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Identification and Characterization of a New *Enterobacter* Onion Bulb Decay Caused by *Lelliottia amnigena* in China

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Abstract

Objectives: *Enterobacter* is a genus with numerous species associated with clinical relevance, plants, foods and environmental sources. However, the taxonomy of *Enterobacter* is complicated and confusing. In order to identify the taxonomy of the causal agent isolated from decay onion bulbs in China and provide an example to identify plant pathogens, we used biochemical technologies in combination with molecular biology to confirm the status of the pathogen.

Methods: In this study, biochemical test, colonial and microscopic morphology analysis, 16S rRNA gene sequencing and multilocus sequences analysis (MLSA) based on partial sequencing of *rpoB*, *atpD*, *gyrB* and *infB* were performed on the isolates obtained from the decayed onion bulbs. According to the biochemical characteristics and genetic relationship, we compared the pathogen with related strains obtained from BLASTn alignment. Finally, pathogenicity test was performed on healthy onion bulbs to verify the Koch's postulates.

Results: Biochemical test, colonial and electron microscopic morphology indicated that the pathogen is gram-negative, belongs to the genus *Lelliottia* and in combination with phylogenetic analysis, it is most similar to *L. amnigena*.

Conclusion: To our knowledge, this is the first report of *L. amnigena* naturally causing soft rot disease on harvested onion.

Keywords: Onion bulb; Decay; Pathogen; *Enterobacter*; *Lelliottia*; MLSA; Identification

Introduction

Enterobacteriacae is a large family of Gram-negative bacteria including many familiar pathogens such as *Escherichia*, *Enterobacter*, *Erwinia*, *Dickeya*, *Citrobacter*, *Klebsiella*, *Pantoea*, *Proteus*, *Salmonella*, *Serratia* and *Shigella*. Members of the Enterobacteriaceae can be **trivially** referred to as enterobacteria or "enteric bacteria" and the type species is *Escherichia coli*, causing intestine disease in animals.

Since created in 1960, *Enterobacter* has become one of the largest genera within Enterobacteriaceae, usually lives in almost all habitats, including in normal intestinal flora, in stool of animals, in plants, in water, in insects and in foods. However, some *Enterobacters* were founded to be phytopathogens, e.g. *E. cloacae* and *E. mori* both caused bacterial wilt of mulberry in China [1,2]. *E. cloacae* also caused some post-harvest plant diseases such as onion bulb rot in the Columbia basin of Washington state [3], ginger rhizome rot in Brazil [4] and papaya necrosis [5]. *L. nimipressuralis* (formerly named as *E. nimipressuralis*) caused elm wet wood [6], *E. cancerogenus* resulted in poplar canker and *E. dissolvens* infected corn and resulted in maceration rot [5].

Enterobacter has a long and complicated taxonomic history and currently includes 19 species [7]. However, with the development of taxonomy and identification methods, many species were reclassified from this genus, e.g. *E. agglomerans* was transferred from *Enterobacter* to the genus *Pantoea* [8]. *E. intermedius* was reassigned to *Kluyvera intermedia* [9]. *E. sakazakii* was transferred to the genus *Cronobacter* [10] and more recently, *E. nimipressuralis* and *E. amnigenus* were proposed to reclassify into a novel genus *Lelliottia* as *L. nimipressuralis* and *L. amnigena*, respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* as *P. gergoviae* and *P. pyrinus*, respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into *Kosakonia* as *K. cowanii*, *K. radicincitans*, *K. oryzae* and *K. arachidis*, respectively and *E. turicensis*, *E. helveticus* and *E. pulveris into Cronobacter* as *C. zurichensis*, *C. helveticus* and *C. pulveris*, respectively [7].

