Characterization, Phylogenetic Analyses, and Pathogenicity of *Enterobacter cloacae* on Rice Seedlings in Heilongjiang Province, China

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Abstract

Rice is used as a staple food in different areas of world, especially in China. In recent years, rice seedlings have been affected seriously by symptoms resembling bacterial palea browning (BPB) in Heilongjiang Province. To isolate and identify the pathogenic bacteria responsible for the disease, 40 bacterial strains were isolated from diseased rice seedlings collected from the four major accumulative-temperature zones of rice fields cultivated in Heilongjiang Province, and these were identified as 13 species based on morphological characteristics and 16S ribosomal RNA (rRNA) gene sequences. Inoculation of all the isolates on healthy rice seedlings showed that the nine *Enterobacter cloacae* isolates were the pathogens causing typical symptoms of BPB, including yellowing to pale browning, stunting, withering, drying, and death. Moreover, the nine *E. cloacae* isolates could also cause symptoms of bacterial disease

on the seedlings of soybean (*Glycine max*), maize (*Zea mays* L.), and tomato (*Solanum lycopersicum*). Phylogenetic analysis based on the 16S rRNA gene sequences and phenotypic and biochemical characteristics indicated that these nine pathogenic isolates were *E. cloacae*. In addition, analysis of the sequences of four housekeeping genes (*rpoB*, *gyrB*, *infB*, and *atpD*) from the selected strain SD4L also assigned the strain to *E. cloacae*. Therefore, *E. cloacae* is the pathogen causing disease of rice seedlings in Heilongjiang Province, which we propose to classify as a form of BPB. To the best of our knowledge, this is the first study to identify *E. cloacae* as a causal agent of BPB in rice.

Keywords: 16S rRNA gene, bacterial palea browning, *Enterobacter cloacae*, rice

Globally, rice (*Oryza sativa* L.) is considered as a major food crop for people. Over 3.5 billion people rely on rice as their staple food and source of daily calories (Cheng et al. 2007), particularly in Asia; nearly 90% of the world's rice is produced and consumed in this region (Gumma et al. 2011). Certainly, rice is also one of the most important food crops in China and plays an important role in agricultural energy. Heilongjiang Province is an important rice-planting area in northeast China. The yield and quality of rice in Heilongjiang Province are directly related to national food security but also affect the stability of the economy. Bacterial palea browning (BPB), typically caused by *Pantoea agglomerans* (Gavini et al. 1983, 1989; Kim et al. 1989; Tabei et al. 1988), *P. ananatis* (Azegami 2013; Cortesi and Pizzatti 2007), and *P. ananas* (Hasegawa et al. 2003; Mergaert et al. 1993), is a devastating and destructive disease of rice. Infection of plants

by the BPB at the heading and flowering stages results in quality reduction, while infection at early stages causes severe damage (Azegami 2013; González et al. 2015; Hasegawa et al. 2003; Kim et al. 1989; Lee et al. 2010). BPB infection may be noticed at the seedling stage. The symptoms are characterized by yellowing of leaves, which starts from the tips of leaves and proceeds downward, with leaves later turning pale brown and dry in paddy fields (Azegami 2013; González et al. 2015; Hasegawa et al. 2003; Lee et al. 2010; Tabei et al. 1988). The disease is visualized with small droplets of bacterial ooze (pale amber in color) found from the affected portions. The most destructive phase of the disease in the wilt phase resulting from early systemic infection in the nursery or from seed infection (Kim et al. 1989; Tabei et al. 1988).

Enterobacter cloacae is a Gram-negative bacterium most frequently isolated from humans and animals (Doijad et al. 2016; Wu et al. 2018, 2019) and commonly found in the environment (e.g., water and soil) (Kim et al. 1998; Subrahmanyam et al. 2011; Yokozeki et al. 1982). In addition to being a human pathogen (Sanders and Sanders 1997), *E. cloacae* is also a pathogen of plants, including the plant hosts coconut (George et al. 1976), onion bulb (Schwartz and Otto 2000; Zaid et al. 2011), edible ginger (Nishijima et al. 2004), mulberry (Wang et al. 2008), and papaya fruit (Keith et al. 2008). Plant-growth-promoting *E. cloacae* strains have been found associated with rice (Shankar et al. 2012) but no *Enterobacter* rice pathogens have been identified.

In this research, we report on the identification and characterization of the *E. cloacae* strains isolated from rice seedlings with browning symptoms in Heilongjiang Province, China, and determine their pathogenic responses on rice and other hosts. Due to similarity in phylogeny and symptoms to the disease caused by *Pantoea* spp., we propose to classify the *E. cloacae* disease as BPB. This pathogen may present a threat to the production of rice in this area. Identifying

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the causative agent of this disease is necessary to help researchers develop viable disease control strategies. This is the first report of *E. cloacae* causing BPB on rice seedlings.

