Isolation and Characterization of Novel *Pantoea stewartii* subsp. *indologenes* Strains Exhibiting Center Rot in Onion

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Abstract

Center rot of onion is an economically important disease caused by three *Pantoea* spp.: *Pantoea ananatis*, *P. agglomerans*, and *P. allii*. Symptoms caused by these three species are similar and include white streaking and necrosis of foliage; and, in some cases, the bacterium may enter the bulb, causing liquefaction and rot of bulb scales. Two bacterial strains were isolated from onion expressing symptoms indicative of center rot from two different outbreaks in Toombs County, GA in 2003 (PNA 03-3) and 2014 (PNA 14-12). These strains were initially identified as *P. ananatis* based on physiological and specific polymerase chain reaction (PCR) assays; however, further 16S ribosomal RNA (rRNA) and multi-locus sequence analysis showed that the strains were more closely related to *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes*. Further characterization using phylogenetic analysis, a *P. stewartii* subsp. *indologenes*-specific PCR assay, indole test, and pathogenicity on onion and pearl millet were conducted. Phylogenetic analyses (16S

rRNA and *atpD*, *gyrB*, *infB*, and *rpoB* genes) revealed that these strains formed a distinct cluster with the type strains of *P. stewartii* subsp. *indologenes* LMG 2632^T and *P. stewartii* subsp. *stewartii* LMG 2715^T separate from *P. ananatis*, *P. agglomerans*, and *P. allii*. Furthermore, onion strains were amplified with the *P. stewartii* subsp. *indologenes*-specific PCR assay. The pathogenicity assays with onion strains showed that they were pathogenic on onion and pearl millet, a known host of *P. stewartii* subsp. *indologenes*. However, the type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T was pathogenic only on pearl millet but not on onion. These results suggest that the onion strains PNA 03-3 and PNA 14-12 can potentially be novel *P. stewartii* subsp. *indologenes* strains capable of producing symptoms on onion. Hence, we recommend the inclusion of *P. stewartii* subsp. *indologenes* as the fourth member in the center rot complex of onion, along with *P. ananatis*, *P. agglomerans*, and *P. allii*.

Center rot of onion is caused by three known *Pantoea* spp.; namely, *Pantoea ananatis*, *P. agglomerans*, and *P. allii* (Brady et al. 2011; Edens et al. 2006; Gitaitis and Gay 1997; Hattingh and Walters 1981). *P. ananatis* was first reported in Georgia in 1997 from symptomatic onion foliage (Gitaitis and Gay 1997). Although *P. agglomerans* was associated with center rot of onion in South Africa in 1981, the bacterium was not identified as a causal agent of center rot in Georgia until 2006 (Edens et al. 2006; Hattingh and Walters 1981). This was the first report of the bacterium causing this disease in the United States (Edens et al. 2006).

P. allii was identified as the third member in *Pantoea* spp. complex causing center rot of onion (Brady et al. 2011). This bacterium was identified from infested onion plant and seed in Georgia (Brady et al. 2011). Initially, the bacterial strains pathogenic on onion were tentatively identified as members of the genus *Pantoea* based on phenotypic tests. However, after further genotypic and phenotypic characterization, including sequencing of 16S ribosomal RNA (rRNA) and housekeeping genes (*gyrB*, *rpoB*, *infB*, and *atpD*), DNA-DNA hybridization, amplified fragment length polymorphism, and substrate utilization tests, the strains were identified as a novel species of *Pantoea* named *P. allii*, distinct from other known species (Brady et al. 2011).

Disease symptoms on foliage and onion bulbs caused by three *Pantoea* spp. are similar (Gitaitis et al. 2002; Walcott et al. 2002). Symptoms include white streaking along the length of the leaf with water soaked-margins. These streaks turn necrotic and appear blighted as the disease progresses. In severe cases, the entire plant may wilt with necrotic foliage. The bacterium may also invade bulb tissues, which can often result in opening avenues for secondary infections (Carr

et al. 2013). The invasion of secondary infections can result in liquefaction and rotting of tissue in storage. If conditions are ideal for disease, this pathogen can cause significant economic losses (Walcott et al. 2002).