Currently, 16S rRNA gene sequence is found insufficient to assign the taxonomy of new species owing to its polyphyletic nature not only in a same family, but also in a same genus. Even the multilocus sequences analysis (MLSA) based on common house-keeping gene sequences such as *rpoA*, *rpoB*, *recN* and *thdF* resulted in confusing conclusions on the taxonomy of the enterobacteria [11]. Thereafter, an effective combination of methods should be proposed to assign a new species in taxonomy based on traditional observation technologies such as phenotypic characteristics and modern molecular biological

means such as DNA-DNA hybrids, 16S rRNA gene sequence analysis, MSLA and conserved gene cluster analysis.

In our study, we identified a pathogen isolated from decayed onion bulbs as *L. amnigena* according to the cultured colonial morphology, microscopic morphology, biochemical characteristics, combining with the analysis of 16S rRNA gene sequence and the MSLA based on the partial sequences of *rpoB*, *atpD*, *gyrB* and *infB*, suggesting to provide a reference to assign the taxonomy of the pathogen identification.

Materials and methods

Pathogen isolation

On Nov 22th, 2014, several rot samples of onion bulbs were collected from a local market in Guangzhou city, Guangdong province, China. Scales from four diseased bulbs were surface-sterilized in 70% ethanol for 30 s and rinsed 3 times in sterilized water and cut into small pieces (1 to 5 mm in length). Tissues were then macerated for 5 min in sterilized water and the lixivium was streaked onto Luria-Bertani (LB) medium plates and incubated at 28°C for 24 hours [12]. Cultures were maintained frozen at -80°C in Luria-Bertani (LB) supplemented with 20% (v/v) glycerol.

DNA extraction and PCR amplification

Bacterial genomic DNA was extracted using the MasterPure™ DNA Purification Kit following the manufacturer's protocol (Epicentre Co., USA) and stored at -20°C. Primers used for amplification of the 16S rRNA gene were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3') with conditions determined by Coenye et al. [13]. Partial sequences of the RNA polymerase β subunit (*rpoB*) was amplified using primers *rpoB* CM7-F (5'-AACCAGTTCCGCGTTGGCCTG-3') and *rpoB* CM31b-R (5'-CCTGAACAACACGCTCGGA-3'), while DNA gyrase subunit B (*gyrB*) using gyrB 01-F (5'-TAARTTYGAYGAYAACTCYTAYAAAGT-3') and gyrB 02-R (5'-CMCCYTCCACCARGTAMAGTT-3'), initiation translation factor 2 (*infB*) using infB 01-F (5'-ATYATGGGHCAYGTHGAYCA-3') and *infB* 02-R (5'-ACKGAGTARTAACGCAGATCCA-3') and ATP synthase β subunit (*atpD*) using atpD 01-F (5'-RTAATYGGMGCSGTRGTNGAYGT-3') and *atpD* 02-R (5'-TCATCCGCMGGWACRTAWAYNGCCTG-3') with conditions previously described [14]. Amplicons were purified with Omega's EZNA. TM Cycle Pure Kit following the manufacturer's protocol (Omega, USA) and sequenced by Invitrogen Company in Guangzhou, China. The sizes of the resultant amplicons were: *rpoB*=637 bp, *gyrB*=742 bp, *infB*=615 bp and *atpD*=642 bp.

Multilocus sequences analysis (MLSA) and phylogenetic analysis

Sequences analyses were performed using DNASTAR Lasergene SeqMan program. Sequence similarities of 16S rRNA gene were determined using EzTaxon-eserver (http://www.ezbiocloud.net/eztaxon) [15] and BLASTn program. Sequences of *rpoB*, *gyrB*, *infB* and *atpD* genes of related strains were obtained from GenBank database. Sequences were aligned with ClustalW and phylogenetic trees were reconstructed using the neighbor-joining method with maximum composite likelihood model. Bootstrap analysis was performed based on 1000 replicates. The MEGA5 package was used for all phylogenetic analyses [16]. The GenBank accession numbers of the 16S rRNA, *rpoB*, *gyrB*, *infB* and *atpD* gene sequences of strain ZJN are KP642511, KP642510, KP642508, KP642509 and KP642507, respectively.

