Pathogenicity and a TaqMan Real-Time PCR for Specific Detection of *Pantoea allii*, a Bacterial Pathogen of Onions

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Abstract

Bacterial diseases of onion are reported to cause significant economic losses. *Pantoea allii* Brady, one of the pathogens causing the center rot on onions, has not yet been reported in Canada. We report the pathogenicity of *P. allii* on commercially available Canadian green onions (scallions). All *P. allii*-inoculated plants, irrespective of the inoculum concentration, exhibited typical leaf chlorotic discoloration on green onion leaves, which can reduce their marketability. Reisolation of *P. allii* from infected scallion tissues and reidentification by sequencing and phylogenetic analyses of the *leuS* gene suggest that the pathogen can survive in infected tissues 21 days after inoculation. This is the first report of *P. allii* as a potential pathogen of green onions. This study also reports the development and validation of a TaqMan real-time PCR assay targeting the *leuS* gene for reliable detection of *P. allii* in pure cultures and in planta. A 642-bp *leuS* gene fragment was targeted because it showed high nucleotide diversity and positively correlated with genome-based average nucleotide identity with respect to percent similarity index and identity of *Pantoea* species. The assay specificity was validated

using 61 bacterial and fungal strains. Under optimal conditions, the selected primers and FAM-labeled TaqMan probe were specific for the detection of nine reference *P. allii* strains by real-time PCR. The 52 strains of other *Pantoea* spp. ($n = 25$), non-*Pantoea* spp. ($n = 20$), and fungi/oomycetes ($n = 7$) tested negative (no detectable fluorescence). Onion tissues spiked with *P. allii*, naturally infested onion bulbs, greenhouse infected green onion leaf samples, as well as an interlaboratory blind test were used to validate the assay specificity. The sensitivities of a 1-pg DNA concentration and 30 CFU are comparable to previously reported real-time PCR assays of other bacterial pathogens. The TaqMan real-time PCR assay developed in this study will facilitate reliable detection of *P. allii* and could be a useful tool for screening onion imports or exports for the presence of this pathogen.

Keywords: disease development and spread, epidemiology, fluorescent probes, pathogen survival, pathogen detection, prokaryotes, techniques, vegetables

Alliums (bulb onion and scallions) are vegetables commonly consumed worldwide (Opara 2003). In Canada, most onions originate from Ontario and Quebec, with 45 and 37% of crop land, respectively (Agriculture and Agri-Food Canada 2012). In 2012, Canada exported about CAD\$140 million and imported CAD\$22 million worth of onions (Agriculture and Agri-Food Canada 2012). The movement of agricultural produce such as onions across international borders has an inherent risk of spreading prevalent or new diseases into new habitats. It is crucial that onion production in Canada is protected against new diseases that might reduce the market quality with economic loss to growers.

Bacterial diseases of onion caused by *Pantoea ananatis* and *P. agglomerans* have been reported in the United States and South Africa (Edens et al. 2006; Gitaitis and Gay 1997; Hattingh and Walters 1981) with potentially significant economic losses (Hattingh and Walters 1981; Walcott et al. 2002). In 2004, a new pathogenic *Pantoea* strain was isolated from onions in South Africa. Taxonomic study based on amplified fragment length polymorphism, 16S rRNA sequence similarity, and whole-genome DNA-DNA relatedness confirmed the

classification of the strain as novel species, *P. allii* (Brady et al. 2011). This study also included *Pantoea* strains from the United States collected from fields with center rot incidence, which were later confirmed as *P. allii*. Center rot incidence associated with *P. allii* has not been reported in Canada. Precautionary measures are required to prevent infected onion imports from introducing *P. allii* into Canada.

P. allii is phylogenetically related to *P. ananatis* (Goszczyńska et al. 2006; Palmer et al. 2017; Tambong et al. 2014). *P. allii* is a facultative anaerobic, Gram-negative, nonspore-forming, noncapsule rod-shaped bacterium (Brady et al. 2010, 2011). Its pathogenicity to green onions and other *Allium* spp. grown in Canada has not been reported, so it is important to determine whether this bacterium can infect commercially available green onions.

Furthermore, there is currently no reliable molecular assay for specific identification and detection of *P. allii* on onion plants as well as in pure cultures, given that it is a relatively new pathogen. A conventional PCR assay based on an 16S to 23S rRNA internal transcribed spacer developed to detect *P. ananatis* also produces a 389-bp amplicon with *P. allii* (Figueiredo and Paccola-Meirelles 2012). This seems to be the only publicly available molecular tool for detection of *P. allii*. The differentiation between *P. ananatis* (389 bp) and *P. allii* (361 bp) strains using this PCR-based assay relies on a 28-bp difference in amplicon length (Figueiredo and Paccola-Meirelles 2012). An agarose gel electrophoretic separation of PCR amplicons that differ by only 28 bp can be a challenge, leading to potential misidentification. In addition, the conventional PCR-based technique requires post-PCR handling to visualize the results for identification and as such is prone to errors. Alternatively, TaqMan real-time PCR minimizes some of these inherent problems while providing accurate detection and identification of specific plant pathogens (Robène et al. 2015; Tambong et al. 2008, 2016; Xu and Tambong 2011). Because post-PCR processing steps are not required, real-time PCR can be easily automated with high sample throughput and specificity (Tambong et al. 2016; Weller et al. 2000).