Strains PNA 03-3 (2003) and PNA 14-12 (2014), isolated from onion foliage from two separate center rot outbreaks in Toombs County, GA, were utilized in this study. The study was aimed at deciphering genotypic relatedness of *P. ananatis* strains in Georgia using various methods, including multilocus sequence analysis (MLSA) and repetitive extragenic palindrome polymerase chain reaction (PCR). Although these strains were initially diagnosed as *P. ananatis* using phenotypic and PCR-based assays, they did not cluster with known *P. ananatis* strains (97-1 and LMG 2665^T) or with other center-rot-causing *Pantoea* spp. such as *P. allii* (LMG 24248^T) and *P. agglomerans* (06-1 and LMG 1286^T) strains when 16S rRNA and four highly conserved housekeeping genes (*atpD*, *gyrB*, *infB*, and *rpoB*) were analyzed. Interestingly, the sequences of abovementioned genes exhibited >99% similarity to *P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii*.

P. stewartii subsp. *indologenes* is known to cause leafspot on foxtail millet (*Setaria italica*) and pearl millet (*Pennisetum americanum*), while *P. stewartii* subsp. *stewartii* causes Stewart's wilt of corn (Mergaert et al. 1993). None of these *P. stewartii* spp. has been reported to cause disease on onion. *P. stewartii* subsp. *stewartii* is unable to utilize indole, whereas *P. stewartii* subsp. *indologenes* exhibits positive indole production. This assay has been a reliable method to differentiate these subspecies. However, it is difficult to differentiate *P. ananatis* and *P. stewartii* subsp. *indologenes* because both species exhibit many of the same phenotypic characteristics, such as being positive for indole production (Mergaert et al. 1993). In addition, published *P. ananatis*-specific primers cannot differentiate *P. ananatis* from *P. stewartii* (Gitaitis et al. 2002). In this article, we report the inclusion of *P. stewartii* subsp. *indologenes* as the fourth member in the center rot complex of onion. Multiple genotypic and phenotypic assays were utilized to confirm the identity and characterization of the bacterial pathogen, including sequence analysis of 16S rRNA and housekeeping genes, species-specific PCR, indole test, and pathogenicity on pearl millet and onion.

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*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary figure is published online.

Materials and Methods

Bacterial strains. Two strains investigated in this study, PNA 03-3 and PNA 14-12, were isolated from symptomatic onion foliage in Georgia in 2003 and 2014, respectively. These strains were stored in sterile 15% aqueous glycerol solution at -80°C . The strains were tentatively identified as *P. ananatis* by their morphological and physiological characteristics such as gram-negative, facultative anaerobic, positive for indole production, and negative for nitrate reductase and phenylalanine deaminase. Furthermore, confirmation was achieved by amplifying a 398-bp fragment using *P. ananatis* species-specific primers (Gitaitis et al. 2002). Additional strains used in this study are outlined in Table 1.

16S rRNA amplification and sequencing. Bacterial suspensions were grown overnight in nutrient broth following transfer of single colonies of each strain from 48-h old cultures on nutrient agar (NA) medium. The nutrient broth was shaken overnight on a rotary

shaker (Inova; New Brunswick Scientific, Edison, NJ) at 150 rpm. After incubation, 1.8 ml of bacterial suspension was used for genomic DNA extraction using an UltraClean Microbial DNA Kit (MO BIO, Carlsbad, CA) according to the manufacturer's instructions. DNA was quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and 50 ng of genomic DNA was used for PCR. Amplifications were carried out in 50 μl of a PCR master mix with the 16S rRNA primer pair (Table 2), as outlined by Weisburg et al. (1991). PCR products were subjected to gel electrophoresis in Tris-acetate-EDTA buffer on a 1.5% agarose gel at 100 V for 45 min. Positive reactions were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions, and were outsourced for sequencing (Eurofins, Louisville, KY).