Colonial and microscopic morphology

Colonial morphology was observed on LB agar plate with bacteria grown for 8 h, the bacterial cells were suspended in sterile distilled water and stained with phosphotungstic acid [3% (v/V), pH 7.0] for 2 min, air-dried and observed by using transmission electron microscope (Hitachi H7650).

Biochemical test

Phenotypic characterization was carried out according to the minimal standards proposed by Brady et al. [7]. Biochemical features of the isolate were studied using standardized procedures [17] of the following tests: Gram staining, oxidase, catalase, cell motility, oxidation and fermentation test, gas and acid production from glucose, indole, methyl red, Voges-Proskauer reaction, utilization of citrate, propanedioic acid, hydrogen sulfide, arginine dihydrolase, lysine and ornithine decarboxylases, urease, O-nitrophenyl-β-D-galactopyranoside, nitrate reduction and gelatine hydrolysis. The Biolog system (GEN III v2.7.1.40.I5G) was used to determine the carbohydrate fermentation profile and readings were made after 22 h of incubation at 33°C.

Pathogenicity test on bulbs

For the pathogenicity test, mature bulbs were chosen as a randomized complete block design with a factorial combination of the red cultivars (Redwing representing common red storage cultivars grown in the Shandong province of China). Three inoculation treatments [strain ZJN, *Escherichia coli* DH5α and Phosphate-buffered saline (PBS) buffer] in three inoculation locations were performed. Strains ZJN and *E. coli* DH5α were grown overnight in 15 ml of LB medium at 30°C and 37°C respectively with 200 rpm oscillation. Cells were harvested by centrifugation (3000 rpm for 5 min), washed with 0.5-fold volume of PBS buffer and resuspended in PBS buffer to an optical density at 600 nm of 0.3-0.5 (approximately 1×10^8 CFU/ml) and 0.2 ml inoculum or PBS buffer was injected into each bulb [18,19]. The inoculated bulbs were kept at 30°C for approximately three weeks. Three replicates of three onion bulbs were used for each treatment.

Results

Symptoms of the onion bulb decay disease

In this study, we collected decay onion bulbs from a local market and found that the naturally infected bulbs showed intact onion on peel, but decayed internal tissues and odorous smells dispersed when cutting open. After disease development, the whole bulbs became soft, watery and decayed (Figure 1A).

Morphological characteristics of the casual agents

Casual agents from the interfaces of health and disease were isolated and grown on LB agar plates. Colonies of the cultured bacteria seemed approximate and eight representative isolates were selected for further characterization. Cultural results showed that the colonies were unpigmented, convex, round and smooth with entire margins. Electron micrograph showed that the bacterial cells are straight rod, 0.7-1.0 μm

× 0.88-2.68 μm in size, with peritrichous flagella and fimbriae and wrinkle cell surfaces (Figure 1B).

Figure 1: Symptoms of natural infection and microscopic morphology of the isolate. (A) Infected bulbs showed intact onion on peel, but decayed internal tissues and odorous smells dispersed when cutting open. (B) Transmission electron micrograph of isolate ZJN showed that cells are straight rod, with peritrichous flagella and fimbriae and wrinkle cell surface. Bar=200 nm.

Phylogenetic analysis on the strain

Then 16S rRNA gene sequence analysis and MLSA were conducted for rapid classification of the strains. Results showed that all sequences of the eight isolates were identical in each gene. Analysis on the 16S rRNA gene sequence revealed strain ZJN belonged to the Enterobacteriaceae, showing the similarities with *Enterobacter aerogenes* (99.43%), *Lelliottia amnigena* (99.35%), *Kluyvera cryocrescens* (99.27%), *Enterobacter soli* (99.07%), *Leclercia adecarboxylata* (98.85%), *L. nimipressuralis* (98.63%) and *Buttiauxella izardii* (97.85%) (Table 1).