Materials and Methods

Sample collection and isolation. Leaves with symptoms from each of 10 randomly selected rice seedlings of each region were cut into pieces of 5 to 10 mm in length and surface sterilized (75% ethanol for 30 s and 1% sodium hypochlorite [NaClO] for 3 min). After that, samples were macerated in sterilized water and placed on semiselective tryptic soy broth (TSB) (Aoboxing Bio-Tech Co., Ltd., Beijing, China) agar medium and incubated for 48 h at 28°C. Single colonies were isolated and restreaked on TSB agar medium at least two consecutive times to purify the strains. Then, the strains were preliminarily identified by morphology and the 16S ribosomal RNA (rRNA) gene sequences. The numbers of bacteria were recorded and the percentage of each species was calculated. Stock cultures were stored in sterile distilled water (SDW) in test tubes at 20°C or at room temperature. Strains selected for long-term storage were stored in 20% glycerol at –80°C.

Pathogenicity tests. Forty isolates were recovered in this study, and all strains were used for pathogenicity studies on rice seedlings (Table 1). From the 40 isolates, *E. cloacae* strains that induced BPB on rice seedlings were also inoculated on the seedlings of soybean (*Glycine max*), maize (*Zea mays* L.), and tomato (*Solanum lycopersicum*) to test their ability to macerate other crops.

All inoculum for this study was prepared from cultures grown on TSB agar medium at 30°C for 3 to 5 days. Bacterial suspensions for each strain were prepared by scraping cells from individual cultures into 15 to 20 ml of SDW (Nishijima et al. 2004). These bacterial suspensions were adjusted spectrophotometrically to approximately 10^8 to 10^9 CFU/ml with SDW to an optical density at 600 nm (OD_{600}) of 0.5 using a UV spectrophotometer (U-3900; Hitachi Co., Tokyo, Japan) and then immediately misted on the surface of fresh,

healthy, and surface-disinfested leaves of the tested crops with a hand-held spray bottle until runoff (Hou et al. 2019). SDW was used as a control.

Inoculation of rice seedlings in greenhouse. Forty isolates were evaluated for their pathogenicity on the rice seedlings (Qijing 10 varieties). The surface of leaves of the rice seedlings was disinfested using 70% ethanol (vol/vol) for 30 s, rinsed with SDW, and air dried prior to inoculation. Two hundred pots (approximately 30 rice seedlings per pot) of 28-day-old rice seedlings were inoculated by spraying bacterial suspensions (10^8 CFU/ml, as determined by viable counts of serial dilutions of the initial suspension at OD_{600} = 0.5) on the surface of leaves with a handheld spray bottle until runoff and covered with plastic bags for 24 h. The rice seedlings were placed in a humidified chamber (>95% relative humidity) at 28°C for 48 h after inoculation, then kept in a growth chamber at 25°C with 12-h day-and-night cycle. Twenty pots of surface-disinfested rice seedlings inoculated with SDW were used as a control. Disease severity was assessed 9 days after inoculation. The experiment was conducted three times.

Inoculation of soybean, maize, and tomato seedlings in greenhouse. Nine *E. cloacae* isolates that induced BPB on rice seedlings were inoculated on soybean seedlings (Hefeng 25 varieties). Forty-five pots (four soybean seedlings per pot) of 35-day old soybean seedlings were inoculated by spraying bacterial suspensions (10^8 CFU/ml) on the surface of disinfested leaves (70% ethanol [vol/vol] for 30 s, rinsed with SDW, and air dried) as described above and covered with plastic bags for 24 h. The soybean seedlings were placed in a humidified chamber (>95% relative humidity) at 28°C for 48 h after inoculation and then kept in a growth chamber at 25°C with 12-h day-and-night cycle. Ten pots of surface-disinfested soybean seedlings that were inoculated with SDW were used as a control. Inoculation tests were also performed on the seedlings of 30-day-old maize (Tiannong 9 varieties, one maize seedling per pot) and 35-day-old tomato (Qiyanaifen varieties, one tomato seedling per pot) as in the method described above. Disease severity was assessed 9 days after inoculation. All the experiments were conducted two times.

Disease severity was rated based on a slightly modified 0-to-4 disease index scale (Liu et al. in press), where 0 = no symptoms; 1 = small lesions on the leaf (covering less than 1/4 of the leaf); 2 = moderate-sized lesions on the leaf (covering about 1/4 to 1/2 of the leaf); 3 = large lesions on the leaf (covering about 1/2 to 3/4 of the leaf); and 4 = dead plants, with lesions covering the whole leaf. The percent disease index for BPB was calculated to determine the pathogenicity of the strains as follows: disease index < 50 = weakly pathogenic, 50 ≤ disease index < 60 = moderately pathogenic, and disease index ≥ 60 = highly pathogenic.