MLSA. In addition to amplification of the 16S gene, four house-keeping genes were amplified (*atpD*, *gyrB*, *infB*, and *rpoB*) (Table 2)

Table 1. Strains used in this study for genetic analysis and pathogenicity assays

Strain name ^a	Host	Place of isolation	Year of isolation
<i>Erwinia amylovora</i> CFBP 1430	Hawthorn	France	1972
<i>Pantoea agglomerans</i> LMG 1286 ^T	Human	Zimbabwe	1888
<i>P. agglomerans</i> 06-1	Onion	Georgia, United States	2006
<i>P. allii</i> LMG 24248 ^T	Onion seed	South Africa	2008
<i>P. ananatis</i> LMG 2665 ^T	Pineapple	Brazil	1965
<i>P. ananatis</i> 97-1	Onion	Georgia, United States	1997
PNA 03-3	Onion	Georgia, United States	2003
PNA 14-12	Onion	Georgia, United States	2014
<i>P. stewartii</i> subsp. <i>indologenes</i> LMG 2630	Guar gum	Unknown	1966
<i>P. stewartii</i> subsp. <i>indologenes</i> LMG 2632 ^T	Foxtail Millet	India	1960
<i>P. stewartii</i> subsp. <i>indologenes</i> LMG 2671	Pineapple	Hawaii	1966
<i>P. stewartii</i> subsp. <i>indologenes</i> CFBP 3517	Foxtail Millet	India	Unknown
<i>P. stewartii</i> subsp. <i>indologenes</i> CFBP 3614	Foxtail Millet	India	1960
<i>P. stewartii</i> subsp. <i>stewartii</i> GSPB 2626	Corn	United States	Unknown
<i>P. stewartii</i> subsp. <i>stewartii</i> LMG 2713	Corn	United States	1963
<i>P. stewartii</i> subsp. <i>stewartii</i> LMG 2715 ^T	Corn	United States	1970
<i>P. stewartii</i> subsp. <i>stewartii</i> LMG 2718	Corn	United States	Unknown
<i>P. stewartii</i> subsp. <i>stewartii</i> PSS 90-1	Corn	United States	1990
<i>P. stewartii</i> subsp. <i>stewartii</i> DS203	Corn	United States	2003

^a Superscript "T" indicates type strain.

Table 2. List of primers used in this study

Gene	Primer name	Primer sequence	References
16S ribosomal RNA	fd1	5'-AGAGTTTGATCCTGGCTCAG-3'	Weisburg et al. 1991
	rd1	5'-AAGGAGGTGATCCAGCC-3'	...
<i>atpD</i>	atpD 01-F	5'-RTAATYGGMGCSGTRGTNGAYGT-3'	Brady et al. 2008
	atpD 02-R	5'-TCATCCGCMGGWACRTAWAYNGCCTG-3'	...
Sequencing primer	atpD 03-F	5'-TGCTGGGAAGTKCAGCARGCAG-3'	Brady et al. 2008
	atpD 04-R	5'-CCMAGYARTGCGGATACTTC-3'	...
<i>gyrB</i>	gyrB 01-F	5'-TAARTTYGAYGAYAACTCYTAYAAAGT-3'	Brady et al. 2008
	gyrB 02-R	5'-CMCCYTCCACCARGTAMAGTT-3'	...
Sequencing primer	gyrB 07-F	5'-GTVCCTTTCTGGCCVAG-3'	Brady et al. 2008
	gyrB 08-R	5'-CTTTACGRCGKGTATWTCAC-3'	...
<i>infB</i>	infB 01-F	5'-ATYATGGGHCAYGTHGAYCA-3'	Brady et al. 2008
	infB 02-R	5'-ACKGAGTARTAACGCAGATCCA-3'	...
Sequencing primer	infB 03-F	5'-ACGGBATGATYACSTTCCTGG-3'	Brady et al. 2008
	infB 04-R	5'-AGYTTAGATTTCTGCTGACG-3'	...
<i>rpoB</i>	rpoB CM7-F	5'-AACCAGTTCGCGTTGGCCTG-3'	Brady et al. 2008
	rpoB CM31b-R	5'-CCTGAACAACACGCTCGGA-3'	...
Sequencing primer	rpoB CM81-F	5'-CAGTTCCGCGTTGGCCTG-3'	Brady et al. 2008
	rpoB CM81b-F	5'-TGATCAACGCCAAGCC-3'	...
	rpoB CM32b-R	5'-CGGACCGCCTGACGTTGCAT-3'	...
<i>galE</i>	3614galE	5'-CGACCTGTTTGCTCTCACC-3'	Gehring et al. 2014
	3614galEc	5'-CATCAGCTTGGAGGTGCCG-3'	...
<i>recA</i>	3614recA	5'-TGACGCTGACGTTGATGCC-3'	Gehring et al. 2014
	3614recAc	5'-TCAGTGCGTTACGCCCGTA-3'	...

