrA* of *P. carotovorum*. In the ML phylogeny, the strains clustered into three main groups, namely the *Pcc*, *Pcb*, and *Pco* subspecies, and no other known *Pectobacterium* species clustered in these groups (Fig. 1). Eighteen strains (including nine strains isolated from Chinese cabbage and nine strains from bok choy) clustered tightly together with the *Pcc*-standard strain DSM 30168^T and other published *Pcc* strains, supported with a bootstrap value of 100%. The *Pcc* strains from China shared 98.5 to 99.6% nucleotide identity with DSM 30168^T from potato collected in Denmark. Twenty-nine strains, including the members from celery (13 strains) and from Chinese cabbage (16 strains), were grouped with the standard strain NCPPB 3839^T and the other *Pco* strain isolated from chicory, supported with a bootstrap value of 100%. These Chinese *Pco* strains shared high identity (99.4 to

Fig. 2. Conventional PCR and quantitative PCR detection limit of *Pectobacterium carotovorum* subsp. *odoriferum* (*Pco*) with the subspecific primers **A**, srIE-F1/srIE-R1 and **B**, srIE-qF1/srIE-qR1, respectively. Lanes 1 to 10 = 100, 10, and 1 ng/μl; 100, 10, 1 pg/μl; and 100, 10, 1, and 0.1 fg/μl of *Pco* BCS7 DNA, respectively; and lane 11 = ddH₂O.

Fig. 3. Conventional PCR and quantitative PCR detection of *Pectobacterium carotovorum* subsp. *odoriferum* (*Pco*) in local and distant sites of infected celery tissues with the subspecific primers **A**, srIE-F1/srIE-R1 and **B**, srIE-qF1/srIE-qR1, respectively. Lanes 1 to 3, 7 to 9, and 13 to 15 = three localized sites with soft rot symptoms from different infected plants after artificial inoculation by *P. carotovorum* subsp. *carotovorum* (*Pcc*) B5, *P. carotovorum* subsp. *brasiliensis* (*Pcb*) BC1, and *Pco* BCS7, respectively; lanes 4 to 6, 10 to 12, and 16 to 18 = three distant sites without soft rot symptoms from different infected plants after artificial inoculation by *Pcc* B5, *Pcb* BC1, and *Pco* BCS7, respectively; lanes 19 to 21 = three different healthy plant samples artificially inoculated by sterilized water; lanes 22 to 24 = DNA of *Pcc* B5, *Pcb* BC1, and *Pco* BCS7, respectively; and lane 25 = ddH₂O.

100%) with the chicory strains NCPPB 3839^T and NCPPB 3841. The sequences of the *Pco* strains isolated from Chinese cabbage shared 99.1 to 100% sequence identity with those of the *Pco* strains isolated from celery.

The remaining 25 strains (nine from Chinese cabbage, 15 from bok choy, and one from celery) grouped into the *Pcb* subspecies group with the standard strain PBR1692^T and other published *Pcb* strains. They were clustered separately from the clade containing the closely related subspecies *Pcc* and *Pco*. The Chinese *Pcb* strains shared 96.2 to 97.9% nucleotide identity with the *Pcb* strain PBR1692^T from potato in Brazil as well as with the *Pcb* strain PcbHPI01 isolated from cucumber (*Cucumis sativus*) in South Africa. The *Pcb* strains isolated from Chinese cabbage in this study had 96.5 to 97.4% sequence identity with the *Pcb* PCC21 strain isolated from Chinese cabbage in Korea. Generally, divisions according to geographical origin and year of isolation were not observed in the case of the strains included in the phylogenetic tree. Furthermore, strains isolated from different hosts also grouped in the same clusters.

Subspecies-specific PCR and the specificity of the two new pairs of primers for *Pco*. The results of the subspecies-specific PCR amplification assays for all strains verified the classification obtained from the phylogenetic tree described above (Supplementary Fig. S1B to D). All 25 *Pcb* strains yielded 322-bp-long fragments with the *Pcb*-specific primers BR1f/L1r, but none of the other strains produced this fragment (Supplementary Fig. S1B). Primers srIE-F1/srIE-R1 for conventional PCR and qPCR primers srIE-qF1/srIE-qR1 were developed based on the gene sequence of *srIE* and were used to identify the Chinese *Pco* strains collected from different hosts and different locations. All *Pco* strains produced the expected 674- and

108-bp products, respectively, regardless of host, geographical origin, and year of collection, whereas no PCR products were generated in the strains from the *Pcc* and *Pcb* groups (Supplementary Fig. S1C and D). Thus, the PCR assay using primers srIE-F1/srIE-R1 as well as the qPCR assay using primers srIE-qF1/srIE-qR1 could be considered as useful tools for identifying *Pco* strains in China.