Reisolation of inoculated bacterial strains. The pathogenic bacteria (*E. cloacae* isolates) were reisolated from symptomatic tissues of inoculated plants (rice, soybean, maize, and tomato) and species identification was performed by the microscopic, morphological, and molecular methods to fulfill Koch's postulates and conducted according to methods described earlier, with some modifications. Leaves with BPB symptoms from all reisolated plants (rice, soybean, maize, and tomato) were cut into pieces of 5 to 10 mm in length (rice and maize) or around 0.5 cm² (soybean and tomato) and surface sterilized (75% ethanol for 30 s, 1% sodium hypochlorite for 3 min, then washed with SDW). After that, disinfested tissues were placed on semiselective TSB agar medium. Isolations from selected plants inoculated with SDW were similarly performed.

Phenotypic and biochemical characterization of *E. cloacae* isolates. The *E. cloacae* isolates (SD4L, SD5L, SD22L, SD23L, SD24L, SD32L, SD33L, SD39L, and SD40L) were characterized and identified according to “The Laboratory Guide for Identification of Plant Pathogenic Bacteria” (Schaad 1988) and “Association of *Enterobacter cloacae* with rhizome rot of edible ginger in Hawaii” (Nishijima et al. 2004). Cell morphology was examined by transmission electron microscopy (Hitachi H-7650; Hitachi Co.). On TSB agar, following incubation for 24 h at 37°C, colonies were circular (1 to 2 mm in diameter), white, opaque, and elevated, with a regular margin. Growth at different temperatures (0 to 45°C, steps

Table 1. Rice varieties in different sampling sites and major accumulative temperature zones

Variety	Sampling site	Different accumulated temperature zone
Qijing 10	Qiqihar city (47° 15' N, 123° 41' E)	I (>2,700°C)
Daohuaxiang 2	Wuchang city (45° 04' N, 127° 18' E)	II (2,500–2,700°C)
Suijing 18	Jiansanjiang (47° 19' N, 133° 26' E)	III (2,300–2,500°C)
Longqing 3	Fuyuan city (48° 16' N, 134° 34' E)	IV (2,100–2,300°C)

Table 2. Amplification and sequencing primers for *gyrB*, *rpoB*, *atpD*, and *infB*

Primers	Sequence (5'–3')
Amplification primers	
<i>rpoB</i> CM7-F	AAC CAG TTC CGC GTT GGC CTG
<i>rpoB</i> CM31b-R	CCT GAA CAA CAC GCT CGG A
<i>gyrB</i> 01-F	TAA RTT YGA YGA YAA CTC YTA YAA AGT
<i>gyrB</i> 02-R	CMC CYT CCA CCA RGT AMA GTT
<i>infB</i> 01-F	ATY ATG GGH CAY GTH GAY CA
<i>infB</i> 02-R	ACK GAG TAR TAA CGC AGA TCC A
<i>atpD</i> 01-F	RTA ATY GGM GCS GTR GTN GAY GT
<i>atpD</i> 02-R	TCA TCC GCM GGW ACR TAW AYN GCC TG
Sequencing primers	
<i>rpoB</i> CM81-F	CAG TTC CGC GTT GGC CTG
<i>rpoB</i> CM32b-R	TGA TCA ACG CCA AGC C
<i>gyrB</i> 07-F	GTV CGT TTC TGG CCV AG
<i>gyrB</i> 08-R	CTT TAC GRC GKG TCA TWT CAC
<i>infB</i> 03-F	ACG GBA TGA TYA CST TCC TGG
<i>infB</i> 04-R	AGY TTA GAT TTC TGC TGA CG
<i>atpD</i> 03-F	TGC TGG AAG TKC AGC ARC AG
<i>atpD</i> 04-R	CCM AGY ART GCG GAT ACT TC

of 1°C below 5°C, then intervals of 5°C, and 37°C also included) was determined in TSB agar medium after incubation for 7 days. Growth tests for pH ranging from 4.0 to 12.0 in 1.0-pH unit intervals and NaCl tolerance (0 to 10% [wt/vol] in 1% intervals) were determined in TSB medium at 37°C for 7 days on a rotary shaker. The buffer systems were pH 4.0 to 5.0, 0.1 M citric acid per 0.1 M sodium citrate; pH 6.0 to 8.0, 0.1 M KH₂PO₄ per 0.1 M NaOH; pH 9.0 to 10.0, 0.1 M NaHCO₃ per 0.1 M Na₂CO₃; and pH 11.0 to 12.0, 0.2 M KH₂PO₄ per 0.1 M NaOH (Zhao et al. 2019). Growth was determined by monitoring the turbidity at OD₆₀₀ using a spectroscopic method (U-3900; Hitachi Co.) and plate counting. Basic biochemical characterization of the isolates was achieved using the API 20E kit following the manufacturer's instructions (bioMérieux). Intrastrain variations were further tested using the API-50CH kit (bioMérieux). Motility tests were performed under the light microscope on cells grown for 24 h at 37°C in nutrient broth. Catalase activity was determined by observing bubble formation after dropping H₂O₂ on 24-h-old biomass on nutrient agar. Oxidase activity was tested by using oxidase reagent (bioMérieux). Antibiotic susceptibility testing was performed using the VITEK 2 compact system with AST N117 cards (bioMérieux).