and concatenated to produce a 2,260-bp fragment. Each PCR was performed in a 50- μ l reaction consisting of 10 μ l of 5 $\times$ PCR buffer, 0.2 mM dNTP, 0.2 μ M each primer, 1.25 U of HotStart Taq Polymerase (Qiagen), and template DNA at 50 ng/ μ l. Amplification conditions included denaturation at 95°C for 5 min; 3 cycles of denaturation at 95°C, annealing at 55°C for 2 min 15 s, and elongation at 72°C for 1 min 15 s; followed by 30 cycles of denaturation at 95°C for 35 s, annealing at 55°C for 1 min 15 s, and elongation at 72°C for 1 min 15 s; and a final step of 7 min of elongation at 72°C (Brady et al. 2008). Amplicons were confirmed using gel electrophoresis and purified as described above. The purified amplicons were outsourced for sequencing. The list of PCR amplification and sequencing primers for each housekeeping genes is provided in Table 2. All sequences of MLSA and 16S rRNA were deposited in the GenBank database under accession numbers MF771244 to MF771259.

Phylogenetic analysis of sequencing data. Sequences were manually trimmed based on peak quality of forward and reverse chromatograms in Geneious 8.1.8 (Biomatters, Auckland, New Zealand) and were aligned using MUSCLE (<https://www.ebi.ac.uk/Tools/msa/muscle/>). The trimmed and aligned 16S rRNA sequences (667 bp) were used to generate a maximum-likelihood (ML) tree based on the Tamura-Nei model of MEGA 6.06, with bootstrapping repetitions of 1,000. Likewise, a phylogenetic tree was generated using trimmed and concatenated sequences of the four housekeeping genes: *atpD* (657 bp), *gyrB* (579 bp), *infB* (615 bp), and *rpoB* (409 bp).

Indole test. Single colonies of bacterial strains *P. stewartii* subsp. *stewartii* LMG 2715^T and *P. stewartii* subsp. *indologenes* type strains LMG 2632^T, along with PNA 03-3, and PNA 14-12, from 48-h-old NA plates were restreaked onto tryptic soy agar amended with 1% DL-tryptophan (Sigma Aldrich, St. Louis). *P. stewartii* subsp. *stewartii*

LMG 2715^T and *P. stewartii* subsp. *indologenes* type strains LMG 2632^T served as negative and positive controls, respectively. After incubation for 12 h at 28°C, single colonies were streaked onto commercially available DrySlide indole cards (Becton, Dickinson and Company, Sparks, MD). A positive reaction for indole production was indicated by the presence of purple color on the card when test cultures were applied. The assay was repeated three times.

***P. stewartii* subsp. *indologenes*-specific PCR.** Primers used in this PCR assay (Table 2) were from Gehring et al. (2014) and were specific to *P. stewartii* subsp. *indologenes* based on single-nucleotide polymorphisms within *recA* and *galE* protein-coding genes. Genomic DNA from *P. stewartii* subsp. *indologenes* LMG 2632^T, *P. stewartii* subsp. *stewartii* LMG 2715^T, PSS 90-1, PSS DS203, PNA 03-3, and PNA 14-12 were included in this assay. The PCR cycle for *recA* and *galE* primer pairs included a two-step series: initial denaturation at 94°C for 2 min; 10 cycles of a denaturation step at 94°C for 20 s, annealing at 68°C for 30 s, and elongation at 72°C for 30 s; 20 cycles of denaturation step at 94°C for 20 s, annealing at 63°C for 30 s, and elongation at 72°C for 30 s; and a final elongation step at 72°C for 4 min. Sterile water was used as a negative control for two primer pairs. PCR products were run on an agarose gel for 45 min at 100 V, as described previously. The entire experiment was repeated once.