	Gene sequence similarity (%) with strain ZJN					
Species	**16S rRNA**	*rpoB*	*atpD*	*gyrB*	*infB*	**Concatenated** *rpoB*, *atpD*, *gyrB* **and** *infB*
Enterobacter aerogenes	99.43	95.29	93.77	88.68	87.97	91.42
Enterobacter cloacae subsp. cloacae	97.84	95.92	94.08	86.39	88.29	91.17
Enterobacter cancerogenus	98.99	95.76	94.7	88.41	89.27	92.04
Kluyvera cryocrescens	99.27	95.13	93.61	87.73	90.08	91.64
Enterobacter soli	99.07	95.45	93.77	86.39	90.41	91.51
Lelliottia amnigena	99.35	92.94	94.55	88.27	92.85	92.15
Lelliottia nimipressuralis	98.63	93.25	94.55	89.08	92.85	92.43
Leclercia adecarboxylata	98.85	96.23	95.02	87.60	92.52	92.84

Table 1: 16S rRNA, *rpoB*, *atpD*, *gyrB* and *infB* gene sequence similarities between strain ZJN and type strains of phylogenetically related species.

Phylogenetic analysis based on 16S rRNA gene sequence with neighbor-joining approach indicated that the isolate ZJN was clustered together with *L. amnigena* and *L. nimipressuralis*, *Enterobacter soli* and *Buttiauxella izardii* (Figure 2). Moreover, the reconstructed phylogenetic tree based on partial *rpoB* (637 bp), *atpD* (642 bp), *gyrB* (742 bp) and *infB* (615 bp) gene sequences (Figure 3) indicated that strain ZJN belonged to *Lelliottia* genus, showing both 94.55% *atpD* gene sequence similarity, 92.94 and 96.23% *rpoB* gene sequence similarity, 88.27% and 89.08% *gyrB* gene sequence similarity and both 92.85% *infB* gene sequence similarity to *L. amnigena* and *L. nimipressuralis*, respectively (Table 1). Accumulatively, we assigned strain ZJN into the genus *Lelliottia*. Additionally, the *Enterobacter aerogenes* strains were clustered in the same clade as *Klebsiella* strains (Figure 3), suggesting that *E. aerogenes* should be reclassified as *K. pneumoniae*, consistent with the reports in Bergey's Manual of Systematic Bacteriology [20] and Brady et al. [7].

