Emergence of Bacterial Soft Rot in Cucumber Caused by *Pectobacterium carotovorum* subsp. *brasiliense* in China

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Abstract

During 2014 to 2015, a devastating bacterial soft rot on cucumber stems and leaves occurred in Shandong, Shanxi, Hebei, Henan, and Liaoning provinces of China, resulting in serious economic losses for cucumber production. The gummosis emerged on the surface of leaves, stems, petioles, and fruit of cucumber. The basal stem color was dark brown and the stem base turned to wet rot. Yellow spots and wet rot emerged at the edge of the infected cucumber leaves and gradually infected the leaf centers. In total, 45 bacterial strains were isolated from the infected tissues. On the basis of phenotypic properties of morphology, physiology, biochemistry, and

16S ribosomal RNA gene sequence analysis, the pathogen was identified as *Pectobacterium carotovorum*. Multilocus sequence analysis confirmed that the isolates were *P. carotovorum* subsp. *brasiliense*, and the pathogens fell in clade II. The pathogenicity of isolated bacteria strains was confirmed. The strains reisolated were the same as the original. The host range test confirmed that strains had a wide range of hosts. As far as we know, this is the first report of cucumber stem soft rot caused by *P. carotovorum* subsp. *brasiliense* in China as well as in the world, which has a significant economic impact on cucumber production.

Cucumber (*Cucumis sativus* L.) is a widely cultivated plant in the gourd family Cucurbitaceae. Statistically, China is the largest cucumber producer in the world, with 1.17 million hm² of harvested area and 54.36 million tons in 2013 (<http://faostat3.fao.org/download/Q/QC/E>). However, during the growth process, cucumber plants are susceptible to many diseases. During 2014 to 2015, an outbreak of cucumber bacterial soft rot occurred in Shandong, Shanxi, Hebei, Henan, and Liaoning provinces in China. The disease incidence varied from 15 to 50% in different fields, causing 20 to 30% yield losses.

Bacterial soft rot was thought to be caused by *Pectobacterium carotovorum*, which can macerate parenchymatous plant tissues by producing a mass of pectic enzymes (Pérombelon and Kelman 1980) and causes soft rot, blackleg, and wilt in many vegetable crops and ornamental plant species (Hauben et al. 1998; Pérombelon 2002; Toth et al. 2003). *P. carotovorum* consist of three valid names: *P. carotovorum* subsp. *carotovorum* (Gardan et al. 2003), *P. carotovorum* subsp. *odoriferum* (Gallois et al. 1992), and *P. carotovorum* subsp. *brasiliense* (Duarte et al. 2004). *P. carotovorum* subsp. *brasiliense* was first reported as pathogenic bacteria of potato black rot in Brazil (Duarte et al. 2004) and, recently, has been found as the pathogen of paprika in Korea (Choi and Kim 2013) and potato in the United States, Israel, Kenya, New Zealand, Switzerland, and South Africa (de Werra et al. 2015; Marquez-Villavicencio et al. 2011; Onkendi et al. 2014; Panda et al. 2012; Pérombelon 2002; van der Merwe et al. 2010). However, there is no report about *P. carotovorum* subsp. *brasiliense* causing bacterial soft rot in cucumber.

The biochemical or genetic characteristics of *P. carotovorum* strains show diversity among the same subspecies (Nabhan et al. 2012); thus, identification of these pathogens is difficult when only using phenotypic, biochemical studies or 16S ribosomal RNA

(rRNA) sequence analysis. In recent years, classification studies based on multilocus sequence analysis (MLSA) were used to identify *P. carotovorum* strains (Nabhan et al. 2011; Onkendi et al. 2014), because MLSA may provide a better understanding of speciation events in bacteria (Hanage et al. 2006) and it was proved to be one of the most reliable methods for differentiating *P. carotovorum* strains (Kim et al. 2009).

This study aimed to isolate and identify the bacterium associated with recently occurred soft rot on cucumber in Shandong, Shanxi, Hebei, Henan, and Liaoning provinces of China. Different types of analyses, such as physiological and molecular analyses, MLSA of housekeeping genes, and pathogenicity assays in cucumber and different plant hosts were used to determine the validity of the pathogen.