In contrast, the predicted 550-bp fragments using primers EXPCCR/EXPCCF were amplified not only from all of the *Pcc* strains but also from the other tested strains, including all of the *Pco* strains and some of the *Pcb* strains isolated in this study (*data not shown*). Therefore, the *Pcc*-specific PCR assay could not clearly distinguish the *Pcc* strains from the strains of the two other closely related subspecies.

Sensitivity and efficiency of the new PCR/qPCR assays for *Pco*. Sensitivity tests were carried out using different concentrations of genomic DNA of the *Pco* BCS7 strain as a template, and the PCR/qPCR amplifications were conducted three times for each DNA concentration. The results of these repeats were congruent, and positive amplifications were detected through agarose gel electrophoresis when 100 pg/μl to 100 ng/μl of template DNA was used, whereas no respective fragments were obtained when a lower concentration of template DNA was used for qPCR analysis (Fig. 2A). In contrast, the conventional PCR analysis could detect as little as 1 pg/μl of bacterial genomic DNA (Fig. 2B).

Furthermore, the PCR/qPCR efficiency in the bacteria and host cell mixture was determined. Water-soaked decay appeared on the infected celery petioles at 24 h after inoculation. Total genomic DNA from the local sites and the distant sites of the macerated tissues that had been artificially inoculated by *Pco* BCS7, *Pcc* B5, *Pcb*

Fig. 4. Detection limit of *Pectobacterium carotovorum* subsp. *odoriferum* (*Pco*) in artificially inoculated celery plants using the **A**, conventional PCR and **B**, quantitative PCR assays. Lanes 1 to 9 = total genomic DNA of *Pco* CFU/mg of plant tissue: 2.5×10^5 , 2.5×10^4 , 2.5×10^3 , 2.5×10^2 , 2.5×10^1 , 2.5 , 2.5×10^{-1} , 2.5×10^{-2} , and 2.5×10^{-3} CFU/mg; lane 10 = total genomic DNA of plants inoculated with sterilized H₂O; and lane 11 = ddH₂O.

Table 2. The diameter of rotting tissue in Chinese cabbage petioles and potato tubers as well as the maceration weight in potato inoculated with *Pectobacterium* strains^z

Species/subspecies	Tested strains (<i>n</i>)	Diameter of rotting tissue per Chinese cabbage petioles (cm)		Diameter of rotting tissue per potato tuber (cm)		Weight of maceration per potato tuber (g)	
		Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
<i>Pcc</i>							
All <i>Pcc</i> strains tested	18	1.65 ± 0.05 cd	0.86–2.14	1.42 ± 0.08 cd	0.91–2.17	0.41 ± 0.07 c	0.13–0.94
<i>Pcc</i> from Chinese cabbage	9	1.57 ± 0.06 d	1.14–1.95	1.48 ± 0.11 cd	0.92–2.17	0.42 ± 0.09 c	0.13–0.76
<i>Pcc</i> from bok choy	9	1.73 ± 0.04 c	0.86–2.14	1.37 ± 0.11 cd	0.91–2.03	0.39 ± 0.06 cd	0.16–0.94
<i>Pcb</i>							
All <i>Pcb</i> strains tested	25	1.60 ± 0.02 cd	0.81–2.52	1.45 ± 0.12 cd	0.74–2.11	0.39 ± 0.06 cd	0.08–0.77
<i>Pcb</i> from Chinese cabbage	9	1.65 ± 0.04 cd	0.81–2.13	1.29 ± 0.17 d	0.74–2.02	0.31 ± 0.09 d	0.08–0.77
<i>Pcb</i> from bok choy	15	1.55 ± 0.04 d	1.01–2.52	1.55 ± 0.12 bc	0.79–2.11	0.44 ± 0.05 c	0.11–0.71
<i>Pcb</i> from celery	1	2.01 ± 0.20 ab	–	1.49 ± 0.37 cd	–	0.43 ± 0.10 c	–
<i>Pco</i>							
All <i>Pco</i> strains tested	29	1.97 ± 0.02 ab	0.48–2.88	1.69 ± 0.09 ab	0.87–2.97	0.77 ± 0.06 ab	0.20–1.61
<i>Pco</i> from Chinese cabbage	16	1.90 ± 0.05 b	0.48–2.74	1.81 ± 0.08 a	0.94–2.97	0.84 ± 0.08 a	0.20–1.61
<i>Pco</i> from celery	13	2.05 ± 0.03 a	1.49–2.88	1.55 ± 0.11 bc	0.87–2.10	0.70 ± 0.06 b	0.29–1.39