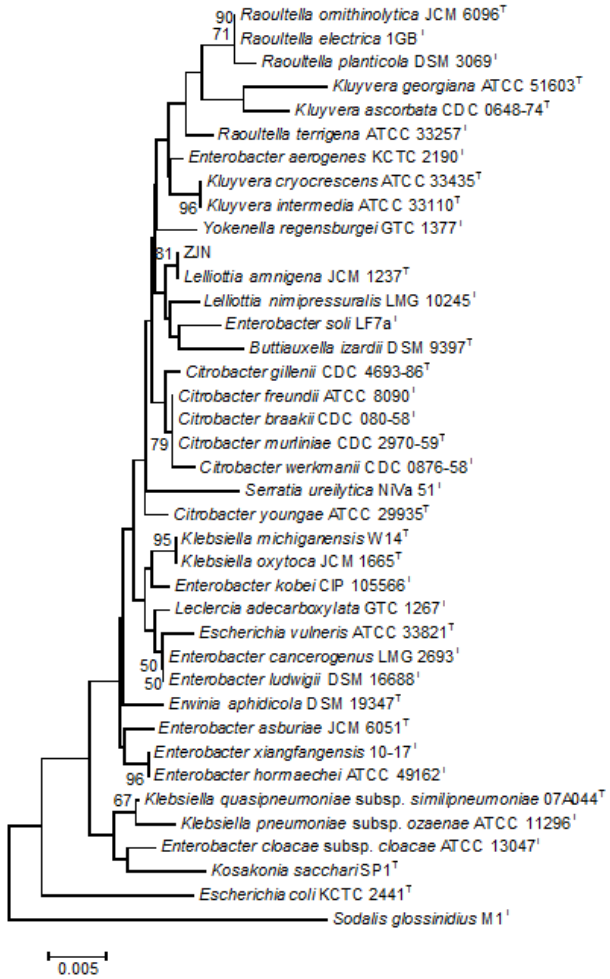

Figure 2: Neighbor-joining tree showing the 16S rRNA gene phylogenetic relationships of strain ZJN and phylogenetically related reference strains. Bootstrap values based on 1000 resamplings are shown at branch nodes. Bar, 0.5% substitution rate per site.

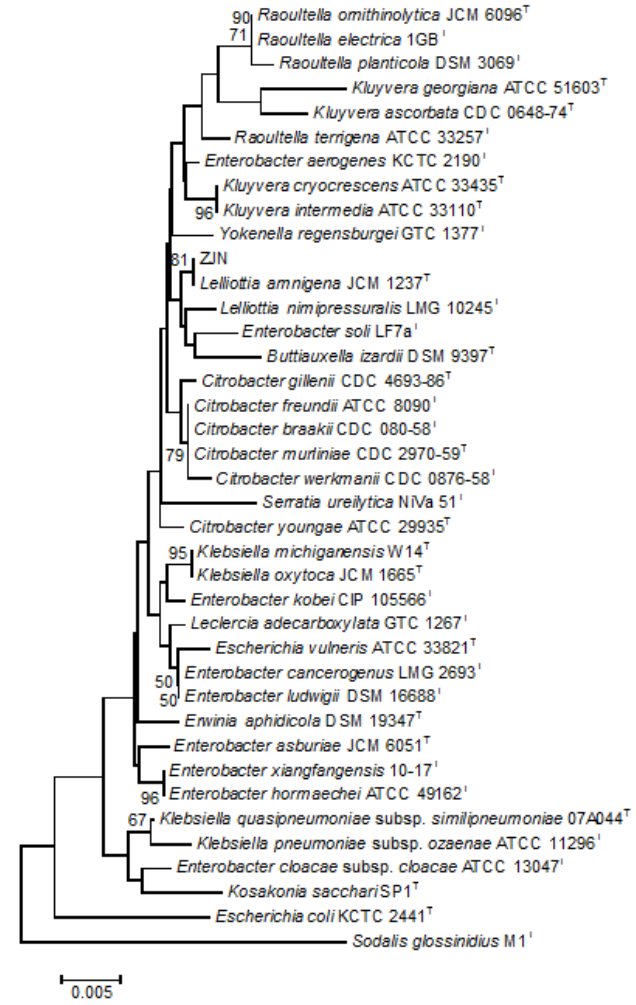

Figure 3: Neighbor-joining tree showing the phylogenetic relationships of strain ZJN and phylogenetically related reference strains based on concatenated partial *rpoB*, *atpD*, *gyrB* and *infB* gene sequences. Bootstrap values based on 1000 resamplings are shown at branch nodes. Bar, 2% substitution rate per site.

Biochemical test on the strain

Further biochemical test showed that strain ZJN is Gram negative (Figure 4), facultatively anaerobic. Optimum temperature for growth is 30°C, grows at 37°C and 41°C, different from the *L. amnigena* and *L. nimipressuralis* (Table 2). Based on the biochemical characteristics in Biolog Gen database (Table 2) and the report by Brady et al. [7], this strain was identified to *Lelliottia* genus. However, it was positive for methyl red and oxidized D-sorbitol (Table 2), more closely related with *L. amnigena* than *L. nimipressuralis* and *Leclercia adecarboxylata*. In combination of the phylogenetic analysis and the results of biochemical test, we assigned strain ZJN as *L. Amnigena*.