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The objectives of this research were to (i) evaluate the reliability of using a 642-bp *leuS* gene fragment for differentiation of *P. allii* from its closest phylogenetic *Pantoea* species relative to genome-based parameters, (ii) develop and validate a TaqMan-based real-time PCR assay for specific detection and/or identification of *P. allii* based on the *leuS* gene, and (iii) conduct a pathogenicity study on commercially available Canadian green onion (scallions) plants. A housekeeping gene, *leuS*, was targeted for the development of the assay because it was reported to be a reliable phylogenetic marker (Tambong et al. 2014). The molecular tool developed in this study could be used by regulatory agencies to screen onion imports for this pathogen.

Materials and Methods

Genomic DNA extraction, PCR gene amplification, and sequencing. Bacterial strains (Table 1) were cultured overnight at 28°C in Luria-Bertani (LB) broth (per liter of H₂O: 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl; BD Canada) and genomic DNA was extracted and purified using the Wizard SV Genomic DNA Purification System Kit (Promega) following the manufacturer's protocols. The DNA was quantified using picogreen chemistry in FLUOstar (BMG Labtech) and stored at -20°C. Genus-based and species-level identification of *P. allii* was performed as previously reported (Tambong et al. 2014) using 16S rRNA and *leuS*

Table 1. Bacterial strains used in this study

Species/strain ^y	Host	Origin	Source ^z
<i>Pantoea</i>			
<i>P. allii</i>			
LMG 24248 ^T	Onion	South Africa	LMG
LMG 24202	Onion	South Africa	LMG
LMG 24203	Onion	South Africa	LMG
200-5	Onion	United States	B. Dutta
200-13	Onion	United States	B. Dutta
PNA200-13-1	Onion	United States	B. Dutta
UG-G	Onion	United States	B. Dutta
UG-G1	Onion	United States	B. Dutta
<i>P. agglomerans</i> pv. <i>herbicola</i>			
LMG 1286 ^T	Knee laceration	Zimbabwe	LMG
LMG 2102	Deer	United States	LMG
<i>P. ananatis</i>			
LMG 2665 ^T	<i>Ananas comosus</i>	Brazil	LMG
<i>P. anthophila</i>			
LMG 2558 ^T	<i>Impatiens balsamina</i>	India	LMG
<i>P. calida</i>			
LMG 25383 ^T	Powdered infant formula	Switzerland	LMG
<i>P. cedenensis</i>			
CFBP 6627 ^T	<i>Olea europaea</i>	France	CFBP
<i>P. beijingsensis</i>			
LMG 27579 ^T	<i>Pleurotus eryngii</i> , fruiting body	China	LMG
<i>P. brenneri</i>			
LMG 05343 ^T	Human, urethra	United States	LMG
<i>P. cyripedii</i>			
LMG 2657 ^T	<i>Cypripedium</i> sp.	United States	LMG
<i>P. deleyi</i>			
LMG 24200 ^T	Eucalyptus	Uganda	LMG
<i>P. dispersa</i>			
LMG 2603 ^T	Soil	Japan	LMG
<i>P. eucalypti</i>			
LMG 24197 ^T	Eucalyptus	Uruguay	LMG
LMG 24198	Eucalyptus	Uruguay	LMG
<i>P. gaviniae</i>			
LMG 25382 ^T	Powdered infant formula	Switzerland	LMG
<i>P. oleae</i>			
CFBP 6632 ^T	<i>Olea europaea</i>	France	CFBP
<i>P. rodasii</i>			
LMG 26273 ^T	Eucalyptus	Colombia	LMG
<i>P. rwandensis</i>			
LMG 26275 ^T	Eucalyptus	Rwanda	LMG
<i>P. septica</i>			
LMG 5345 ^T	Human, stool	United States	LMG
<i>P. stewartii</i> subsp. <i>indogenes</i>			
LMG 2632 ^T	<i>Setaria italica</i>	India	LMG
<i>P. stewartii</i> subsp. <i>stewartii</i>			
LMG 2715 ^T	Corn	United States	LMG

(Continued on next page)

^y The superscript "T" denotes type strain.

^z Strains were obtained from the following culture collections: LMG = Laboratorium voor Microbiologie at Rijks Universiteit (Gent, Belgium), CFBP = Plant Associated Bacteria Collection (France), NCPPB = National Collection of Plant Pathogenic Bacteria (United Kingdom), GSPB = Braunschweig, Germany, DSM = German Collection of Microorganisms and Cell Cultures GmbH, ATCC = American Type Culture Collection, and CCFC = Canadian Collection of Fungal Cultures.