Molecular characterization. *DNA extraction.* DNA of all the strains isolated from this study was obtained from single colonies that grew for 24 h in TSB medium at 37°C, using the TIANamp Bacteria DNA Kit (TIANGEN Biotech, Co. Ltd., Beijing, China) following the manufacturer's instructions.

16S rDNA sequencing. Universal primers 27F and 1492R (Stackebrandt and Goodfellow 1991) were used to amplify and sequence the 16S rRNA gene from the isolates. PCR was carried out in a final volume of 50 µl, with 1× PCR Buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.6 µM each oligonucleotide, 1.5 U of Taq polymerase, and 10 ng of template DNA. PCR conditions were initial denaturation at 95°C for 5 min; followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 57°C for 30 s, and extension at 72°C for 90 s; and a final extension at 72°C for 5 min (Eden et al. 1991). Aliquots of 5 µl were analyzed by electrophoresis in 1.5% (wt/vol) agarose in TBE buffer. Gels were stained with ethidium bromide and visualized in a gel documentation system. The PCR products were purified and sequenced using an Applied Biosystems DNA sequencer (model 3730XL; Applied Biosystems Inc., Foster City, CA, U.S.A.) and software provided by the manufacturer.

Phylogenetic analysis. The 16S rRNA gene sequences were identified via Basic Local Alignment Search Tool (BLAST) matches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) in the NCBI database. Reference sequences used for phylogenetic analysis were downloaded from the NCBI's GenBank sequence database. The 16S rRNA gene sequences were aligned using the ClustalW algorithm and manually adjusted to allow maximum sequence similarity. The aligned file was analyzed using the criterion of neighbor-joining algorithm of Saitou and Nei (1987) by Molecular Evolutionary Genetics Analysis (MEGA) software, version 7.0 (Kumar et al. 2016). The stability of the topology of the phylogenetic tree was assessed using the bootstrap method with 1,000 repetitions (Felsenstein 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura 1980).

gyrB, rpoB, infB, and atpD sequencing. To further confirm the taxonomic status of the nine *E. cloacae* isolates, one representative isolate (SD4L) was randomly selected from the nine strains to sequence the four individual housekeeping genes (DNA gyrase [*gyrB*], RNA polymerase β subunit [*rpoB*], translation initiation factor 2 [*infB*], and, ATP synthase β subunit [*atpD*]) using the primers as shown in Table 2 and conditions as described by Brady et al. (2013). PCR and sequencing setup and purification steps were carried out at the Comate Biosciences Co., Ltd., China. Sequences were identified via BLAST matches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) in the NCBI database. Consensus sequences obtained in this research were all deposited in GenBank.

Results

Isolation and identification of pathogenic bacteria. In April to May 2018 and 2019, during field trips to different fields of

Heilongjiang Province, it was observed that rice seedlings showed areas of yellow leaves, drying, and death (Fig. 1). Due to the characteristics of these symptoms, it was hypothesized that the diseased rice seedlings could have bacterial leaf blight caused by *Xanthomonas oryzae* or BPB caused by *Pantoea* spp. The symptomatic rice seedlings were collected from the four major accumulative-temperature zones

Fig. 1. Symptoms observed on rice seedlings in Heilongjiang Province, China, in 2018.

Table 3. Bacterial species and their frequencies identified from rice bacterial palea browning samples from Heilongjiang Province, China

Species	Number of isolates	Frequency (%)
<i>Stenotrophomonas maltophilia</i>	2	5.0
<i>Kosakonia cowanii</i>	3	7.5
<i>Enterobacter roggenkampii</i>	4	10.0
<i>S. pavanii</i>	5	12.5
<i>Bacillus altitudinis</i>	2	5.0
<i>Klebsiella oxytoca</i>	2	5.0
<i>K. pneumoniae</i>	2	5.0
<i>Leclercia adecarboxylata</i>	3	7.5
<i>Achromobacter spanius</i>	2	5.0
<i>B. safensis</i>	3	7.5
<i>Paenibacillus polymyxa</i>	2	5.0
<i>Pantoea dispersa</i>	1	2.5
<i>E. cloacae</i>	9	22.5

Fig. 2. Bacterial distribution among the four different rice varieties collected from Heilongjiang Province.