Pathogenicity on onion. To confirm pathogenicity on onion, a known *P. ananatis* strain from onion (PNA 97-1) (Gitaitis and Gay 1997), along with test strains PNA 03-3 and PNA 14-12, were grown overnight as described above and inoculum was adjusted to an optical density of 0.3 at 600 nm (approximately 1×10^8 CFU/ml using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY). Onion seedlings (cultivar Century) were established in plastic pots (10 by 8 cm, diameter by height; Hummert International, Earth

Fig. 1. Maximum-likelihood tree based on the Tamura-Nei model of *Pantoea* strains derived from partial 16S ribosomal RNA sequences. Bootstrap values are based on 1,000 repetitions and values greater than 50% are shown. *Erwinia amylovora* CFBP 1430^T was used as an outgroup. Superscript "T" denotes type strain of the species.

City, MO) containing commercial potting mix. The seedlings ($n = 5$ seedlings/strain tested) were kept in greenhouse and maintained at 25 to 28°C and 80 to 90% relative humidity with a photoperiod of 12 h of light and 12 h of darkness. Three-month-old plants were inoculated by cutting the central leaf 1 cm from the apex with a sterile pair of scissors, as described earlier (Dutta et al. 2014, 2016). Briefly, using a micropipette, a 10- μ l drop of bacterial suspension was placed at the cut end of the leaf. Seedlings were incubated for 3 days postinoculation (dpi) and were scored for center rot foliar symptoms (white streaking and necrosis of foliage). Seedlings inoculated with 0.1 M phosphate-buffered saline (PBS) served as a negative control.

Additionally, strains were assayed for their ability to cause symptoms on bulbs (cv. Century), as described previously by Schroeder and du Toit (2010). Prior to inoculation, tunic layers around the bulb were removed and bulbs were surface sterilized with 70% ethanol. A 50- μ l aliquot of inoculum was injected into the shoulder region of the bulb ($n = 5$ bulbs/strain) using a syringe and 22-gauge needle. The needle was sterilized between replications and separate syringes and needles were used for each strain. The test strains (PNA 03-3 and PNA 14-12) along with a positive control (PNA 97-1) were used. Bulbs inoculated with 0.1 M PBS served as a negative control. After 3 dpi, onion bulbs were cut open and evaluated for symptom expression (rotting and liquefaction). Two independent experiments were conducted for both foliar and bulb assays. Bulbs inoculated with 0.1 M PBS served as a negative control. Seedlings and onion bulbs displaying putative center rot symptoms on respective tissues in the above experiments were sampled for pathogen isolation and identification as described earlier for *P. ananatis* (Dutta et al. 2016; Stumpf et al. 2017). Isolation of pathogens and further identification as *P. stewartii* subsp. *indologenes* on seedlings and onion bulbs were conducted using subspecies-specific primers, as described above (Gehring et al. 2014).

Pathogenicity on pearl millet. Pearl millet cultivar Tifgrain 102 was established in a greenhouse in the same manner as onion seedlings described above. Inoculum from bacterial suspensions of *P. stewartii* subsp. *indologenes* LMG 2632^T and test strains PNA 03-3 and PNA 14-12 was grown and prepared as previously described. Three-week-old plants were used for inoculation. A syringe was used to infect healthy appearing leaves ($n = 3$ leaves/strain) on the abaxial surface with 0.5 ml of inoculum (1×10^8 CFU/ml). PBS was used as a negative control. Seedlings with symptoms on foliage were sampled for pathogen isolation and identification, as described earlier for *P. ananatis* and *P. stewartii* subsp. *indologenes*. Five plants were used in a single experiment and the experiment was repeated once.

Results

16S rRNA amplification and sequencing. An ML tree was constructed from 16S rRNA sequences of type strains of *Pantoea* spp. along with two tested strains (PNA 03-3 and PNA 14-12). *Erwinia amylovora* CFBP 1430 served as an outgroup. Strains PNA 03-3 and PNA 14-12 formed a distinct cluster with the type strains of *P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii* with a bootstrap value of 92 (Fig. 1). These strains also clustered separately from the type strains of *P. ananatis*, *P. allii*, *P. agglomerans*, and *P. dispersa* (Fig. 1).