Primers and TaqMan probe design and real-time PCR conditions. The *leuS* gene was identified as a candidate for TaqMan probes based on high nucleotide variation across the genus *Pantoea* (Tambong et al. 2014). The *leuS* sequences of all publicly available *Pantoea* species were downloaded or extracted from GenBank and aligned using the Cluster W method (Thompson et al. 1994) as implemented in MegAlign (version 7.0; DNASTAR). Specific primers and dual-labeled TaqMan probes were designed (Tambong et al. 2016; Xu and Tambong 2011). A similarity search was performed using BLAST (Blastn, nucleotide versus nucleotide comparison) with the primers as queries against all publicly available GenBank nucleotide databases targeting the type strains as previously described (Tambong et al. 2006). In addition, a customized database comprising over 150 genomes of known bacterial and fungal pathogens of onion (<https://www.apsnet.org/edcenter/resources/commonnames/Pages/OnionandGarlic.aspx>) was used to perform a similarity search using nucleotide sequences of the *leuS* fragment as the query. Primer-BLAST (Ye et al. 2012; <https://www.ncbi.nlm.nih.gov/tools/primer-blast>) with default parameters and the NCBI nucleotide (nr) database were also used to check the specificity of the primers and probe. The primers and probe were designed and analyzed for dimer or hairpin loop structures by using Oligo 6.71 software (Molecular Biology Insights, Cascade, CO). The following three primers were designed and evaluated for specificity: allii-leuS181F (5'-GTCCGGACACCTTCTATGGCGCAA-3'), allii-leuS235R (5'-GGTTGCTTGCGGAAGCCTGCAGT-3'), and allii-leuS320R (5'-ATGTCAGCTTCAGCCACTTTGGTG-3'). A dual-labeled TaqMan probe, allii-leuS205pb (5'-[6 FAM]-TACGTTGCCGTCGCGCGGGGCCA-[BHQ1]-3'), was designed and tested for specificity by BLAST (Tambong et al. 2016; Xu and Tambong 2011). Primers and probes were synthesized by Sigma-Aldrich (Oakville, ON, Canada), and the probe was labeled with the corresponding fluorescent reporter dyes. Preliminary evaluation of the two reverse primers (allii-leuS235R and allii-leuS320R) showed that the former produced more consistent fluorescent sigmoidal curves than the latter. As such, the reverse primer allii-leuS235R was selected for downstream study.

The optimal conditions for the TaqMan real-time PCR reaction, in a Chromo4 real-time PCR system (MJ Research Bio-Rad Laboratories, Mississauga, ON, Canada), start with an initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Primer pair allii-leuS181F and allii-leuS235R and probe

allii-leuS205pb were used in real-time PCR reaction containing 5 µl of 2× SsoAdvanced Universal Probes Supermix (Bio-Rad), 0.15 µl of 20 µmol/liter of each primer, 0.15 µl of 10 µmol/liter of probe, 1 µl of template DNA, and 3.4 µl of sterile ultrapure water to a total volume of 10 µl (Tambong et al. 2008). Detection of the fluorescent emission was read after every cycle and data were assembled as previously reported (Tambong et al. 2008, 2016). Samples or standards were processed in triplicate including negative (water or no DNA template) and/or positive (type strain of *P. allii*) controls where necessary.

Sensitivity and specificity. Genomic DNA of *P. allii* strain LMG 24248^T was serially diluted 10-fold from 10 ng to 10 fg (10⁻⁵ ng) in sterile nuclease-free water in order to determine the sensitivity (detection limit). The minimum number of colonies required for positive detection was determined using bacterial suspensions serially diluted in phosphate-buffered saline (PBS) nuclease-free water or from 10⁸ to 10² CFU/ml. The assay specificity was validated on genomic DNA of 61 bacterial and fungal strains, including 34 *Pantoea* strains, 20 non-*Pantoea* strains, and seven fungi/oomycetes (Table 1). DNA of all of the strains was diluted to 1 ng/µl and TaqMan real-time PCR was performed using the same conditions indicated above. Each strain was run in triplicate and repeated once including negative controls. In addition, four blind-coded samples (two *P. allii* [samples 1 and 2], one *P. ananatis* [sample 3], and one *P. stewartii* subsp. *stewartii* [sample 4]) were processed by a second laboratory (Canadian Food Inspection Agency, Charlottetown, PEI, Canada) to independently verify the reliability of the assay and to test its reproducibility on a different real-time PCR machine using the same conditions. This was done on a Rotor-Gene Q real-time PCR machine (Qiagen) and data were captured using Rotor-Gene Q series software version 2.3.1 (Qiagen) and repeated once.

Pathogenicity test and in planta TaqMan real-time PCR detection of *P. allii*. Commercially available green onion seedlings (*A. fistulosum* L. 'Green Bunching-Flavor King') were planted in potting soil (0.5:1:1 in Promix/black top soil/sand) in a growth chamber at 28°C with a 16-h/8-h light/dark photoperiod. One seedling was planted per square fiber pot (13.125 cm wide × 8.78 cm high; Ritchie, Canada). After 3 weeks, 15 plants having three to four leaves were selected for syringe inoculation at the lowest leaf close to the whorl region. *P. allii* LMG 24248^T was grown in LB as indicated above. The bacterial broth was centrifuged at 10,625 × *g* for 5 min and the supernatant was discarded. The pellet was resuspended in PBS.