^z *Pcc* = *P. carotovorum* subsp. *carotovorum*, *Pcb* = *P. carotovorum* subsp. *brasilense*, and *Pco* = *P. carotovorum* subsp. *odoriferum*. Chinese cabbage petioles and potato tubers were inoculated with the indicated strain at 28°C for 24 h postinoculation before the diameter of the rotten tissue and the maceration weight were measured. Values on Chinese cabbage are means ± SD of three experiments with three replicate petioles per strain per experiment, and values on potato tubers are means ± SD of four replicates. The different letters indicate a significant difference ($P < 0.05$, Duncan's test).

BC1, and sterilized water was used to perform PCR and qPCR with the subspecific primers srlE-F1/srlE-R1 and srlE-qF1/srlE-qR1, respectively. The results of the two assays were consistent and both showed that the specific fragments could be amplified from the local sites as well as the distant sites of the plants infected by *Pco*, whereas no PCR products were amplified from the DNA extracted from any tissues of *Pcc*, *Pcb*, or the sterilized water-inoculated plants (Fig. 3). The sequence of each amplicon generated was 100% identical to that of the expected target fragment, thus confirming the applicability of the tests on contaminated plant materials even before any external symptoms appeared.

Detection limit of *Pco* in plant samples. The suitability of the two novel primer pairs in detecting and quantifying bacteria directly in plant samples was further evaluated with celery samples that had been artificially contaminated with serial dilutions of *Pco* ranging from 2.5×10^{-3} CFU/mg (i.e., 2.5×10^{-3} CFU of *Pco* per milligram of plant tissue estimated) to 2.5×10^5 CFU/mg. Both the conventional PCR and qPCR assays could detect as low as 2.5×10^{-2} CFU/mg according to agarose gel electrophoresis (Fig. 4).

Pathogenicity assay. All of these newly isolated *P. carotovorum* strains could cause soft rot symptoms on Chinese cabbage and potato at 24 h after inoculation. The details of the pathogenicity of each strain are shown in Supplementary Table S1. Moreover, the abilities of the *Pco* strains and strains from the other subspecies to macerate Chinese cabbage petioles and potato tubers were compared by ANOVA (Table 2). The mean diameter of the rotting tissue was significantly greater for strains belonging to the subspecies *Pco* (1.97 cm) than for the *Pcc* (1.65 cm) and *Pcb* (1.60 cm) strains ($P < 0.05$), whereas no significant difference was observed between *Pcc* and *Pcb* (Fig. 5A; Table 2). Furthermore, the rotting in Chinese cabbage petioles caused by the *Pco* strains was significantly greater for strains isolated from celery than for strains isolated from Chinese cabbage (Table 2) ($P < 0.05$). Maceration was also significantly greater for *Pcc* strains isolated from bok choy than for *Pcc* strains isolated from Chinese cabbage, and no obvious difference in virulence was observed between the *Pcb* strains from bok choy and from Chinese cabbage.

In addition, maceration development in Chinese cabbage by the 72 *P. carotovorum* strains was determined at 6, 10, 24, and 30 h postinoculation (hpi) (Supplementary Table S2). No strains were observed to cause disease symptoms on the petioles at 6 hpi. Most strains (66.7, 52.0, and 69.0% of the *Pcc*, *Pcb*, and *Pco* strains, respectively) could induce water-soaked lesions on the petioles at 10 hpi. Lesions appeared on all of the inoculated petioles for the tested strains at 24 hpi, and the tissue maceration was highest at 30 hpi. No maceration was observed on the water-inoculated control petioles. Maceration was significantly greater for the *Pco* strains

than for *Pcc* and *Pcb* at 24 hpi as well as 30 hpi (Fig. 6A), whereas no significant difference was observed between *Pcc* and *Pcb*. There was no obvious difference among the three subspecies at 10 hpi. The lesion growth rate was significantly greater for *Pco* than for *Pcc* and *Pcb* during the period from 10 to 24 hpi; however, from 24 to 30 hpi, the lesion growth rates of the three subspecies did not differ significantly (Fig. 6B) ($P < 0.05$).