MLSA. Four housekeeping genes from *Pantoea* spp. were sequenced and concatenated to form a phylogenetic tree where *E. amylovora* CFBP 1430^T served as an outgroup (Fig. 2). *P. dispersa* LMG 2603^T did not cluster closely with any of the strains in this study. As observed in the 16S rRNA phylogenetic tree, PNA 03-3 and PNA 14-12 formed a distinct cluster with the type strains of *P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii*, with bootstrap value of 100 (Fig. 2). These strains also clustered separately from the type strains of *P. ananatis*, *P. allii*, *P. agglomerans*, and *P. dispersa* (Fig. 2).

Fig. 2. Maximum-likelihood tree based on the Tamura-Nei model of *Pantoea* strains derived from concatenation of four housekeeping genes (*atpD*, *gyrB*, *infB*, and *rpoB*). Bootstrap values are based on 1,000 repetitions and values greater than 50% are shown. *Erwinia amylovora* CFBP 1430^T was used as an outgroup. Superscript "T" denotes type strain of the species.

Indole test. As expected, the type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T produced a positive reaction by producing a purple color change on DrySlide indole cards. The strains PNA 03-3 and PNA 14-12 also produced a purple color change on indole cards. The type strain of *P. stewartii* subsp. *stewartii* LMG 2715^T did not produce a color change.

***P. stewartii* subsp. *indologenes*-specific PCR.** The type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T was amplified using both *galE*- and *recA*-based primer pairs, with amplicon sizes of 267 and 422 bp, respectively. Amplifications with these primer pairs were also observed for the strains PNA 03-3 and PNA 14-12, with

expected band sizes of 267 bp (*galE*) and 422 bp (*recA*), respectively. Amplifications were not observed for the negative controls and also for none of the tested *P. stewartii* subsp. *stewartii* strains (PSS 90-1 and DS 203), including type strain LMG 2715^T (Fig. 3).

Pathogenicity on onion. In total, 100% of the seedlings whose foliage was inoculated with *P. ananatis* 97-1 displayed typical foliar center rot symptoms (white streak and tissue necrosis) at 3 dpi. Also, all seedlings inoculated with PNA 03-3 and PNA 14-12 displayed symptoms similar to those above at 3 dpi. The severity of symptoms with PNA 03-3 and PNA 14-12 was similar to that of symptoms caused by PNA 97-1 because the mean lesion lengths were around 4 to 4.5 cm for all

Fig. 3. *Pantoea stewartii* subsp. *indologenes*-specific polymerase chain reaction (PCR) assay. Primers based on single-nucleotide polymorphism in *galE* and *recA* genes were utilized in stepdown PCR. The lanes in the gel image denote the following: lane 1, 100-bp ladder (New England Biolabs); lane 2, negative control (*galE*); lane 3, *P. stewartii* subsp. *stewartii* (PSS 90-1; *galE*); lane 4, *P. stewartii* subsp. *stewartii* (DS203; *galE*); lane 5, *P. stewartii* subsp. *stewartii* (LMG 2715^T; *galE*); lane 6, *P. stewartii* subsp. *indologenes* (LMG 2632^T; *galE*); lane 7, *P. stewartii* subsp. *indologenes* (PNA 03-3; *galE*); lane 8, *P. stewartii* subsp. *indologenes* (PNA 14-12; *galE*); lane 9, negative control (*recA*); lane 10, *P. stewartii* subsp. *stewartii* (PSS 90-1; *recA*); lane 11, *P. stewartii* subsp. *stewartii* (DS203; *recA*); lane 12, *P. stewartii* subsp. *stewartii* (LMG 2715^T; *recA*); lane 13, *P. stewartii* subsp. *indologenes* (LMG 2632^T; *recA*); lane 14, *P. stewartii* subsp. *indologenes* (PNA 03-3; *recA*); and lane 15, *P. stewartii* subsp. *indologenes* (PNA 14-12; *recA*).

Fig. 4. Center rot symptoms on onion foliage. Onion blades from plants were clipped 1 cm from the apex and were inoculated by depositing a 10- μ l drop (1×10^8 CFU/ml) at the cut end. **A**, Phosphate-buffered saline (PBS) (0.1 M); **B**, *Pantoea ananatis* 97-1; **C**, *P. stewartii* subsp. *indologenes* (PNA 03-3); and **D**, *P. stewartii* subsp. *indologenes* (PNA 14-12). Symptoms of center rot were observed 3 days after inoculation. Total two experiments were conducted with five replicates per treatment. Arrows in B to D denote necrotic lesions on the inoculated foliage and the arrow in A denotes a leaf inoculated with PBS.

tested strains. Center rot symptoms were not observed in negative control seedlings (Fig. 4).