Table 2. Values of genome-based average nucleotide identity (ANI) and *leuS*-based pairwise percent identity (LNI) of *P. allii* relative to 13 other *Pantoea* species

<i>Pantoea</i> species ^z	Genome accession no.	Genome size (Mb)	Coding sequences (n)	Contigs (n)	Pseudogenes (n)	LNI (%)	Genome-based ANI (%)	Reference
<i>P. allii</i> LMG 24248 ^T	NTMH000000000	5.24	4,722	57	159	100.00	100.00	This study
<i>P. allii</i> LMG 24248 ^T	MLFE000000000	5.01	4,114	92	519	100.00	99.96	Palmer et al. (2017)
<i>P. ananatis</i> LMG 2665 ^T	JFZU000000000	4.98	4,486	27	114	89.41	88.65	Adam et al. (2014)
<i>P. stewartii</i> subsp. <i>indologenes</i> LMG 2632 ^T	JPKO000000000	4.68	3,936	35	247	88.01	84.33	Du Plessiss (<i>unpublished data</i>)
<i>P. stewartii</i> subsp. <i>stewartii</i> DC283	AHIE000000000	5.23	4,791	65	164	87.69	84.66	Wang et al. (2012)
<i>P. vagans</i> C9-1	CP002206	4.89	4,353	4	98	82.87	79.78	Smits et al. (2010)
<i>P. agglomerans</i> DSM3493 ^T	FYAZ000000000	4.66	4,210	33	88	81.31	79.50	Prakash (<i>unpublished data</i>)
<i>P. deleyi</i> LMG 24200 ^T	MIPO000000000	4.61	3,666	361	737	80.37	79.45	Palmer et al. (2017)
<i>P. septica</i> LMG 5345 ^T	MLJJ000000000	4.31	3,898	89	107	80.37	78.04	Palmer et al. (2017)
<i>P. cyripedii</i> LMG 2657 ^T	MLJI000000000	6.55	5,914	3	126	80.22	77.41	Palmer et al. (2017)
<i>P. conspiciua</i> LMG 24534 ^T	MLFN000000000	4.31	3,616	128	388	71.16	79.92	Palmer et al. (2017)
<i>P. brenneri</i> LMG 5343 ^T	MIEI000000000	4.93	4,058	241	532	71.03	79.70	Palmer et al. (2017)
<i>P. rwandensis</i> LMG 26275 ^T	MLFR000000000	5.78	5,007	69	242	69.03	76.55	Palmer et al. (2017)
<i>P. rodasi</i> DSM 26611 ^T	PIQI000000000	5.75	5,236	31	133	68.47	76.90	Palmer et al. (2017)
<i>P. wallisii</i> LMG 26277 ^T	MLFS000000000	4.65	3,765	149	511	68.09	77.72	Palmer et al. (2017)

^z The superscript "T" denotes type strain.

Inoculum suspensions containing 10^7 ml $^{-1}$ or 10^4 CFU/ml were prepared in PBS to investigate the effect of different inoculum concentrations. Plate counting on LB agar plates was used to determine the CFU number as previously described (Tambong et al. 2008). A 100- μ l aliquot from each inoculum concentration was used to inoculate seedlings (five seedlings/inoculum concentrations). For the negative control, five green onion seedlings were inoculated with 100 μ l of sterile PBS. The seedlings were monitored daily for 21 days. The plants were manually irrigated as required and no fertilizer was applied. To test for the viability of the bacteria-infected tissues, leaves were harvested and chopped in 100 μ l of PBS and the suspension was plated on LB agar medium. Colony identification was based on morphology (yellow, round with irregular edges) and 16S rRNA amplification using primer pair 16S27 (5'-AGAGTTTGTATCMTGGCTCAG-3') and 1492R (5'-TACGGY TACCTTGTTACGACTT-3') (Lane 1991) for genus-level identification. Species-level identification was achieved using *leuS* gene amplification with the LeuS3/LeuS4 primer pair (5'-CAGACCGTGGCTGGCCAACGARCARGT-3'/5'-CGGCGCGCC CCARTARCGCT-3') (Delétoile et al. 2009) followed by DNA sequencing and BLAST. In addition, DNA sequences of the bacterial colonies were analyzed by phylogenetic tree reconstruction after alignment with the MUSCLE algorithm (Edgar 2004) and maximum likelihood with 1,000 bootstrap replicates as implemented in MEGA6 (Tamura et al. 2013). The pathogenicity experiments were repeated twice.

In addition, the collected tissues were processed and subjected to TaqMan real-time PCR to test the reliability of the developed assay to detect *P. allii* in planta with or without DNA extraction. The collected tissue samples (50 mg) were chopped into pieces (1 cm $\times$ 1 cm) using a sterile surgical blade, added to 100 μ l of PBS, and vortexed in order for the bacteria to ooze into the suspension. The approximate bacterial population was determined by culture plating, after serial dilution, of an 100- μ l aliquot on nutrient agar and incubated at 28°C for 48 h. Alternatively, total DNA was extracted from tissues by using the Power Soil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA) according to the manufacturer's recommendations. One microliter of the bacterial suspension obtained from the chopped leaf tissues or purified DNA was subjected to real-time PCR to detect *P. allii* targeting *leuS*. The samples were run in triplicate in two independent setups and fluorescence was captured as indicated above. Samples were evaluated for TaqMan PCR inhibition using a lambda DNA-based internal control system with the IC-65for/IC-65rev primer pair (5'-TAAAGAGTCGA ATGATGTTGGC-3'/5'-GGTCGCTAATACGCTAAAAGAT-3') and a hex-labeled probe (IC-88pb; 5'-Hex-AAATCACATCGT CACCCATT-BHQ1-3') as previously described (Tambong et al. 2008).