A potato tuber assay was also conducted and both the maceration diameter and weight were measured in this assay. All of the strains induced light brown to black soft rot accompanied by a nauseating odor on the potato at 24 hpi (details shown in Supplementary Table S1). No symptoms developed on the water-inoculated controls. The average maceration diameter and weight were both significantly higher for *Pco* strains (1.69 cm, 0.77 g) than for the *Pcb* (1.45 cm, 0.39 g) and *Pcc* (1.42 cm, 0.41 g) strains ($P < 0.05$), whereas no difference between the *Pcc* and *Pcb* strains was detected based on the maceration diameter or weight ($P > 0.05$) (Fig. 5B and C; Table 2). A positive correlation was observed between the diameter and the percentage of macerated tissue caused by these strains (Supplementary Table S1). Furthermore, the rotting of the potato tubers caused by *Pco* was significantly greater for strains isolated from Chinese cabbage (1.81 cm, 0.84 g) than for strains isolated from celery (1.55 cm, 0.70 g), both in terms of maceration weight and diameter.

Biochemical traits of the *P. carotovorum* strains. To further analyze the features of the *P. carotovorum* ssp., five biochemical traits that are known to be distinctive among the *Pcc*, *Pcb*, and *Pco* subspecies were examined for all strains used in this study (Perombelon and van der Wolf 2002; Schaad et al. 2001). All tested *Pco* strains could produce acid from D-sorbitol, palatinose, α -methylglucoside, D-arabitol, and inulin, whereas negative results were obtained for the isolated *Pcc* strains and *Pcb* strains, except for a few weakly positive results for some individual *Pcc* and *Pcb* strains (Table 3; Supplementary Table S3).

Diversity of *Pectobacterium* subspecies on vegetables. All 72 strains in this study were identified as *P. carotovorum*, including 34 strains isolated from Chinese cabbage, 24 strains from bok choy, and 14 strains from celery. The results showed that three *P. carotovorum* subspecies (nine *Pcc*, nine *Pcb*, and 16 *Pco*) were identified as the causative agents of soft rot on Chinese cabbage in the field, and *Pcc* (nine strains) and *Pcb* (15 strains) caused soft rot on bok choy (Supplementary Fig. S2). In contrast with the miscellaneous distribution of pectolytic bacteria on the two closely related *B. rapa* vegetables (Chinese cabbage and bok choy), 13 strains isolated from different soft rot samples of celery were identified as *Pco*, whereas the one remaining strain was identified as *Pcb*.

Fig. 5. Maceration diameters of the *Pectobacterium* strains in the **A**, Chinese cabbage petioles and **B**, potato tubers, and **C**, maceration weight of the potato tubers. *Pcc* = *P. carotovorum* subsp. *carotovorum*, *Pcb* = *P. carotovorum* subsp. *brasiliense*, and *Pco* = *P. carotovorum* subsp. *odoriferum*. Chinese cabbage petioles and potato tubers were inoculated with the indicated strain at 28°C for 24 h before the diameter of the rotten tissue and the weight of maceration were measured. White horizontal lines at the center of each box show median values. The bounds of each black box show the quartiles, and the upper and lower bars show the maximum and minimum values, respectively. This image was drawn using matplotlib in Python. Numbers above the violin plot indicate the means $\pm$ SD calculated from three experiments with **A**, three replicate petioles per strain per experiment in the Chinese cabbage assay and **B** and **C**, four replicates in the potato assay. The different letters indicate a significant difference ($P < 0.05$, Duncan's test).

Discussion

This study identified and characterized the *Pcc*, *Pcb*, and *Pco* strains collected from three economically important vegetables in China. This study improves our current understanding of the occurrence of bacterial soft rot caused by *P. carotovorum* on Chinese

cabbage, bok choy, and celery in China. *Pcc* has long been considered as the primary causal agent of soft rot on Chinese cabbage (Ren et al. 2001a). This study confirmed that *Pco* and *Pcb* are also primary causative agents of soft rot on this economically important vegetable (Park et al. 2012; Zhu et al. 2010). Because most soft rot