(I, >2,700°C; II, 2,500 to 2,700°C; III, 2,300 to 2,500°C; and IV, 2,100 to 2,300°C) of Heilongjiang Province to isolate the pathogenic bacteria. In total, 40 bacterial strains were isolated. Based on the high similarity of morphology and 16S rRNA gene sequence (99 to 100%), these isolates were identified as the following 13 species (Table 3): 9 *E. cloacae* isolates (22.5%), 5 *Stenotrophomonas pavanii* isolates (12.5%), 4 *E. roggenkampii* isolates (10.0%), 3 *Kosakonia cowanii* isolates (7.5%), 3 *Leclercia adecarboxylata* isolates (7.5%), 3 *Bacillus safensis* isolates (7.5%), 2 *S. maltophilia* isolates (5.0%), 2 *B. altitudinis* isolates (5.0%), 2 *Klebsiella oxytoca* isolates (5.0%), 2 *K. pneumonia* isolates (5.0%), 2 *Achromobacter spanius* isolates (5.0%), 2 *Paenibacillus polymyxa* isolates (5.0%), and 1

Pantoea dispersa isolate (2.5%). Among the 40 bacterial strains, 11 isolates (8 bacterial species) were isolated from rice seedlings of Qijing 10, 13 isolates (9 bacterial species) were isolated from rice seedlings of Daohuxiang 2, 9 isolates (6 bacterial species) were isolated from rice seedlings of Suijing 18, and 7 isolates (5 bacterial species) were isolated from rice seedlings of Longqing 3 (Fig. 2). Thus, *E. cloacae* was identified as the dominant bacterium, which was isolated from all four rice varieties surveyed in the current study.

Symptoms and pathogenicity. *Pathogenicity tests on rice seedlings.* Pathogenicity tests showed that *E. cloacae* was the most aggressive species. *S. maltophilia*, *E. roggenkampii*, *K. pneumonia*, *A. spanius*, *B. safensis*, *S. pavanii*, *K. oxytoca*, *L. adecarboxylata*,

Table 4. Pathogenicity and geographical location of bacteria isolates from rice in Heilongjiang Province

Group, number	Isolate	Accession number	Pathogenicity ^a	Region
<i>Enterobacter cloacae</i>				
1	SD4L	MN647552	P (S)	Qiqihar
2	SD5L	MN647553	P (S)	Qiqihar
3	SD22L	MN647556	P (S)	Wuchang
4	SD23L	MN647665	P (S)	Wuchang
5	SD24L	MN647741	P (S)	Wuchang
6	SD32L	MN647739	P (S)	Jiansanjiang
7	SD33L	MN647667	P (S)	Jiansanjiang
8	SD39L	MN647736	P (S)	Fuyuan
9	SD40L	MN647742	P (S)	Fuyuan
<i>Leclercia adecarboxylata</i>				
10	SD18L	MN647952	N (W)	Wuchang
11	SD30L	MN649211	N (W)	Jiansanjiang
12	SD38L	MN649212	N (W)	Fuyuan
<i>E. roggenkampii</i>				
13	SD2L	MN647546	N (W)	Qiqihar
14	SD3L	MN647555	N (W)	Qiqihar
15	SD12L	MN647547	N (W)	Wuchang
16	SD13L	MN647554	N (W)	Wuchang
<i>Klebsiella oxytoca</i>				
17	SD16L	MN647786	N (W)	Wuchang
18	SD37L	MN647924	N (W)	Fuyuan
<i>K. pneumonia</i>				
19	SD7L	MN647735	N (W)	Qiqihar
20	SD17L	MN647588	N (W)	Wuchang
<i>Kosakonia cowanii</i>				
21	SD26L	MN648220	N (-)	Jiansanjiang
22	SD27L	MN648221	N (-)	Jiansanjiang
23	SD34L	MN648222	N (-)	Fuyuan
<i>Pantoea dispersa</i>				
24	SD11L	MN647743	N (-)	Qiqihar
<i>Achromobacter spanius</i>				
25	SD8L	MN647606	N (W)	Qiqihar
26	SD19L	MN647627	N (W)	Wuchang
<i>Stenotrophomonas maltophilia</i>				
27	SD1L	MN647536	N (W)	Qiqihar
28	SD25L	MN647557	N (W)	Jiansanjiang
<i>S. pavanii</i>				
29	SD14L	MN647752	N (W)	Wuchang
30	SD28L	MN647761	N (W)	Jiansanjiang
31	SD29L	MN647765	N (W)	Jiansanjiang
32	SD35L	MN647766	N (W)	Fuyuan
33	SD36L	MN647784	N (W)	Fuyuan
<i>Paenibacillus polymyxa</i>				
34	SD20L	MN648192	N (W)	Wuchang
35	SD21L	MN649214	N (W)	Wuchang
<i>Bacillus altitudinis</i>				
36	SD6L	MN647559	N (-)	Qiqihar
37	SD15L	MN647666	N (-)	Wuchang
<i>B. safensis</i>				
38	SD9L	MN647651	N (W)	Qiqihar
39	SD10L	MN647738	N (W)	Qiqihar
40	SD31L	MN647740	N (W)	Jiansanjiang