Detection of *P. allii* in spiked and naturally infected onion tissues. The two outermost scales of onion bulbs were discarded and the remaining tissues were cut into pieces of approximately 1 cm 2 area. The diced onion tissues (0.25 g) were spiked, in triplicate, with 100 μ l of 10-fold serial dilutions (10^2 to 10^9 CFU/ml) of *P. allii* strain LMG 24248^T. The spiked onion tissues were transferred into the PowerSoil DNA isolation kit (MO BIO Laboratories Inc.) and homogenized using a Precellys 24 Tissue Homogenizer (Bertin Technologies) with default parameter settings. Total DNA of spiked onion tissues was extracted and eluted in 100 μ l of DNA-free sterile water following the manufacturer's instructions. Onion tissues not spiked with the bacteria were used as negative controls. Detection of *P. allii* DNA in the spiked onion tissues was done by real-time PCR assay, in triplicate and in two independent experiments, targeting *leuS* as indicated above using 3 μ l of the total DNA.

The assay was also validated using naturally infected onion plants collected in 2018 in Georgia in the United States. Three onion (*A. cepa* 'Century') scales showing typical water-soaking and tissue-discoloring symptoms were excised from the bulb using a sterile scalpel. Based on the visual disease severity score, eight technical replicates (onion 1: UG-1, UG-1a, and UG-1b; onion 2: UG-2, UG-2a, and UG-2b; and onion 3: UG-3a and UG-3b) were prepared

from the bulbs. The tissue (30 mg) was ground in a sterile mortar and pestle using liquid nitrogen. Total DNA of the ground tissue was extracted from each of the eight samples using an UltraClean Microbial DNA Kit (MO BIO Laboratories Inc.) according to the manufacturer's instructions. The extracted DNA were shipped to the Bacteriology Laboratory in Ottawa, Ontario, Canada, and processed with DNA from healthy field onion as a negative control, in triplicate, using the developed TaqMan real-time PCR assay. In parallel, isolation of the *Pantoea*-like colonies was performed. Symptomatic bulbs were sprayed with 70% ethanol and wiped with sterile paper towel. Small pieces (4 mm 2) of bulb tissue bordering the symptomatic and asymptomatic areas were macerated using a sterile scalpel with a no. 3 blade (DR Instruments, Palos Hills, IL) in 20 μ l of 0.1 M PBS. Tissue macerates were streaked on nutrient agar plates and incubated for 48 h at 28°C. Eight colonies showing typical *Pantoea* colony morphology and color were purified by restreaking and assayed using the *P. allii*-specific TaqMan real-time PCR reported here. In addition, two *Pantoea*-like strains (2229 and 2221) isolated from symptomatic onion in Quebec, Canada, were also processed by real-time PCR. All samples were evaluated for PCR inhibition using the lambda DNA-based internal control system indicated above.

Sequence accession numbers. The nucleotide sequences were deposited in the GenBank database with accession numbers MN059882 to MN059888 and MN065819 to MN065825 for 16S rRNA and *leuS*, respectively.

Results

Relationship between genome-based ANI and LNI. The draft genome of *P. allii* LMG 24284^T (NTMH00000000) obtained in this study has 57 contigs, a size of 5.24 Mb with 4,722 coding sequences, and 159 pseudogenes (Table 2). This sequence was used to compute the ANI values relative to other *Pantoea* species. ANI values between *P. allii* and the other *Pantoea* species ranged from 76.55 to 88.65, whereas LNI values ranged from 68.09 to 89.41% (Table 2). *P. allii* shared the highest ANI and LNI values with *P. ananatis* (88.64 and 89.41%, respectively) and *P. stewartii* (84.33 or 84.66 and 88.01 or 87.69% respectively), depending on the subspecies (Table 2). LNI correlated positively ($\rho = 0.8392$) and significantly ($t = 5.3457$, $df = 12$, $P < 0.001$) with genome-based ANI. Figure 1 shows a linear relationship between LNI and ANI with a highly significant coefficient of determination, suggesting that nucleotide variations in the 642-bp *leuS* gene fragment can provide reliable differentiation of *P. allii* from the other *Pantoea* species.

Verification of primers and probe by BLAST. Figure 2 shows the partial alignment of the *leuS* gene fragment with the position of the selected primers and probe used in this study. Preliminary evaluation of the two reverse primers identified *allii-leuS*-235R to be better than *allii-leuS*-320R based on the consistency of the sigmoidal graph (Fig. 3). Figure 3A shows consistent amplifications (increments in threshold cycle [C_T] values of 3.0 to 3.7) using primer *allii-*

Fig. 1. Plotted values of genome-based average nucleotide identity versus *leuS*-based pairwise percent identity of *Pantoea allii* relative 13 other *Pantoea* species. ** $P < 0.001$.

leuS235R, with a clear distinction between the serial dilutions of *P. allii* DNA. In contrast, the reverse primer allii-leuS320R exhibited inconsistent fluorescence kinetics (Fig. 3B).