Fig. 6. Maceration development of *Pectobacterium carotovorum* strains on Chinese cabbage. **A**, Lesion diameters were captured at 6, 10, 24, and 30 h postinoculation (hpi). Blue = *P. carotovorum* subsp. *carotovorum* (*Pcc*), orange = *P. carotovorum* subsp. *brasiliense* (*Pcb*), and gray = *P. carotovorum* subsp. *odoriferum* (*Pco*). Horizontal lines at the center of each box show median values. The bounds of each box show the quartiles, and the upper and lower bars show the maximum and minimum values, respectively. This image was drawn using matplotlib. Numbers above the box indicate the means ± SD calculated from three experiments in the Chinese cabbage assay. The different letters indicate a significant difference ($P < 0.05$, Duncan's test). **B**, Lesion development was monitored based on the lesion measurements (in centimeters) from 6 to 30 hpi. Each spot indicates the average value of each strain within a taxon. The regression lines were calculated using python-kapteyn (<https://www.astro.rug.nl/software/kapteyn/>) and the shading of the line indicates the 95% confidence interval for the rotting tissue diameter of the strains within each subspecies.

pathogens are not host specific (Perombelon and Kelman 1980), it is not unexpected that all three *P. carotovorum* subspecies were found on Chinese cabbage. However, one significant finding was that the majority of the strains isolated from celery in this study were identified as *Pco*, which indicates that *Pco* is the predominant pathogen for bacterial soft rot of celery in Beijing, China. Among the *Pectobacterium* strains, *Pco* strains also seem to be more frequently isolated from celery tissues with soft rot symptoms in many other countries (Gallois et al. 1992; Waleron et al. 2014). Although a few cases exist where other *Pectobacterium* strains, including the *Pcb* strain (WQC3) in this work, have been isolated from celery, these bacterial species/subspecies are typically minor celery pathogens. While the strains of these subspecies were not reported to exhibit obvious host specificity, there is potential for their host adaptability to be explored, which may further contribute to investigations on plant–pathogen interactions and the genetic basis of plant disease resistance.

The results of the Chinese cabbage and potato assays were congruent, and both showed that *Pco* had significantly greater virulence than the other closely related *Pcb* and *Pcc* strains, whereas no significant difference was observed between the *Pcc* and *Pcb* strains. This is in accordance with our previous comparative genomics study result that the *Pco* strains possessed subspecific genes/clusters associated with virulence, such as an additional T6SS and the *srl* operon, which are required for pathogenicity and host colonization by phyto-bacteria (Aldridge et al. 1997; Li et al. 2018). In contrast, Waleron et al. (2014) reported no significant difference in virulence between *Pco* and *Pcc* or between *Pco* and *Pcb* based on an assessment of the diameter of rotting tissue in potato tubers at 72 hpi under 28°C. It is still not clear whether the superior virulence of *Pco* at 24 and 30 hpi in this study would change over time and finally disappear at 72 hpi, or whether the difference was not statistically significant attributable, in part, to the variability among strains within taxa. However, we noted that the lesion growth rate was significantly greater for *Pco* than for *Pcc* and *Pcb* during the period from 10 to 24 hpi, whereas the lesion growth rates of the three subspecies from 24 to 30 hpi did not differ significantly. This gives an indication that the superior virulence of *Pco* was possibly attributable to its high aggressiveness during early infection. Moreover, the maceration ability of strains associated with different hosts within the same taxon showed conflicting results in the two assays. Thus, whether the virulence among strains is associated with their hosts still requires further clarification.

Many methods have been studied to detect, identify, and differentiate soft rot-causing bacteria at the species and subspecies level. These include methods based on biochemical characters, serology, molecular techniques that rely on DNA sequence amplification (Czajkowski et al. 2015), and whole-genome sequencing. Our results confirmed that several biochemical tests, including acid production

from D-sorbitol, palatinose, α -methylglucoside, and D-arabitol, allow for the easy differentiation of *Pco* strains from those of two closely related subspecies (*Pcc* and *Pcb*), which is in accordance with Perombelon and van der Wolf (2002) and Schaad et al. (2001). However, the positive results obtained for these Chinese *Pco* strains with inulin are in contrast with those of the *Pco* strains reported by Perombelon and van der Wolf (2002), possibly owing to the natural variability within taxa. Thus, the biochemical tests are not entirely satisfactory to differentiate *P. carotovorum* ssp. and run the risk of closely related subspecies being misidentified (Czajkowski et al. 2015). Furthermore, they are not rapid and cannot be used for direct detection from plants.