Materials and Methods

Sample collection and strain isolation. A field symptom survey was carried out on cucumber in 20 greenhouses in Shandong, Shanxi, Hebei, Henan, and Liaoning provinces in China from November 2014 to March 2015. Approximately 68 samples (including 15 cucumber fruit, 22 stems, and 31 leaves) which showed gummosis, soft rot, or yellow spots were collected from 18 greenhouses. In all, 45 samples (9 fruit, 16 stems, and 24 leaves) were used for isolation and identification, and the other 17 samples (4 fruit, 6 stems, and 7 leaves) were deposited at the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences as specimens for future reference. To isolate the pathogens, infected tissues were surface sterilized for 30 s in 75% ethanol, rinsed in sterile distilled water, and then ground with a sterile scalpel in 1 ml of sterile distilled water. The ground tissue was soaked for 3 min, and 10 µl of liquid suspension was streaked on nutrient agar medium. In total, 45 isolates with the same colony morphology were obtained from infected tissues, and nine strains were randomly selected for the following studies (Table 1).

Phenotypic and biochemical characterization. Carbon source utilization studies were carried out using the Microlog system (Biolog Inc.). Nine selected strains were assayed for the oxidation of the 95 carbon sources in the GN2 microplates, as suggested by the manufacturer. Microplates were inoculated with 150 µl of suspension per well at 27°C. Additionally, all strains were characterized by standard tests commonly used for pectolytic *Pectobacterium* spp. and included a test for pectolytic activity on crystal violet pectate

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(CVP) medium, acid production from maltose and α -methyl-D-glucoside, reducing substances from sucrose, growth at 37°C, erythromycin sensitivity (Duarte et al. 2004), tolerance to 5% NaCl, lactose fermentation, and gas production from D-glucose (Nabhan et al. 2012).

16S rRNA gene sequence analysis. The universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-ACG

GCT ACC TTG TTA CGA CTT-3') were used to amplify the 16S rRNA gene sequences of nine isolated strains. Polymerase chain reaction (PCR) was performed using a 50- μ l reaction mix containing 2 μ l of each primer, 25 μ l of master mix (Biomed), 19 μ l of H₂O, and 2 μ l of DNA template (5 μ g μ l⁻¹). The PCR amplification program consisted of an initial denaturation at 95°C for 10 min; 35

Table 1. Source of strains isolated from cucumber in China and its pathogenicity assays for different plant species

Isolates	Source of isolation				Pathogenicity ^a		
	Tissue	Cultivar	Loc ^b	Data collected	Zucchini	Watermelon	Balsam pear
HG1501090302	Leaf	Zhongnong No. 6	SD	January 2015	+	+	+
HG1501090303	Fruit	Zhongnong No. 6	SD	January 2015	+	+	+
HG1501090304	Stem	Fengyan No. 3	SX	November 2014	+	+	+
HG1501090305	Stem	Fengyan No. 3	SX	November 2014	+	+	+
HG1501090306	Fruit	Deruite 721	SX	February 2015	+	+	+
HG1501090307	Stem	Deruite 233	HN	December 2014	+	+	+
HG1501090308	Leaf	Deruite 233	HN	December 2014	+	+	+
HG1501090309	Fruit	Unknown	HB	February 2015	+	+	+
HG1501090310	Stem	Unknown	LN	March 2015	+	+	+

(continued on next page)

^a Symbols: + represents infection and disease symptoms and – represents no disease symptoms.

^b Locations: SD = Shandong; HB = Hebei; SX = Shanxi; HN = Henan; and LN = Liaoning province.

Table 2. GenBank accession numbers for the eight genes (*acnA*, *gabA*, *icdA*, *mdh*, *mtlD*, *pgi*, *proA*, and *rpoS*) of nine randomly selected strains and reference *Pectobacterium* spp. strains