Nucleotide sequences of the primers and probe were analyzed by BLAST to evaluate their specificity. A comprehensive sequence primer-BLAST and BLASTn results confirmed the specificities of the primers and probe. Of the more than 3,300 NCBI bacterial strains analyzed by primer-BLAST for the allii-leuS181F/allii-leuS235R primer set, only *P. allii* LMG 24248^T (KF482623) showed a perfect match for the target PCR template. No other bacterial species, including *Enterobacter* spp., *Xanthomonas* spp., *Pectobacterium* spp., and *Stenotrophomonas* spp., showed target PCR templates. In addition, no target templates were found in over 6,600 fungal blast hits analyzed by primer-BLAST. With respect to BLASTn, for example, the forward primer (allii-leuS181F) showed a perfect hit with only *P. allii* LMG 24248^T (KF482623) with an e-value of 0.002. The next best hit was *Paraburkholderia phytofirmans* PsJN (CP001053) at 83% coverage with an e-value of 0.54. A similar pattern was observed for the reverse primer (allii-leuS235R), with mannan polymerase complex of *Capronia epimyces* CBS 606.96 (XM_007732321) as the closest match with an e-value of 1.6. The probe nucleotide sequence had a perfect match (100%, e-value = 0.007) to the type strain of *P. allii* LMG 24248^T. No significant primer and probe hits were recorded with any other *Pantoea* species or distant relatives such as *Pectobacterium* or *Erwinia* species. In addition, no significant BLAST hits were recorded with over 150 genomes of known bacterial and fungal pathogens of onion.

Sensitivity and specificity of detection of *P. allii* DNA. TaqMan real-time PCR was performed with primer set allii-leuS181F/allii-leuS-235R and dual-labeled probe (allii-leuS-205pb) on *P. allii* DNA. Figure 3A shows the fluorescence kinetics of the 10-fold serially diluted *P. allii* DNA (10 ng to 10 fg) assayed using the optimized TaqMan real-time PCR assay. The assay exhibited consistent amplification of *leuS* to 1 pg of *P. allii* DNA (Fig. 3A). The C_T increased with each DNA dilution and C_T value increments of about 3.5 were recorded for each 10-fold dilution of *P. allii* DNA (Fig. 3A and C). A high negative correlation was established between the C_T values and purified *P. allii* DNA concentration, with a coefficient of determination (*r*²) of 0.9901 (Fig. 3C).

The specificity of the TaqMan PCR assay we developed for *P. allii* was evaluated using bacterial strains comprising 34 *Pantoea* (nine *P. allii*) and 20 non-*Pantoea* strains and seven fungal pathogens. The increase in fluorescence crossing the threshold value at a specific cycle indicated positive amplification of the target gene. All *P. allii* strains exhibited a positive detection threshold at a mean C_T of 20.64 ± 2.0 (Fig. 4; Table 3). The presence of the expected band

was confirmed by agarose gel electrophoresis (*data not shown*). Strains (*n* = 52) of the other *Pantoea* species, non-*Pantoea* spp., and fungi/oomycetes as well as the negative controls (water) did not record any detectable fluorescence using the TaqMan assay (Fig. 4; Table 3). Processing of blind-coded samples independently verified the reliability and reproducibility of the assay on a different real-time PCR machine at similar C_T values. Only the blind-coded *P. allii* samples (numbers 1 and 2) gave a positive reaction with a slight difference in the mean C_T values between Chromo4 (20.64 ± 2.0) and Rotor-Gene Q (15.32 ± 0.32). Blind-coded samples of *P. ananatis* (number 3) and *P. stewartii* subsp. *stewartii* (number 4) did not exhibit any detectable fluorescence. These results suggest the specificity of the assay using purified DNA.

Pathogenicity of *P. allii* and real-time PCR detection on infected green onions. Fifteen commercially available green onions were planted and inoculated with *P. allii* under optimal conditions. All *P. allii*-inoculated green onions showed similar yellow discoloration (Supplementary Fig. S1) of the entire inoculated leaf irrespective of the inoculum concentration. No discoloration was observed on PBS-inoculated control plantlets 21 days after inoculation. All total DNA purified or bacterial suspensions from *P. allii*-inoculated green onion plants, 21 days after inoculation, exhibited a positive TaqMan real-time PCR reaction for the presence of *P. allii* (*data not shown*). The fluorescence kinetic curves generated from all inoculated green onions appeared above the preset (0.1) threshold fluorescence at a mean C_T of 30 ± 2.4, indicating the presence of the target DNA. C_T values obtained without DNA extraction were about three cycles higher than purified DNA. The lambda DNA-based internal control showed a detectable and consistent fluorescence at a mean C_T value of 14.8 ± 0.8, suggesting the absence of significant TaqMan real-time PCR inhibition (*data not shown*). Twenty-one days after inoculation, the viability of *P. allii* cells in infected onion tissues was verified by colony isolation. Ten *Pantoea*-like colonies isolated from infected green onions were confirmed to be *P. allii* based on 16S rRNA and *leuS* analyses. *LeuS*-phylogeny inference clustered the bacterial isolates from infected green onions within the *P. allii* LMG 24248^T clade, which is well supported by 100% bootstrap values (Fig. 5). All noninoculated plantlets did not show detectable fluorescence.