This study showed that direct PCR analysis could be used for the rapid identification of *Pco* subspecies. In combination with the previously published specific PCR markers for *Pcb* and *Pcc* (Duarte et al. 2004; Kang et al. 2003), three subspecies of *P. carotovorum* causing soft rot can now be rapidly differentiated from each other. Moreover, we also established a qPCR assay for *Pco*, which can be further used in professional diagnostic laboratories. Both assays could detect the pathogen directly from infected plant tissues even before external symptoms appear, suggesting that these assays can also potentially be used to detect the disease in latently infected plants.

Both the *Pco*-specific PCR and qPCR primers were developed from *srlE*, which encodes a sorbitol-specific phosphotransferase enzyme IIB component and plays an important role in the function of the *srl* operon (related to sorbitol metabolism in bacteria) (Boyd et al. 2000). The conventional PCR assay can be used for the fast identification of pathogens, because it has lower instrument, reagent, and operation requirements, and its larger amplicon size (674 bp) means that it can be more easily distinguished by agarose gel electrophoresis than that of the qPCR analysis (108 bp). The PCR and qPCR methods presented herein thus satisfy the needs of different researchers for different goals.

Overall, these results identified the pathogens causing soft rot on economically important vegetables in China. The pathogenicity differences among the subspecies of the devastating phytopathogen *P. carotovorum* were explored and a protocol was established to detect, identify, and differentiate the soft rot-causing bacteria at the subspecies level, providing a foundation for the diagnosis and management of the disease.

Acknowledgments

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Literature Cited

- Aldridge, P., Metzger, M., and Geider, K. 1997. Genetics of sorbitol metabolism in *Erwinia amylovora* and its influence on bacterial virulence. *Mol. Gen. Genet.* 256:611-619.
- Boyd, D. A., Thevenot, T., Gumbmann, M., Honeyman, A. L., and Hamilton, I. R. 2000. Identification of the operon for the sorbitol (glucitol) phosphoenolpyruvate: Sugar phosphotransferase system in *Streptococcus mutans*. *Infect. Immun.* 68: 925-930.
- Czajkowski, R., Grabe, G. J., and Wolf, J. M. V. D. 2009. Distribution of *Dickeya* spp. and *Pectobacterium carotovorum* subsp. *carotovorum* in naturally infected seed potatoes. *Eur. J. Plant Pathol.* 125:263-275.
- Czajkowski, R., Jafra, S., Lojkowska, E., Potrykus, M., Van, D. W. J., and Sledz, W. 2015. Detection, identification and differentiation of *Pectobacterium* and *Dickeya* species causing potato blackleg and tuber soft rot: A review. *Ann. Appl. Biol.* 166:18-38.
- Darrasse, A., Priou, S., Kotoujansky, A., and Bertheau, Y. 1994. PCR and restriction fragment length polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. *Appl. Environ. Microbiol.* 60:1437-1443.
- Davidsson, P. R., Kariola, T., Niemi, O., and Palva, E. T. 2013. Pathogenicity of and plant immunity to soft rot pectobacteria. *Front. Plant Sci.* 4:191.
- De Boer, S. H., Li, X., and Ward, L. J. 2012. *Pectobacterium* spp. associated with bacterial stem rot syndrome of potato in Canada. *Phytopathology* 102:937-947.
- De Boer, S. H., and Ward, L. J. 1995. PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology* 85:854-858.
- Dees, M. W., Lysøe, E., Rossmann, S., Perminow, J., and Brurberg, M. B. 2017. *Pectobacterium polaris* sp. nov., isolated from potato (*Solanum tuberosum*). *Int. J. Syst. Evol. Microbiol.* 67:5222-5229.

Table 3. Distinctive metabolic traits between *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*), *P. carotovorum* subsp. *brasiliense* (*Pcb*), and *P. carotovorum* subsp. *odoriferum* (*Pco*)

Tests	Positive/weak positive/ negative strains in this study (n)*			Standard and reference strains ^y			
	<i>Pcc</i>	<i>Pcb</i>	<i>Pco</i>	<i>Pcc</i>	<i>Pco</i>	<i>Pa</i>	<i>Pb</i>
Genomes tested (n)	18	25	29				
Acid produced from							
α -Methyl glucoside	0/0/18 ^z	0/2/23	29/0/0	–	+	+	+
D-Arabitol	0/3/15	0/0/25	29/0/0	–	+	–	–
Sorbitol	0/0/18	0/0/25	29/0/0	–	+	–	–
Palatinose	0/4/14	0/0/25	29/0/0	–	+	+	+
Inulin	0/4/14	0/2/23	29/0/0	–	–	–	+

* Number of strains tested.