Bulbs ($n = 5$ bulbs/strain/experiment) inoculated with *P. ananatis* 97-1 and *P. ananatis* LMG 2665^T exhibited typical center rot symptoms (liquefaction and rotting) at 3 dpi. These symptoms were also present in onion bulbs inoculated with PNA 03-3 and PNA 14-12 at 3 dpi. The type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T did not cause liquefaction or rotting of onion bulbs. Bulbs subjected to negative control did not express symptoms (Fig. 5; Supplementary Figure S1). Isolates from the infected foliage and bulb tissues were reisolated and reconfirmed as *P. stewartii* subsp. *indologenes* using a species-specific PCR assay, as described above (Gehring et al. 2014). In some instances, other noninoculated foliage in the same plant displayed leaf-drying symptoms (Fig. 4B). Isolations were made from the same foliage, which did not result in bacterial colonies on agar medium.

Pathogenicity on pearl millet. White streaks with necrotic lesions were observed with the type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T (positive control) at 5 dpi for all of the replicates in two

experiments. In two independent experiments, plants infected with PNA 03-3 and PNA 14-12 strains displayed symptoms as above at 5 dpi. Plants inoculated with PBS (negative control) did not show symptoms on foliage (Fig. 6). Isolates from the infected foliage were reisolated and reconfirmed as *P. stewartii* subsp. *indologenes*, as described above.

Discussion

Currently, three known closely related *Pantoea* spp. (*P. ananatis*, *P. agglomerans*, and *P. allii*) are associated with center rot of onion. These bacterial species induce similar symptoms on onion such as leaf blight and bulb rot (Brady et al. 2011; Edens et al. 2006; Gitaitis and Gay 1997). Physiological and molecular tests can reliably differentiate these three *Pantoea* spp. (Delétoile et al. 2009). Apart from various genotypic and physiological assays that can differentiate *P. agglomerans* from *P. ananatis* and *P. allii*, indole production is used as an easy and quick test to differentiate these species. This test relies on the ability of a microorganism to degrade the amino acid

Fig. 5. Center rot symptoms on onion bulbs. Onion bulbs were surface sterilized and inoculated at the shoulder by injecting a 50- μ l drop of bacterial suspension (1×10^8 CFU/ml) using a syringe. Bulbs were dissected 3 days after inoculation and symptoms (liquefaction and rotting) were observed. **A**, Phosphate-buffered saline (0.1 M); **B**, *Pantoea ananatis* 97-1; **C**, *P. stewartii* subsp. *indologenes* (PNA 03-3); and **D**, *P. stewartii* subsp. *indologenes* (PNA 14-12). Total two experiments were conducted, with five replicates per treatment. Circled areas denote liquefaction and softening of bulb tissue.

Fig. 6. Pathogenicity assay on Tifgrain 102 pearl millet. Foliage of pearl millet (3 weeks old) was infected by infiltrating bacterial suspensions (1×10^8 CFU/ml) using a syringe. Symptoms (streaking and necrosis) were observed 5 days post inoculation. **A**, Phosphate-buffered saline (0.1 M); **B**, *Pantoea stewartii* subsp. *indologenes* LMG 2632^T; **C**, *P. stewartii* subsp. *indologenes* (PNA 03-3); and **D**, *P. stewartii* subsp. *indologenes* (PNA 14-12). Two experiments were conducted, with three repeats per experiment.

tryptophan and produce indole (Mergaert et al. 1993). Although *P. agglomerans* is indole negative, *P. ananatis* and *P. allii* are indole positive. Moreover, physiologically, *P. allii* can be distinguished from *P. ananatis* and *P. agglomerans* on the basis that the former produces acid from amygdalin and is capable of utilizing adonitol (Brady et al. 2011). The three *Pantoea* spp. can also be differentiated using molecular assays. Molecular detection assays such as PCR with specific primers are available for *P. ananatis* but not for *P. agglomerans* and *P. allii* (Asselin et al. 2016; Gitaitis et al. 2002). Hence, researchers routinely rely on sequence analysis of both 16S rRNA and housekeeping genes (Hauben et al. 1998; Maiden et al. 1998; Naum et al. 2008). Being a highly conserved gene, 16S rRNA sequences do not reliably differentiate closely related *Pantoea* spp., including *P. ananatis*, *P. agglomerans*, and *P. allii*. Rather, sequencing of multiple housekeeping genes has been reliably used to differentiate and deduce phylogenetic relationships among closely related *Pantoea* spp. (Delétoile et al. 2009).