Characteristic	*Leclercia adecarboxylata*	*Lelliottia amnigena*	*Lelliottia nimipressuralis*	ZJN
Growth at temp				
37°C	+	+	+	+
41°C	ND	-	-	+
Motility	+	+	+	+
Growth in KCN	+	+	+	ND
Yellow pigment	+	-	-	-
Indole production	+	-	-	-
H_2S production	-	-	-	-
Methyl red	+	V	-	+
Voges-Proskauer	-	+	+	+
Arginine dihydrolase	-	+	+	-
Ornithine decarboxylase	-	V	+	-
Lysine decarboxylase	-	-	-	-
Malonate	+	+	+	+
Citrate	-	+	+	+
D-Serine	-	V	V	-
Nitrate to nitrite	ND	+	+	+
Urease	ND	+	+	-
Gelatinase	-	-	-	-
β-galactosidase	+	+	+	+
Turanose	-	V	V	-
p-Hydroxy-phenylacetic acid	+	-	-	-
Fermentation of:				
Tween 40	-	-	-	V
Tween 80	-	-	-	V
D-Arabinose	+	+	+	+
Maltose	ND	+	+	+
D-Glucose	+	+	+	+
D-Lactose	+	+	+	+
D-Galactose	ND	+	+	+
L-Rhamnose	+	+	+	+
D-Mannose	ND	+	+	+
D-Fructose	ND	+	+	+
α-methyl-D-glucoside	-	V	ND	+

D-Trehalose	ND	+	+	+
D-Raffinose	V	+	+	V
Melibiose	+	+	+	+
D-Sucrose	V	V	-	-
D-Mannitol	+	+	+	+
meso-Inositol	-	-	-	V
D-Cellobiose (acid)	+	+	+	+
D-Cellobiose (gas)	+	+	+	+
D-Glycerol (gas)	ND	+	+	+
D-Sorbitol	V	V	-	+
Salicin	+	+	ND	+
D-Arabitol	+	-	-	V

Table 2: Phenotypic features of strain ZJN and phylogenetically related species.

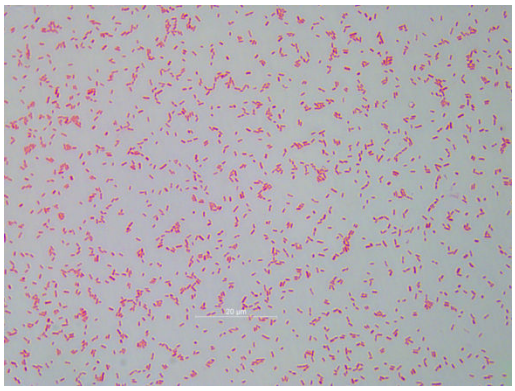

Figure 4: Gram staining of strain ZJN.

Pathogenicity test of the strain

Subsequently, triplicates of onion bulbs were then injected at the neck, the base and the shoulder, with strain ZJN, *E. coli* DH5α and PBS buffer, respectively. After incubating at 30°C for 3 weeks, symptoms similar to the natural infections were observed on the ZJN inoculated bulbs, whereas, bulbs injected with PBS buffer and *E. coli* DH5α remained symptomless (Figure 5). However, different speeds and symptoms in disease development were indicated at different injection sites. When inoculating at the shoulder, the infected tissues of the bulbs became darker brown discoloration, dried out, collapsed and lesions extended through the site of inoculation, with intact peel (Figures 5A, 5C, 5D), whereas, when infecting at the neck or base, the internal fleshy scales of the bulbs were water soaking, with tissues maceration, necrosis and lesions extended outside (Figure 5B). Bacteria were reisolated from diseased tissues and further identified as *L. amnigena* ZJN by identical 16S rRNA gene sequence.

At this moment, we confirm that strain ZJN, which caused an *Enterobacter* bulb decay of onion, is *L. amnigena* according to the 16S rRNA gene sequence analysis, multilocus sequence analysis of *rpoB*, *gyrB*, *infB* and *atpD* gene sequences and biochemical features.