^a Pathogenicity of the strains: pathogenic (P) or not pathogenic (N), disease index < 50 indicates weak pathogenicity (W), 50 ≤ disease index < 60 indicates moderate pathogenicity (M), and 60 ≤ disease index indicates strong pathogenicity (S).

and *Paenibacillus polymyxa* did not cause a clear disease response, while *Kosakonia cowanii*, *Pantoea dispersa*, and *B. altitudinis* could not infect rice seedlings (Table 4). Nine days after inoculation, the plants inoculated with *E. cloacae* developed typical symptoms of yellowing to pale browning, stunting, withering, drying, and death (Fig. 3, only showing inoculation with *E. cloacae* SD4L), which were similar to those which occur under natural conditions in the rice field in Heilongjiang Province, China (Fig. 1), whereas other weak pathogenic strains did not show the typical symptoms (data not shown). Control samples were free of symptoms (Fig. 3). The *E. cloacae* isolates were the pathogens causing BPB in the four different accumulated-temperature zones of cultivated rice fields (representing four rice varieties) located in Heilongjiang Province, northeastern China. The inoculated *E. cloacae* bacteria were successfully reisolated from the inoculated rice seedlings.

To test the ability to macerate other crops, *E. cloacae* isolates were also inoculated on the seedlings of soybean (*G. max*), maize (*Z. mays* L.), and tomato (*Solanum lycopersicum*).

Pathogenicity tests on soybean, maize, and tomato seedlings. The *E. cloacae* isolates showed moderate, strong, and moderate pathogenicity on soybean, maize, and tomato seedlings, respectively (Table 5). Nine days after inoculation, symptoms caused by *E. cloacae* included yellow leaves, stunting, and withered leaves on soybean seedlings; withered leaves, stunting, and death on maize seedlings; and yellow and withered leaves and stunting on tomato seedlings (Fig. 4A to C, only showing inoculation with *E. cloacae* SD4L). None of the SDW controls showed disease symptoms and the

inoculated *E. cloacae* bacteria were all successfully reisolated from the inoculated soybean, maize, and tomato seedlings.

Phenotypic and biochemical characterization. The nine *E. cloacae* isolates are Gram-negative, motile, nonspore forming, and facultatively anaerobic. Colonies are circular, white, translucent, raised, and smooth after 24 h of incubation at 37°C on TSB agar. Cells are motile rods (0.4 to 0.8 µm in width and 0.8 to 1.9 µm in length) with peritrichous flagella (Fig. 5). Growth occurs at 4 to 40°C, with optimal growth at 35 to 37°C. Growth occurs between pH 5.0 and 10.0 and optimal pH is 6.0 to 7.0. Cells grow at 37°C in the presence of 0 to 7% (wt/vol) NaCl in TSB medium. Acid is produced from

Fig. 3. Symptoms caused by *Enterobacter cloacae* on the rice seedlings 9 days after inoculation.

Table 5. Pathogenicity on other crops of the *Enterobacter cloacae* isolates from diseased rice in Heilongjiang Province^a

Isolates	Soybean	Maize	Tomato
SD4L	M	S	M
SD5L	M	S	M
SD22L	M	S	M
SD23L	M	S	M
SD24L	M	S	M
SD32L	M	S	M
SD33L	M	S	M
SD39L	M	S	M
SD40L	M	S	M

^a M indicates moderate pathogenicity and S indicates strong pathogenicity.

Fig. 4. Symptoms observed 9 days after inoculation on **A**, soybean (*Glycine max*); **B**, maize (*Zea mays* L.); and **C**, tomato (*Solanum lycopersicum*).

arbutin, D-adonitol, cellobiose, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, raffinose, D-ribose, D-sorbitol, trehalose, D-xylose, aesculin, gentiobiose, glycerol, melibiose, L-arabinose, L-rhamnose, salicin, and sucrose but not from lactose,

Fig. 5. Transmission electron microscopy of *Enterobacter cloacae* on tryptic soy broth agar after incubation for 24 h at 37°C. Bar = 0.5 μm.