Bacterial strains PNA 03-3 and PNA 14-12 isolated from onion, identified initially as *P. ananatis*, were re-examined using sequencing of 16S rRNA gene and MLSA. These two strains did not cluster with reference strains of *P. ananatis*, *P. allii*, and *P. agglomerans* and exhibited >99% sequence similarity to *P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii*. Rather, they formed a distinct cluster with the type strains of *P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii*, with bootstrap value more than 92 (Fig. 1). These results indicate that strains PNA 03-3 and PNA 14-12 are closely related to *P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii*, and distinct from known center-rot-causing *Pantoea* spp. (*P. ananatis*, *P. allii*, and *P. agglomerans*).

Indole production has been a reliable biochemical test used to differentiate *P. stewartii* subsp. *stewartii* from *P. stewartii* subsp. *indologenes*. It was observed that the type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T, along with strains PNA 03-3 and PNA 14-12, produced a positive indole reaction whereas *P. stewartii* subsp. *stewartii* LMG 2715^T did not. These observations indicate that strains PNA 03-3 and PNA 14-12 may belong to the subspecies “indologenes” of *P. stewartii*. Further confirmation was achieved by *P. stewartii* subsp. *indologenes*-specific PCR assays (Gehring et al. 2014). The type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T and strains PNA 03-3 and PNA 14-12 were amplified using *galE* and *recA* primer pairs. In contrast, *P. stewartii* subsp. *stewartii* strains were not amplified by the same primer pairs. These results further confirm that strains PNA 03-3 and PNA 14-12 are not *P. stewartii* subsp. *stewartii* but, rather, *P. stewartii* subsp. *indologenes*.

Center rot of onion produces typical leaf blight and bulb rot symptoms on onion. Several researchers have successfully demonstrated methods of conducting pathogenicity assays on onion leaves and bulbs (Carr et al. 2013; Schroeder and du Toit 2010). The strains were used for the pathogenicity assays on onion foliage and onion bulbs. Of the seedlings whose foliage was inoculated with *P. ananatis* 97-1 along with our strains in question (PNA 03-3 and PNA 14-12), 100% displayed typical foliar and bulb symptoms at 3 dpi. Interestingly, the type strain of *P. stewartii* subsp. *indologenes* LMG 2632^T did not produce any visible foliar and bulb symptoms. These results suggest that the strains PNA 03-3 and PNA 14-12 can potentially be novel *P. stewartii* subsp. *indologenes* strains capable of producing symptoms on onion. In addition, *P. stewartii* subsp. *indologenes* LMG 2632^T and the strains PNA 03-3 and PNA 14-12 were pathogenic on pearl millet, indicating that onion strains responded typically to their described subspecies (Mergaert et al. 1993). Together, the results suggest that these onion strains could potentially represent a new pathovar of *P. stewartii* subsp. *indologenes* that is pathogenic on both onion and pearl millet. Further detailed pathogenicity assays are required to determine the host range of these onion strains, especially on other *Allium* spp.

This is the first instance where *P. stewartii* subsp. *indologenes* has been identified as a pathogen of onion. Hence, we recommend the inclusion of *P. stewartii* subsp. *indologenes* as the fourth member in the

center rot complex of onion, along with *P. ananatis*, *P. agglomerans*, and *P. allii*. For diagnostic purposes, it is important to assay symptomatic center rot plants for *P. stewartii* subsp. *indologenes* in addition to *P. ananatis*, *P. agglomerans*, and *P. allii*. Moreover, further research should investigate the vector transmissibility, distribution, prevalence, and epidemiology of this pathogen to determine whether it behaves in a manner similar to other center-rot-causing *Pantoea* spp. A better understanding of the above biology, epidemiology, and distribution will facilitate better management practices for center rot of onion.

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