Strain ^a	GenBank accession numbers							
	<i>acnA</i>	<i>gabA</i>	<i>icdA</i>	<i>mdh</i>	<i>mtlD</i>	<i>pgi</i>	<i>proA</i>	<i>rpoS</i>
<i>brasiliense</i>								
8	JF926768.1	JF926778.1	JF926788.1	JF926798.1	JF926808.1	JF926818.1	JF926828.1	JF926838.1
213	JF926771.1	JF926781.1	JF926791.1	JF926801.1	JF926811.1	JF926821.1	JF926831.1	JF926841.1
371	JF926770.1	JF926780.1	JF926790.1	JF926800.1	JF926810.1	JF926820.1	JF926830.1	JF926840.1
212 ^T	JF926769.1	JF926779.1	JF926789.1	JF926799.1	JF926809.1	JF926819.1	JF926829.1	JF926839.1
SCRI1073	HM156787	HM156848	HM156910	HM156971	HM157033	HM157090	HM157157	HM157201
1001	JF926767.1	JF926777.1	JF926787.1	JF926797.1	JF926807.1	JF926817.1	JF926827.1	JF926837.1
1009	JF926766.1	JF926776.1	JF926786.1	JF926796.1	JF926806.1	JF926816.1	JF926826.1	JF926836.1
A17	HM156764	HM156824	HM156885	HM156946	HM157008	HM157070	HM157132	HM157194
C18	HM156768	HM156828	HM156889	HM156950	HM157012	HM157074	HM157136	HM157198
<i>carotovorum</i>								
JKI4.3.8	HM156790	HM156849	HM156911	HM156972	HM157036	HM157092	HM157158	HM157204
C412.4	HM156784	HM156843	HM156905	HM156966	HM157028	HM157091	HM157152	HM157227
C137	HM156769	HM156829	HM156890	HM156951	HM157013	HM157075	HM157137	HM157210
C142.2	HM156773	HM156833	HM156894	HM156955	HM157017	HM157079	HM157141	HM157214
C267	HM156777	HM156836	HM156898	HM156959	HM157021	HM157083	HM157145	HM157218
C3	HM156767	HM156827	HM156888	HM156949	HM157011	HM157073	HM157135	HM157197
C338	HM156780	HM156839	HM156901	HM156962	HM157024	HM157086	HM157148	HM157221
A10.1	HM156761	HM156821	HM156882	HM156943	HM157005	HM157067	HM157129	HM157191
A18	HM156765	HM156825	HM156886	HM156947	HM157009	HM157071	HM157133	HM157195
M30	HM156785	HM156844	HM156906	HM156967	HM157029	HM157099	HM157153	HM157199
C144	HM156775	HM156835	HM156896	HM156957	HM157019	HM157081	HM157143	HM157216
CFBP2046 ^T	JF926762.1	JF926772.1	JF926782.1	JF926792.1	JF926802.1	JF926812.1	JF926822.1	JF926832.1
SCRI2	HM156788	HM156846	HM156908	HM156969	HM157031	HM157104	HM157155	HM157202
<i>odoriferum</i>								
CFBP1878 ^T	JF926763.1	JF926773.1	JF926783.1	JF926793.1	JF926803.1	JF926813.1	JF926823.1	JF926833.1
JKI582	HM156799	HM156857	HM156919	HM156980	HM157042	HM157102	HM157165	HM157229
NB1892	HM156798	HM156858	HM156920	HM156981	HM157043	HM157103	HM157166	HM157230
Cucumber								
HG1501090302	KX010008	KX010017	KX010026	KX010035	KX010044	KX010062	KX010071	KX010053
HG1501090303	KX010009	KX010018	KX010027	KX010036	KX010045	KX010063	KX010072	KX010054
HG1501090304	KX010010	KX010019	KX010028	KX010037	KX010046	KX010064	KX010073	KX010055
HG1501090305	KX010011	KX010020	KX010029	KX010038	KX010047	KX010065	KX010074	KX010056
HG1501090306	KX010012	KX010021	KX010030	KX010039	KX010048	KX010066	KX010075	KX010057
HG1501090307	KX010013	KX010022	KX010031	KX010040	KX010049	KX010067	KX010076	KX010058
HG1501090308	KX010014	KX010023	KX010032	KX010041	KX010050	KX010068	KX010077	KX010059
HG1501090309	KX010015	KX010024	KX010033	KX010042	KX010051	KX010069	KX010078	KX010060
HG1501090310	KX010016	KX010025	KX010034	KX010043	KX010052	KX010070	KX010079	KX010061

^a Strain designations: *brasiliense* = *Pectobacterium carotovorum* subsp. *brasiliense*, *carotovorum* = *P. carotovorum* subsp. *carotovorum*, *odoriferum* = *P. carotovorum* subsp. *odoriferum*, and Cucumber = strains isolated from cucumber.

cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final 10-min elongation step at 72°C in a S1000 Thermal Cycler (Bio-Rad). Amplified fragments were detected by electrophoresis on a 1.5% agarose gel and purified from the gel using a gel extraction kit (TIANGEN). Sequencing was performed by Biomad (BigDye

Terminator ready reaction mix v31; Applied Biosystems). Sequences were submitted to GenBank under accession numbers KU997676 to KU997684. The 16S rRNA sequences of 9 isolated strains and 18 reference strains were aligned in MEGA 6.0 software. Phylogenetic analysis was performed using the maximum-likelihood method

Table 1. (continued from preceding page)

Pathogenicity ^a										
Loofah	Chinese cabbage	Mustard	Broccoli	Radish	Cabbage	Pepper	Tomato	Potato	Spinach	Celery
–	–	+	+	+	+	+	+	+	+	+
–	–	+	+	+	+	+	+	+	+	+
–	–	+	+	+	+	+	+	+	+	+
–	–	+	+	+	+	+	+	+	+	+
–	–	+	+	+	+	+	+	+	+	+
–	–	+	+	+	+	+	+	+	+	+
–	–	+	+	+	+	+	+	+	+	+
–	–	+	+	+	+	+	+	+	+	+
–	–	+	+	+	+	+	+	+	+	+

Table 3. Primers for amplifying eight conserved housekeeping genes

Gene	Full name	Sequence length (bp)	Primer name	Primer sequence	Reference
<i>acnA</i>	Aconitate hydratase 1	300	acnA3F	CMAGRGTRTTRATGCARGAYTTTAC	Ma et al. 2007
	...	...	acnA3R	GATCATGGTGGTRTGSARTCVGT	...
<i>gapA</i>	Glyceraldehyde-3-phosphate dehydrogenase A	450	gapA326F	ATCTTCCTGACCGACGAAACTGC	Ma et al. 2007
	...	...	gapA845R	ACGTCATCTTCGGTGTAACCCAG	...
<i>icdA</i>	Isocitrate dehydrogenase, specific for NADP+	520	icdA400F	GGTGGTATCCGTTCTCTGAACG	Ma et al. 2007
	...	...	icdA977R	TAGTCGCCGTTTCAGGTTTCATACA	...
<i>mdh</i>	Malate dehydrogenase	460	mdh86F	CCCAGCTTCCTTCAGGTTTCAGA	Ma et al. 2007
	...	...	mdh628R	CTGCATTCTGAATACGTTTGGTCA	...
<i>mtlD</i>	Mannitol-1-phosphate dehydrogenase	390	mtlD146F	GGCCGTAATATCGGCCGTGG	Ma et al. 2007
	...	...	mtlD650R	CATTTCGCTGAAGGTTTCCACCGT	...
<i>pgi</i>	Glucose-6-phosphate isomerase	520	pgi815F	TGGGTCGGCGGCCGTTACTC	Ma et al. 2007
	...	...	pgi1396R	TGCCTTCGAATACTTTGAACGGC	...
<i>proA</i>	γ-Glutamylphosphate reductase	630	proAF1	CGGYAATGCGGTGATTCTGCG	Ma et al. 2007
	...	...	proAR1	GGGTACTGACCGCCACTTC	...
<i>rpoS</i>	RNA polymerase subunit σ factor 38	880	rpoS1	ATGAGCCAAAGTACGCTGAA	Waleron et al. 2002
	...	...	rpoS2	ACCTGAATCTGACGAACACG	...

Fig. 1. Symptoms of the soft rot disease of cucumber caused by *Pectobacterium carotovorum* subsp. *brasiliense*. **A**, **B**, and **C**, Whitish liquid produced on the surface of the stems, fruit, and leaves, respectively; **D**, **E**, and **F**, symptoms of the infected fruit, leaves, and stems, respectively.

based on the Tamura-Nei model with the number of bootstrap replicates set at 1,000 (Tamura and Nei 1993).