D-mannose, methyl α-D-glucopyranoside, N-acetylglucosamine, amygdalin, starch, dulcitol, D-arabinose, melezitose, D-lyxose, erythritol, glucogen, inositol, inulin, L-sorbose, L-xylose, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, potassium 5-ketogluconate potassium gluconate, or xylitol. All isolates have positive reactions for arginine dihydrolase, β-galactosidase, and ornithine decarboxylase but are negative for lysine decarboxylase, deaminase, and gelatinase. They are also negative for urease activity and indole production but are positive for the Voges-Proskauer reaction. They can utilize lactose but not produce H₂S. They are catalase positive and oxidase negative. They can produce pectate lyase, polygalacturonase, cellulase, and proteases. Almost all of the biochemical and physiological characters were matched with *E. cloacae* as described by Richard (1984), except for a negative reaction for production of gelatinase (Table 6). All nine *E. cloacae* isolates were resistant to ampicillin, cefazolin, cefotetan, cefuroxime, and cephalothin and susceptible to amikacin, gentamicin, tobramycin, aztreonam, cefepime, cefotaxime, cefpodoxime, ceftazidime, ceftizoxime, ceftriaxone, doripenem, ertapenem, imipenem, meropenem, piperacillin, ticarcillin, ciprofloxacin, levofloxacin, moxifloxacin, nalidixic acid, norfloxacin, tetracycline, and tigecycline. These results demonstrated that the nine isolates should be *E. cloacae*.

Molecular characterization. *Phylogenetic analysis.* The GenBank accession numbers for the 16S rRNA gene sequences from each strain are listed in Table 4. In total, 53 strains (including 40 isolates in this study and 13 typical reference strains) were used to construct the phylogenetic tree based on the 16S rRNA gene sequences (Fig. 6). The phylogenetic analysis clustered the 40 isolates into the following 12 main clades: *E. cloacae* clade contained 9 isolates (SD4L, SD5L, SD22L, SD23L, SD24L, SD32L, SD33L, SD39L, and SD40L); *L. adedecarboxylata* clade

Table 6. Biochemical and physiological characteristics of *Enterobacter cloacae* (Ec) in this study and the nine type strains of *E. cloacae*^a

Characteristics	Ec ^b	Strains								
		SD4L	SD5L	SD22L	SD23L	SD24L	SD32L	SD33L	SD39L	SD40L
β-Galactosidase	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	–	–	–	–	–	–	–	–	–	–
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	+	+	+	+	+	+	+
H ₂ S production	–	–	–	–	–	–	–	–	–	–
Urease hydrolysis	–	–	–	–	–	–	–	–	–	–
Deaminase	–	–	–	–	–	–	–	–	–	–
Indole production	–	–	–	–	–	–	–	–	–	–
Voges-Proskauer reaction	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	–	–	–	–	–	–	–	–	–
D-Glucose	+	+	+	+	+	+	+	+	+	+
M-Mannitol	+	+	+	+	+	+	+	+	+	+
Inositol	–	–	–	–	–	–	–	–	–	–
D-Sorbitol	+	+	+	+	+	+	+	+	+	+
L-Rhamnose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–	–	–
Nitrate reduction	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	W	+	+
Other tests										
Anaerobic growth	+	+	+	+	+	+	+	+	+	+
Phenylalanine deaminase	–	–	–	–	–	–	–	–	–	–
Pectate degradation	–	–	–	–	–	–	–	–	–	–
Motility	+	+	+	+	+	+	+	+	+	+
Acid from										
Cellobiose	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	+	+	+
α-Methyl-D-glucoside	+	+	+	+	+	+	+	+	+	+

^a Symbols: + indicates positive reactions, – indicates negative reactions, and W indicates weak reactions.

^b Data from Richard (1984).

contained 3 isolates (SD18L, SD30L, and SD38L); *E. roggkampii* clade contained 4 isolates (SD2L, SD3L, SD12L, and SD13L); *Klebsiella oxytoca* clade contained 2 isolates (SD16L and SD37L); *K. pneumoniae* clade contained 2 isolates (SD7L and SD17L); *Kosakonia cowanii* clade contained 3 isolates (SD26L, SD27L, and SD34L); *P. dispersa* clade contained 1 isolate (SD11L); *A. spanius* clade contained 2 isolates (SD8L and SD19L); *Stenotrophomonas maltophilia* and *S. pavanii* clade contained 7 isolates (SD1L, SD14L, SD25L, SD28L, SD29L, SD35L, and SD36L); *Paenibacillus polymyxa* clade contained 2 isolates (SD20L and SD21L); *B. altitudinis* clade contained 2 isolates (SD6L and SD15L); and *B. safensis* clade contained 3 isolates (SD9L, SD10L, and SD31L).

gyrB, *rpoB*, *infB*, and *atpD* sequencing. The resulting nucleotide sequences lengths of isolate SD4L were *gyrB* = 742 bp, *rpoB* = 637 bp, *atpD* = 642 bp, and *infB* = 615 bp. Partial sequences of the four housekeeping genes of *gyrB*, *rpoB*, *atpD*, and *infB* of the strain have been deposited in GenBank under accession numbers MN325087

to MN325090. BLAST analysis revealed that these four housekeeping gene sequences of isolate SD4L were 97.9, 99.5, 99.6, and 97.9%, respectively, similar to those of *E. cloacae* strain colR/S (CP010512). This result also confirmed that SD4L belongs to *E. cloacae*.