Figure 5: Symptoms of artificial infection with isolate ZJN. Bulbs were inoculated with PBS buffer (A), *E. coli* DH5α (B), and strain ZJN at different injection sites (red arrow) such as upper shoulder (C), neck and base (D), down shoulder (E) and base (F). All bacterial inoculums were 0.2 ml with OD600=0.3-0.5 (approximately 1×10^8 CFU/ml). The inoculated bulbs were kept at 30 for approximately three weeks. Three replicates of three onion bulbs were used for each treatment.

Discussion

Allium cepa L, also known as onion, is a popular vegetable and traditional medicine as a seasonal and storage crop. Whereas, onion bulb decay was recently reported as a storage disease caused by Enterobacteriaceae, including *Enterobacter cloacae* in Jordan, Australia, California, Colorado, Washington, New York and China [3,18,21-25] and *Pantoea ananatis* and *Klebsiella pneumoniae* in New York and China [26-28].

In this study, we identified another bacterial pathogen called *Lelliottia amnigena* which caused onion bulb decay in postharvest stage using a combination of taxonomic techniques such as morphological observations, biochemical test and MSLA analysis. Although the 16S rRNA gene sequence of strain ZJN showed the highest similarity with *E. aerogenes* (99.43%) (Table 1) and the concatenated partial *rpoB*, *atpD*, *gyrB* and *infB* gene sequence was most similar to that of *Leclercia adecarboxylata* (92.84%), strain ZJN was assigned in the same clade of *Lelliottia* in both phylogenetic trees (Figure 2 and Figure 3). However, there are some differences in the biochemical phenotypes between strain ZJN and *L. amnigena* and *L. nimipressuralis*, e.g. strain ZJN could grow at 41°C, while the other two species could not [7]; different from *L. amnigena* and *L. nimipressuralis*, strain ZJN was negative for arginine dihydrolase and urease (Table 2), suggesting that these characteristics may be variable in different strains. By comparing the other biochemical features, we found that strain ZJN was positive for methyl red and negative for

ornithine decarboxylase, fully different with that of *L. nimipressuralis*, whereas, these features of *L. amnigena* are variable in different strains [7,29-31]. Accumulatively, we propose to assign this strain as *L. amnigena*.

After pathogenicity test of strain ZJN on healthy onion bulbs, we found that symptoms on different inoculation sites are different, which were darker and dried out at the shoulder with intact peel, whereas, water soaking, macerating and with lesions extended outside at the neck or base. We believed that the internal fleshy scales are more tender and watery, easier to be damaged and macerated, while the external scales are dry with little nutrient to feed the bacteria, leading little symptoms development outside.

Currently, *Lelliottia* is a newly built genus which includes just two species, e.g. *L. nimipressuralis* and *L. amnigena*, which were previously considered to genera *Erwinia* and *Enterobacter*, respectively. Brenner et al. proposed that *Erwinia nimipressuralis* should be transferred to the genus *Enterobacter* as *E. nimipressuralis* [29]. Whereas, Brady et al. proposed *E. nimipressuralis* and *E. amnigenus* to reclassify into *L. nimipressuralis* and *L. amnigena* respectively based on MLSA [7]. *L. amnigena* was multidrug-resistant Enterobacteriaceae strain [32,33], reported to infect heart transplant patient, causing septicemia and endophthalmitis [34-37]. Moreover, a *L. amnigena* isolate from agricultural soil could effectively reduce nitrate to ammonia [20] and was reported to have a good potential for use as an antifungal biocontrol agent. Vipul et al. isolated *L. amnigenus* from the sea dumps [38]. They found that the strain showed high chitinase production and produced proteases, inhibiting the growth of *Fusarium* sp. and *Macrophomina phaseolina*. Up till now, *L. nimipressuralis* were previously reported to naturally infect elm trees and exhibiting symptoms of wetwood disease on plants [6] and could cause wilt of pyracanth [39]. To our knowledge, this is the first report of *L. amnigena* naturally causing soft rot disease on plant.

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