MLSA. Eight informative housekeeping genes (*mtlD*, *acnA*, *icdA*, *mdh*, *pgi*, *gabA*, *proA*, and *rpoS*) were used in the MLSA. Publically available housekeeping gene sequences of 25 *P. carotovorum* strains (Ma et al. 2007; Nabhan et al. 2011) were retrieved from the National Center for Biotechnology Information GenBank (Table 2). For the nine strains isolated from cucumber in China, PCR amplification of eight conserved housekeeping gene fragments was carried out following previously described methods (Nabhan et al. 2011). The primers of eight conserved housekeeping genes are listed in Table 3. Sequencing was carried out by Biomad (BigDye Terminator ready reaction mix v31; Applied Biosystems) and sequences were submitted to GenBank under accession numbers KX010008 to KX010079 (Table 2). The housekeeping gene sequences were aligned using CLUSTAL-W in MEGA, version 6.0 (Tamura et al. 2011). The phylogenetic analysis was conducted using all nucleotide sites of each partial gene sequence: *mtlD* (390 bp), *acnA* (300 bp), *icdA* (520 bp), *mdh* (460 bp), *pgi* (520 bp), *gabA* (450 bp), *proA* (630 bp), and *rpoS* (880 bp). The informative maximum-likelihood method based on the Tamura-Nei model (Tamura and Nei 1993) was constructed in MEGA with a bootstrapping test of 1,000 replications.

Pathogenicity and host range test. To confirm Koch's postulates, fruit, stems, and leaves of cucumber plants ('Zhongnong No. 16') were surface sterilized with 1% NaOCl and inoculated with *P. carotovorum* subsp. *brasiliense* strains. Cucumber fruit were stab inoculated with 10 µl of bacterial suspensions (10^7 CFU/ml) of HG1501090303, HG1501090306, and HG1501090309 (isolated from fruit) obtained from Luria-Bertani (LB) broth (containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl, pH 7.4, in 1 liter of water). Cucumber stems were stab inoculated with 10 µl of bacterial

suspensions (10^7 CFU/ml) of HG1501090304, HG1501090305, HG1501090307, and HG1501090310 (isolated from stems). Cucumber leaves were spray inoculated with 10 ml of bacterial suspensions (10^7 CFU/ml) of HG1501090302 and HG1501090308 (isolated from leaves). Each fruit or stem was stabbed at three points, and the distance between two adjacent stabbed points was 1 cm. Each leaf was sprayed on the surface using the bacterial suspension. Sterilized distilled water was used for negative control inoculations. Inoculated fruit and seedlings were maintained in a glass cabinet at 26 to 30°C with 98% humidity for 48 h. After that, the inoculated fruit and seedlings were moved out and incubated in a greenhouse maintained at 15 to 17°C (night) and 26 to 28°C (day) under natural daylight conditions and monitored daily for presence or absence of symptoms. The fruit and seedlings with typical symptoms of soft rot were reisolated on LB agar mediums, and the morphological and physiological characteristics of the strains were compared with the original bacteria. The inoculation experiment was repeated three times, and each repeat included four fruit and seedlings.

The host ranges of nine randomly selected strains were tested with different plant species. Each inoculum was stabbed into seedlings or tubers of 15 species of plants, including 5 species of Cucurbitaceae (cucumber, zucchini, watermelon, balsam pear, and loofah), 5 species of Brassicaceae (Chinese cabbage, mustard, broccoli, radish, and cabbage), 3 species of Solanaceae (green pepper, tomato, and potato), 1 species of Amaranthaceae (spinach), and 1 species of Apiaceae (celery) (Table 1). Individual seedlings of potted plants (3 weeks old) or tubers from harvested plants were stab inoculated with 10 µl of bacterial suspensions (10^7 CFU/ml) of nine randomly selected strains obtained from LB broth. Each seedling or tube was stabbed at three points, and the distance between two adjacent stabbed points was 1 cm. Controls were inoculated with a sterile toothpick dipped with sterilized water. The inoculum plants were treated as described above.