Based on pathogenicity and phenotypic and molecular characterization, the causal agent of BPB that affects rice seedling in Heilongjiang Province, China is *E. cloacae*.

Discussion

Rice BPB has been responsible for severe decreases in quality in many countries. Previous studies have shown that BPB was mainly caused by *Pantoea agglomerans* (Gavini et al. 1983, 1989; Kim et al. 1989; Tabei et al. 1988), *P. ananatis* (Azegami 2013; Cortesi and Pizzatti 2007), and *P. ananas* (Hasegawa et al. 2003; Mergaert et al. 1993). In this study, 13 species were isolated from diseased rice seedlings collected from the four major accumulative-temperature zones of cultivated rice fields of Heilongjiang Province in 2018-2019

Fig. 6. Phylogenetic tree based on the 16S ribosomal RNA gene sequences constructed using the neighbor-joining method of the MEGA7. Bacterial strains analyzed in the current study are shown with their organism name, isolate code, and accession number in parentheses. Sequences derived from the NCBI are shown with their accession number and organism name. Bootstrap values (1,000 replicates) above 50% are shown at the nodes, and the scale bar indicates 2 changes per 100 nucleotide positions.

(Table 3; Fig. 2). However, none of the species were previously associated with rice disease in China. Of these 13 species, *E. cloacae* has been reported as a pathogen of coconut (George et al. 1976), onion bulb (Schwartz and Otto 2000; Zaid et al. 2011), edible ginger (Nishijima et al. 2004), mulberry (Wang et al. 2008), and papaya fruit (Keith et al. 2008); *Klebsiella pneumonia* has been reported as a pathogen causing bulb disease on onion in China (Liu et al. 2015). *P. dispersa* was recorded on rice in Malaysia as a leaf blight pathogen (Toh et al. 2019) but our studies showed that it was not pathogenic to rice seedlings. *Paenibacillus polymyxa* has been reported as a causal agent of bacterial stem rot on *Hylocereus undulatus* in China (Zhang et al. 2017). In this research, *E. cloacae* isolates ($n = 9$, 22.5%) were isolated from all four rice varieties (Table 4) and were identified as the pathogen. The pathogen *E. cloacae* caused typical symptoms of browning, stunting, withering, and death of rice seedlings (Fig. 3), which started from the tips of leaves and proceeded downward. These results are similar to the symptoms of BPB (Kim et al. 1989; Tabei et al. 1988). Therefore, we hypothesize that *E. cloacae* invades the plant through hydathodes at the leaf tip and leaf margin; then, the lesions enlarge in size, turn yellow to pale brown and dry, and ultimately lead to death of the plant, similar to the infection process of *Pantoea* disease (Tabei et al. 1988). Interestingly, although *Pantoea agglomerans*, *P. ananatis*, and *P. ananas* have been reported as pathogens causing BPB on rice, the three species were not recovered from the diseased rice seedling samples surveyed in the present research, and there were also no related reports in Heilongjiang Province in previous studies. Moreover, *E. cloacae* was also inoculated on soybean, maize, and tomato seedlings and could induce the symptoms of withered leaves, stunting, drying or yellowing, or death. These results indicated that this strain of *E. cloacae* has a broad host range.

The pathogenicity of *E. cloacae* isolates is perhaps based on the ability to produce a range of enzymes such as pectate lyase, polygalacturonase, cellulase, and proteases that degrade structural components of the cell wall and infect the host (Lee et al. 2014; Toth and Birch 2005). In addition, mucoid colonies of the *E. cloacae* isolates were observed on TSB agar medium. The importance of mucoid forms of *E. cloacae* has been reported by Nishijima et al. (2004), although extracellular polysaccharide production may provide the bacteria with some protection in harsh environments (Meade et al. 1993) or may be a factor in causing disease symptoms in plant hosts. The main goal of this research was to identify the causal agent of BPB. Future studies should focus on the genetic variability and virulence and aggressiveness of the *E. cloacae* isolates.

To the best of our knowledge, this is the first report of *E. cloacae* as a rice seedling pathogen in China. It is unknown whether *E. cloacae* has coevolved with rice seedlings as a pathogen over a long period of time in Heilongjiang Province or whether the bacteria have been in China for a long time. Pathogenicity of *E. cloacae* in many hosts represents a risk for agriculture in China. In addition, this data may also provide useful information for implementing effective management strategies in the future.

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