^y Standard and reference strains: data are from Perombelon and van der Wolf (2002). *Pa* = *P. atrosepticum* and *Pb* = *P. betavascularum*. Plus signs (+) = positive and minus signs (–) = negative.

^z The biochemical results of the strains used in this study are shown as numbers of strains positive/weak positive/negative.

- Duarte, V., de Boer, S. H., Ward, L. J., and de Oliveira, A. M. 2004. Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. *J. Appl. Microbiol.* 96:535-545.
- Gallois, A., Samson, R., Ageron, E., and Grimont, P. A. D. 1992. *Erwinia carotovora* subsp. *odorifera* subsp. nov., associated with odorous soft rot of chicory (*Cichorium intybus* L.). *Int. J. Syst. Bacteriol.* 42:582-588.
- Gao, B., Wang, R. Y., Chen, S. L., Ma, J., and Li, X. H. 2016. First report of *Pectobacterium carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum* causing bacterial soft rot of sweet potato in China. *Plant Dis.* 100:1776.
- Gardan, L., Gouy, C., Christen, R., and Samson, R. 2003. Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavascularum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *Int. J. Syst. Evol. Microbiol.* 53:381-391.
- Kang, H. W., Kwon, S. W., and Go, S. J. 2003. PCR-based specific and sensitive detection of *Pectobacterium carotovorum* ssp. *carotovorum* by primers generated from a URP-PCR fingerprinting-derived polymorphic band. *Plant Pathol.* 52:127-133.
- Kettani-Halabi, M., Terta, M., Amdan, M., El Fahime, E. M., Bouteau, F., and Ennaji, M. M. 2013. An easy, simple inexpensive test for the specific detection of *Pectobacterium carotovorum* subsp. *carotovorum* based on sequence analysis of the *pmrA* gene. *BMC Microbiol.* 13:176.
- Khayti, S., Cigna, J., Chong, T. M., Quêtu-Laurent, A., Chan, K. G., Hélias, V., and Faure, D. 2016. Transfer of the potato plant isolates of *Pectobacterium wasabiae* to *Pectobacterium parmentieri* sp. nov. *Int. J. Syst. Evol. Microbiol.* 66: 5379-5383.
- Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol. Biol. Evol.* 35:1547-1549.
- Li, X. Y., Ma, Y. L., Liang, S. Q., Tian, Y., Yin, S. J., Xie, S. S., and Xie, H. 2018. Comparative genomics of 84 *Pectobacterium* genomes reveals the variations related to a pathogenic lifestyle. *BMC Genomics* 19:889.
- Łojkowska, E., and Kelman, A. 1994. Comparison of the effectiveness of different methods of screening for bacterial soft rot resistance of potato tubers. *Am. J. Potato Res.* 71:99-113.
- Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S. V., and Machado, M. A. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol. Plant Pathol.* 13: 614-629.
- Nabhan, S., de Boer, S. H., Maiss, E., and Wydra, K. 2012a. Taxonomic relatedness between *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *odoriferum* and *Pectobacterium carotovorum* subsp. *brasiliense* subsp. nov. *J. Appl. Microbiol.* 113:904-913.
- Nabhan, S., Wydrab, K., Linde, M., and Debener, T. 2012b. The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *Pectobacterium carotovorum*. *Plant Pathol.* 61:498-508.
- Naum, M., Brown, E. W., and Masongamer, R. J. 2008. Is 16S rDNA a reliable phylogenetic marker to characterize relationships below the family level in the Enterobacteriaceae? *J. Mol. Evol.* 66:630-642.
- Onkendi, E. M., and Moleleki, L. N. 2014. Characterization of *Pectobacterium carotovorum* subsp. *carotovorum* and *brasiliense* from diseased potatoes in Kenya. *Eur. J. Plant Pathol.* 139:557-566.
- Osborne, C. A., Maja, G., Parveen, S., and Janssen, P. H. 2005. PCR-generated artefact from 16S rRNA gene-specific primers. *FEMS Microbiol. Lett.* 248:183-187.