Table 4. Phenotypic characteristics of *Pectobacterium carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *carotovorum* reference strains and nine randomly selected strains isolated from cucumber in China

Characteristic	Responses of strains ^a										
	212 ^T	21 ^T	1	2	3	4	5	6	7	8	9
Phosphatase	–	–	–	–	–	–	–	–	–	–	–
Acid from α-methyl glucoside	+	–	+	+	+	+	+	+	+	+	+
Tolerance of 5% NaCl	+	+	+	+	+	+	+	+	+	+	+
Growth at CVP medium ^b	+	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+
Acid production from glucose	+	–	+	+	+	+	+	+	+	+	+
Reducing substances from sucrose	+	–	+	+	+	+	+	+	+	+	+
Acid production from maltose	+	–	+	+	+	+	+	+	+	+	+
Utilization of											
Acetic acid	+	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+
D-arabitol	–	–	–	–	–	–	–	–	–	–	–
2'-Deoxyadenosine	–	+	–	–	–	–	–	–	–	–	–
D-glucosaminic acid	–	–	–	–	–	–	–	–	–	–	–
D-glucuronic acid	–	–	–	–	–	–	–	–	–	–	–
D,l-lactic acid	+	+	–	–	–	–	–	–	–	–	–
D-melibiose	+	+	+	+	+	+	+	+	+	+	+
D-sorbitol	+	–	+	+	+	+	+	+	+	+	+
Glucose-1-phosphate	–	+	–	–	–	–	–	–	–	–	–
Inosine	–	+	–	–	–	–	–	–	–	–	–
L-glutamic acid	–	+	–	–	–	–	–	–	–	–	–
Maltose	–	+	–	–	–	–	–	–	–	–	–
Succinamic acid	+	+	+	+	+	+	+	+	+	+	+
Thymidine	+	+	+	+	+	+	+	+	+	+	+
Tween-40	–	+	–	–	–	–	–	–	–	–	–
Tween-80	–	+	–	–	–	–	–	–	–	–	–
Uridine	+	+	+	+	+	+	+	+	+	+	+

^a Numbers 212^T and 21^T are reference strains of *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *carotovorum*, respectively. Numbers 1 to 9 represent nine randomly selected strains isolated from cucumber in China: 1 = HG1501090302, 2 = HG1501090303, 3 = HG1501090304, 4 = HG1501090305, 5 = HG1501090306, 6 = HG1501090307, 7 = HG1501090308, 8 = HG1501090309, and 9 = HG1501090310. Symbols: + and – indicate positive and negative responses, respectively, for the tested strain.

^b Crystal violet pectate medium.

The inoculation experiment was repeated three times, and each repeat included four plants or tubes.

Results

Symptoms of infected cucumber plants and bacterial isolation.

Surveys of cucumber soft rot disease were conducted from November 2014 to March 2015 in Shandong, Shanxi, Hebei, Henan, and Liaoning provinces of Chin. This disease was found in 90% of 20 surveyed greenhouses, and the disease incidence ranged from 20 to 50% in different regions. Symptoms on cucumber plants initially appeared as whitish, thick liquid produced on the surface of the infected tissues (Fig. 1A, B, and C) and water soaking in the infected stems. Later symptoms present as brown rot in the fruit (Fig. 1D) and wet rot on the edge or center of the leaves (Fig. 1E). With the development of the infection, the stems gradually cracked longitudinally (Fig. 1F) and showed soft rot symptoms. In total, 45 morphologically similar isolates were obtained from 45 diseased tissue samples, and the frequency of recovery of isolates from plated tissues was 100%. Nine strains were randomly selected for later studies (Table 1).

Biochemical and physiological characterizations. The biochemical and physiological characterizations of nine strains are listed in Table 4. All strains were able to grow at 28 and 37°C, showed tolerance of 5% NaCl, and formed pits on CVP media. All strains are gram-negative and facultative anaerobes. Based on biological analysis, 35 of the 95 carbon sources tested can be metabolized by all of the strains. All strains can produce acid from α -methyl glucoside, glucose, and maltose and utilize acetic acid, acetic acid, cellobiose,

d-melibiose, d-sorbitol, succinamic acid, and thymidine. These characteristics are consistent with published data for *P. carotovorum* subsp. *brasiliense* (Duarte et al. 2004; van der Merwe et al. 2010).

16S rRNA gene analysis. The DNA sequences of the partial 16S rRNA region from these nine representative isolates were sequenced and aligned for phylogenetic analysis. Amplification of the 16S rRNA gene generated a 1,400-bp sequence of the nine isolate strains. The phylogenetic analysis based on 16S rRNA divided the 27 strains into four levels of similarity (Fig. 2). The selected strains isolated from cucumber in China share 99% identity with *P. carotovorum* subsp. *brasiliense*, and grouped together with strains of *P. carotovorum* subsp. *brasiliense* and a partial strain of *P. carotovorum* subsp. *carotovorum*. There was no apparent relation between the clustering of isolates and strains of *P. wasabiae*, *P. atrosepticum*, *P. carotovorum* subsp. *odoriferum*, *Dickeya zeae*, or *Pseudomonas syringae* pv. *lachrymans*. This result indicates that the strain isolates from cucumber in China belong to *Pectobacterium carotovorum*.