Detection of *P. allii* in cultures, spiked onion tissues, and naturally infested onion scale samples. The TaqMan real-time PCR we developed reliably detected *P. allii* in 10-fold serial dilutions of 24-h grown cultures to 10³ CFU/ml (30 CFU per reaction) (Fig. 6A). The fluorescence curves of the positive spiked samples appeared above the preset threshold fluorescence of 0.1 (Fig. 6A), an indication of the presence of the target DNA. In addition, the assay accurately

Fig. 2. Alignment of partial *leuS* nucleotide sequences showing the location of the selected primers and probe sequences and base mismatches. 1 = *Pantoea allii* LMG 24248^T (KF482623), 2 = *P. allii* LMG 24202, 3 = *P. allii* LMG 24203, 4 = *P. allii* isolate UG-G1, 5 = *P. allii* UG-G3, 6 = *P. ananatis* LMG 2665^T (K482626), 7 = *P. stewartii* subsp. *stewartii* LMG 2715^T (K4826218), 8 = *P. stewartii* subsp. *indologenes* LMG 2632^T (K482622), 9 = *P. agglomerans* LMG 1286^T (K482638), 10 = *P. vagans* LMG 24199^T (K4826236), 11 = *P. gaviniae* LMG 25382^T (K482641), 12 = *P. eucalypti* LMG 24197^T (K482635), 13 = *P. anthophila* LMG 2558^T (KF482631), 14 = *P. deleyi* LMG 24200^T (KF482631), 15 = *P. dispersa* LMG 2603^T (K482630), 16 = *P. cyripedii* LMG 2657^T (KF482629), 17 = *P. brenneri* LMG 5343^T (MJEI00000000), 18 = *P. rodasii* DSM 26611^T (PIQI00000000), 19 = *P. septica* LMG 5345^T (KF482640), 20 = *P. calida* LMG 25383^T (KF482639), 21 = *P. nyandensis* LMG 26275^T (MIFR00000000), 22 = *P. wallisii* LMG 26277^T (MLFS00000000), 23 = *P. conspicua* LMG 24534^T (MLFN00000000), 24 = *Tatumella citrea* ATCC 31623^T (KF482643), 25 = *T. terreus* CIP 105600^T (KF482642). LMG = Laboratorium voor Microbiologie at Rijks Universiteit (Gent, Belgium), and ATCC = American Type Culture Collection.

detected the DNA of *P. allii* in onion samples spiked with 10^4 CFU/ml or higher (Fig. 6B). This corresponds to a detection limit of approximately 120 CFU/g of onion tissue. The assay did not show any detectable fluorescence with unspiked onion tissues and negative control samples (Fig. 6B).

Assay validation was done using naturally infected onion bulbs collected in 2018. All of the onion samples exhibited detectable fluorescence. Six samples (UG-1, UG-1a, UG-1b, UG-2, UG-3a, and UG-3b) showed a mean C_T at 12.50 (Fig. 7). Two field infested onion samples, UG-2 and UG-2a, had relatively lower C_T values of 28.10 and 30.56, respectively (Fig. 7). Healthy onion plants and no DNA template used as negative controls did not exhibit any detectable fluorescence (Fig. 7). In addition, all three *Pantoea*-like colonies obtained from the same onion bulbs gave detectable fluorescence with a mean C_T value of 19.20, suggesting that these strains could be affiliated taxonomically with *P. allii*. On the contrary, two *Pantoea*-like strains (2229 and 2221) isolated from diseased onion bulbs

in Quebec, Canada, did not exhibit detectable fluorescence. No PCR inhibition was detected based on the internal control TaqMan real-time PCR assay previously reported (Tambong et al. 2008).

Discussion

This study reports the development and validation of a TaqMan real-time PCR assay for reliable detection of *P. allii* in pure cultures, artificially infected green onions, and diseased onion bulbs from the field targeting the *leuS* gene fragment. *leuS* was targeted because it has high nucleotide variability for differentiation of *P. allii* from other *Pantoea* species. For example, within this 642-bp *leuS* gene fragment, *P. allii* differed from *P. ananatis* or *P. stewartii* (its closest phylogenetic relatives) by 68 or 79 nucleotides, respectively. In this study, we demonstrated that a 642-bp *leuS* gene fragment correlated positively and significantly with a widely used genome-based similarity index, the ANI. This is not surprising, given that our previous study identified *leuS* as a reliable phylogenetic marker for the genus *Pantoea* (Tambong et al. 2014).

The TaqMan real-time PCR assay developed here is the first reliable assay for detection of *P. allii* in pure cultures, naturally infected onion bulbs, and artificially inoculated green onions. The only molecular tool reported thus far is that of Figueiredo and Paccola-Meirelles (2012), a conventional PCR assay that differentiates *P. allii* from *P. ananatis* based on agarose gel electrophoretic separation of a 28-nucleotide difference. That assay was designed to detect *P. ananatis*, the causal agent of maize spot disease (Figueiredo and Paccola-Meirelles 2012; Paccola-Meirelles et al. 2001), but caused cross-reactions with *P. allii*. This is not surprising, because *P. ananatis* is phylogenetically the closest species to *P. allii*. The assay developed by Figueiredo and Paccola-Meirelles (2012) could lead to misidentification, given that the published data indicate that 24

Fig. 3. Consistency and sensitivity of detection of *Pantoea allii* DNA in 10-fold serial dilutions by TaqMan real-time PCR targeting *leuS* based on fluorescence kinetics using two reverse primers: **A**, *allii*-leuS235R and **B**, *allii*-leuS320R. Serial dilutions were as follows: 1 = 10 ng/μl, 2 = 1 ng/μl, 3 = 0.1 ng/μl, 4 = 0.01 ng/μl, 5 = 0.001 ng/μl, 6 = 0.0001 ng/μl, 7 = 0.00001 ng/μl, and 8 = water blank. **C**, Standard curve, generated using the *allii*-leuS181F/*allii*-leuS235R primer set and probe *allii*-leuS205pb, showing the log of DNA dilutions and the corresponding threshold cycle. The regression equation and the coefficient of determination (r^2) are indicated. Each dot represents mean data of triplicate TaqMan PCR amplifications of three independent runs.