- Oskiera, M., Kaluzna, M., Kowalska, B., and Smolinska, U. 2017. *Pectobacterium carotovorum* subsp. *odoriferum* on cabbage and Chinese cabbage: Identification, characterization and taxonomic relatedness of bacterial soft rot causal agents. *J. Plant Pathol.* 99:149-160.
- Park, T. H., Choi, B. S., Choi, A. Y., Choi, I. Y., Heu, S., and Park, B. S. 2012. Genome sequence of *Pectobacterium carotovorum* subsp. *carotovorum* strain PCC21, a pathogen causing soft rot in Chinese cabbage. *J. Bacteriol.* 194: 6345-6346.
- Pédrón, J., Bertrand, C., Taghouti, G., Portier, P., and Barny, M. A. 2019. *Pectobacterium aquaticum* sp. nov., isolated from waterways. *Int. J. Syst. Evol. Microbiol.* 69:745-751.
- Perombelon, M. C. M., and Kelman, A. 1980. Ecology of the soft rot erwinias. *Annu. Rev. Phytopathol.* 18:361-387.
- Perombelon, M. C. M., and van der Wolf, J. M. 2002. Methods for the Detection and Quantification of *Erwinia carotovora* on Potatoes: A Laboratory Manual. Scottish Crop Research Institute Occasional Publication, Dundee, Scotland.
- Pritchard, L. P. L., Glover, R. G. R. H., Humphris, S. H. S., Elphinstone, J. E. J. G., and Toth, I. T. I. K. 2016. Genomics and taxonomy in diagnostics for food security: Soft-rotting enterobacterial plant pathogens. *Anal. Methods* 8:12-24.
- Ren, J. P., Petzoldt, R., and Dickson, M. H. 2001a. Genetics and population improvement of resistance to bacterial soft rot in Chinese cabbage. *Euphytica* 117:197-207.
- Ren, J. P., Petzoldt, R., and Dickson, M. H. 2001b. Screening and identification of resistance to bacterial soft rot in *Brassica rapa*. *Euphytica* 118:271-280.
- Sarfraz, S., Riaz, K., Oulghazi, S., Cigna, J., Sahi, S. T., Khan, S. H., and Faure, D. 2018. *Pectobacterium punjabense* sp. nov., isolated from blackleg symptoms of potato plants in Pakistan. *Int. J. Syst. Evol. Microbiol.* 68:3551-3556.
- Schaad, N. W., Jones, J. B., and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd ed. American Phytopathological Society, St. Paul, MN.
- Staley, J. T. 2006. The bacterial species dilemma and the genomic-phylogenetic species concept. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 361:1899-1909.
- Terta, M., El, K. A., Ait, M. R., Achbani, E., Barakate, M., Amdan, M., Annajar, B., El, H. M., Val, F., Bouteau, F., and Ennaji, M. M. 2010. Occurrence of *Pectobacterium carotovorum* strains isolated from potato soft rot in Morocco. *Cell Mol. Biol. (Noisy-le-Grand)* 56:1324-1333.
- Waleron, M., Misztak, A., Waleron, M., Franczuk, M., Wielgomas, B., and Waleron, K. 2018. Transfer of *Pectobacterium carotovorum* subsp. *carotovorum* strains isolated from potatoes grown at high altitudes to *Pectobacterium peruviense* sp. nov. *Syst. Appl. Microbiol.* 41:85-93.
- Waleron, M., Waleron, K., and Łojkowska, E. 2013. Occurrence of *Pectobacterium wasabiae* in potato field samples. *Eur. J. Plant Pathol.* 137: 149-158.
- Waleron, M., Waleron, K., and Łojkowska, E. 2014. Characterization of *Pectobacterium carotovorum* subsp. *odoriferum* causing soft rot of stored vegetables. *Eur. J. Plant Pathol.* 139:457-469.
- Waleron, M., Waleron, K., and Łojkowska, E. 2015. First report of *Pectobacterium carotovorum* subsp. *brasiliense* causing soft rot on potato and other vegetables in Poland. *Plant Dis.* 99:1271.
- Xie, H., Li, X. Y., Ma, Y. L., and Tian, Y. 2018. First report of *Pectobacterium aroidearum* causing soft rot of Chinese cabbage in China. *Plant Dis.* 102:674.
- Zhu, L., Xie, H., Chen, S., and Ma, R. 2010. Rapid isolation, identification and phylogenetic analysis of *Pectobacterium carotovorum* spp. *J. Plant Pathol.* 92:479-483.