MLSA analysis. To further characterize the subspecies level of the selected strains isolated from cucumber in China, eight house-keeping genes of *acnA*, *gapA*, *icdA*, *mdh*, *mltD*, *pgi*, and *proA* from nine isolate strains and 25 related taxa were concatenated and used for MLSA (Fig. 3). The MLSA revealed that the *P. carotovorum* subsp. *brasiliense* strains grouped into two clades, I and II. Clade I comprised strains from Brazil and Peru, including the type strain 212^T, while strains in clade II were from Europe, Asia, and North America, which was consistent with a previous study (Nabhan et al. 2012). The nine selected strains isolated from cucumber in

Fig. 2. Phylogenetic relationships among *Pectobacterium* spp. and *Pseudomonas syringae* pv. *lachrymans* using the maximum-likelihood method. Numbers above branches represent bootstrap value support based on maximum-likelihood analyses of 1,000 bootstrap replications. Accession numbers of *Pectobacterium* spp. and *P. syringae* pv. *lachrymans* strains are shown in bold font.

China were clearly assigned to clade II. The *P. carotovorum* subsp. *carotovorum* strains also grouped into two clusters (clades III and IV) identified in the previous MLSA study (Nabhan et al. 2011). Strains of *P. carotovorum* subsp. *odoriferum* were grouped into clade V and formed a separate homogeneous cluster. These results confirmed that nine selected strains isolated from cucumber in China were different from *P. carotovorum* subsp. *carotovorum* as well as *P. carotovorum* subsp. *odoriferum*, and belong to *P. carotovorum* subsp. *brasiliense*.

Pathogenicity assays. All nine selected strains isolated from cucumber tissues in China were evaluated on their ability to cause soft rot on their original cucumber tissues. Soft rot symptoms were observed on the cucumber seedling stems 24 h after inoculation (Fig. 4C and D), which is consistent with symptoms observed in field. Symptoms on the cucumber fruit (Fig. 4A and B) and leaves (Fig. 4E and F), which were observed 4 days after inoculation, were consistent with symptoms observed in field. The control seedlings inoculated with sterile water were asymptomatic. Bacteria reisolated from lesions of inoculated tissues exhibited the same traits as the original isolates inoculated.

Host range test. Different host plant species were inoculated with nine randomly selected strains. All the tested strains caused soft rot in species of Brassicaceae (Chinese cabbage, mustard, broccoli, radish, and cabbage), Solanaceae (green pepper, tomato, and potato), spinach, celery, and most Cucurbitaceae (cucumber, zucchini, and watermelon),

except for balsam pear and loofah, which belong to the family Cucurbitaceae (Table 1; Fig. 5).

Discussion

Based on morphology, biochemical and physiological characteristics, and molecular analysis, *P. carotovorum* subsp. *brasiliense* was identified to cause soft rot diseases on cucumber in Shandong, Shanxi, Hebei, Henan, and Liaoning provinces in China. Cucumber bacterial soft rot is a worldwide, serious bacterial disease on cucumber. During 2014 to 2015, this disease broke out in Shandong, Shanxi, Hebei, Henan, and Liaoning provinces in China, and caused serious economic losses to the local cucumber production. Due to similar symptoms, this disease was previously thought to be caused by *Pseudomonas syringae* pv. *lachrymans*. However, this study confirmed that the causal pathogen of cucumber bacterial soft rot in China was *Pectobacterium carotovorum* subsp. *brasiliense*.

Pathogenicity tests showed that the strains isolated from cucumber lesions can cause soft rot in stems and fruit and wet rot on the leaves. Bacterium overflow was also observed on the surface of infected tissues under high humidity. The biochemical and physiological characteristics of the isolate strains are consistent with previous special descriptions for *P. carotovorum* subsp. *brasiliense* (van der Merwe et al. 2010). The strains reisolated from lesions of inoculated tissues exhibited the same traits as the original isolates inoculated and validated Koch's postulates.

Fig. 3. Phylogenetic reconstruction showing evolutionary relationships among *Pectobacterium carotovorum* subspecies and isolated strains from cucumber based on multilocus sequence analysis of eight housekeeping gene fragments by the maximum-likelihood method. The bootstrap consensus tree is inferred from 1,000 replicates. There were a total of 3,877 positions in the final data set. Bootstrapping values <50% were cut off.

Based on analysis of the 16S rRNA gene sequence, nine randomly selected strain isolates from cucumber in China are clearly different from *Pseudomonas syringae* pv. *lachrymans* but the analysis failed to differentiate them from other *Pectobacterium carotovorum* strains, which is consistent with the previous study, in which the analysis of partial 16S rRNA gene sequences could not be used to discriminate among the subspecies of *Pectobacterium* (Nabhan et al. 2012; Naum et al. 2008). Due to the high degree of diversity among subspecies of *P. carotovorum*, MLSA, which has higher sensitivity than DNA hybridization and 16S rRNA gene phylogeny, was employed to differentiate *P. carotovorum* subspecies (Nabhan et al. 2012). On the basis of our MLSA result, *P. carotovorum* subsp. *brasiliense* was differentiated as a separate phylogenetic group from *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum*. Strains of *P. carotovorum* subsp. *brasiliense* grouped into two clades, I and II, and the nine randomly selected strains isolated from cucumber in China were grouped in clade II. Clade II comprised strains from Europe, Asia, and North America. Otherwise, *P. carotovorum* subsp. *carotovorum* strains also grouped into two clades, and *P. carotovorum* subsp. *odoriferum* strains formed a separate homogeneous cluster, assigned to clade V. This confirmed that the strains isolated

from cucumber in China were identified as *P. carotovorum* subsp. *brasiliense*.

The strains grouped in clade II in MLSA spread worldwide and the host plants are not only potato but also other plants (Nabhan et al. 2012). Host range tests showed that, among the candidate plants, all of the plants were infected by the strains isolated from cucumber and showed symptoms of soft rot on stems, except balsam pear and loofah. This result was consistent with published data for *P. carotovorum* subsp. *brasiliense* (Marquez-Villavicencio et al. 2011), which indicates that the pathogen of cucumber soft rot in China has a wide host range. This characteristic may assist pathogens to survive the harsh environment by parasitizing other plants or plant residues. The surviving pathogens may infect cucumber as the primary source in the next year.

From the results above, through morphology, biochemical and physiological characteristics, 16S rRNA sequence analysis, MLSA, and pathogenicity analysis, we confirmed that the strains isolated from cucumber in China were identified as *P. carotovorum* subsp. *brasiliense*. As far as we know, this is the first report about *P. carotovorum* subsp. *brasiliense* causing cucumber soft rot disease. The disease is expected to have a significant economic impact on cucumber and other crop production.

Fig. 4. Effect of the inoculation of *Pectobacterium carotovorum* subsp. *brasiliense* strains on **A** and **B**, cucumber fruit; **C** and **D**, stems; and **E** and **F**, leaves of Zhongnong No. 16. CK = inoculated with sterile water; 03, 06, and 09 = cucumber fruit inoculated with strains of HG1501090303, HG1501090306, and HG1501090309, respectively; 04, 05, 07, and 10 = cucumber stems inoculated with strains of HG1501090304, HG1501090305, HG1501090307, and HG1501090310, respectively; and 02 and 08 = cucumber leaves inoculated with strains of HG1501090302 and HG1501090308, respectively.

Fig. 5. Host range test for *Pectobacterium carotovorum* subsp. *brasiliense*. Plants were inoculated with bacterial cells at 10^8 CFU ml $^{-1}$, placed in greenhouse, and incubated at room temperature for 3 days. **A**, Chinese cabbage; **B**, spinach; **C**, tomato; **D**, cabbage; **E**, broccoli; **F**, cucumber; **G**, senry; **H**, balsam pear; **I**, pepper; **J**, radish; **K**, potato; **L**, celery; **M**, towel gourd; **N**, watermelon; and **O**, pumpkin. Lowercase letters indicate plants inoculated with strains **a**, HG1501090302; **b**, HG1501090303; and **c** and **d**, HG1501090304.

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