Fig. 4. Fluorescence kinetics showing the specific detection of purified genomic DNA of *Pantoea allii* strains using TaqMan real-time PCR targeting the *leuS* gene fragment. To note, only strains of *P. allii* exhibited detectable fluorescence above the preset threshold. Nine strains of *P. allii* were run in triplicate and repeated twice.

Table 3. Specificity of detection of purified genomic DNA of *Pantoea allii* strains using TaqMan real-time PCR targeting the *leuS* gene fragment

Bacterial species/strain ^y	Strains detected/ tested, n (%)	Mean $C_T \pm SD^z$
<i>Pantoea</i> strains (n = 33)		
<i>P. allii</i>	9/9 (100)	20.64 $\pm$ 2.0
Other <i>Pantoea</i> spp.	0/25 (100)	N/A
Non- <i>Pantoea</i> (n = 22)	0/20 (0)	
<i>Pectobacterium</i>	0/2 (0)	N/A
<i>Xanthomonas</i>	0/3 (0)	N/A
<i>Bacillus</i>	0/2 (0)	N/A
<i>Dickeya</i>	0/2 (0)	N/A
<i>Clavibacter</i>	0/4 (0)	N/A
<i>Pseudomonas</i>	0/4 (0)	N/A
<i>Burkholderia</i> spp.	0/1 (0)	N/A
<i>Streptomyces</i>	0/2 (0)	N/A
Fungi/oomycetes	0/7 (0)	N/A

^y Only strains of *P. allii* exhibited detectable fluorescence.

^z C_T = threshold cycle and N/A = not applicable.

strains of *P. ananatis* exhibit an identical amplicon size of 361 bp as *P. allii* strains. Another inherent problem of conventional PCR assays is that they require post-PCR handling to visualize the results for pathogen identification and as such are prone to errors. The TaqMan real-time PCR assay reported here would minimize these problems, resulting in accurate and reliable detection of *P. allii*.

The primers and probe targeting the *leuS* gene fragment were specific in differentiating *P. allii* from 51 strains of other *Pantoea* and non-*Pantoea* species, including *P. ananatis*, *P. stewartii* subsp. *stewartii*, and representative strains of the genus *Pectobacterium* as well as fungal pathogens. The potential high specificity and discriminatory power could be attributable to the six nucleotide mismatches distributed along the length of the probe (Fig. 2). In addition, assay reliability and reproducibility were confirmed on another real-time PCR machine (Rotor-gene; Qiagen) using the same conditions and protocol without optimization. This indicates easy transferability of the assay with consistent results across different laboratories and real-time PCR platforms with minimal adjustments. The slight

difference in the mean C_T values between Chromo4 (20.64 ± 2.0) and Rotor-Gene Q (15.32 ± 0.32) could be attributed to differences in the optical fluorescence detection systems required for the range spanning from ultraviolet to infrared wavelengths.

The TaqMan assay sensitivity of 1 pg of *P. allii* DNA compares favorably relative to conventional PCR (Coplin et al. 2002). A similar detection limit has been reported for other bacterial pathogens (Panicker and Bej 2005; Tambong et al. 2016). In addition, the TaqMan assay reliably detected 30 CFU per PCR reaction in pure cultures of *P. allii*. Tambong et al. (2008) reported consistent detection of 10 CFU per reaction of *P. stewartii* by TaqMan real-time assay, whereas Fegan et al. (1998) reported detection of 200 cells of *Ralstonia solanacearum* using conventional PCR. In *P. allii*-spiked onion tissues, our assay showed reliable detectable fluorescence at a 10-fold lower detection limit (300 CFU per reaction) compared with pure cultures. This could be a result of the inefficiency of the DNA extraction method leading to loss of *P. allii* DNA during processing. Second, the high onion DNA amounts

Fig. 5. *leuS*-phylogenetic tree, with the highest log likelihood ($-4,953.2660$), of bacterial isolates recovered from *Pantoea allii*-infected green onions, naturally infested bulb onion, the type strain of *P. allii* LMG 24248^T, and other *Pantoea* species, with *Tatumella* spp. as the outgroup. Maximum likelihood was used with 1,000 bootstrap replicates after sequence alignment using the MUSCLE algorithm (Edgar 2004) as implemented in MEGA6 (Tamura et al. 2013). Bootstrap values below 50% are not shown. Only four of the 10 colonies isolated from infected green onions and identified as *P. allii* as well as two isolates from field bulb onion are used in the phylogenetic analysis. Asterisks indicate *P. allii* strains isolated in 2018 from naturally infested onion bulbs.

could physically interfere with the amplification of the relatively low *P. allii* DNA in the samples. However, based on the data from lambda internal control assays, only minimal and nonsignificant PCR inhibition was observed.

The assay was validated using naturally diseased onion bulbs. All field diseased onion samples with typical symptoms exhibited detectable fluorescence within the acceptable C_T range (<35 cycles). However, samples UG-1, UG-1a, UG-1b, UG-2, UG-3a, and UG-3b had similar mean C_T values that were about 16 or 18 cycles lower than the mean C_T values of samples UG-2a and UG-2b, respectively. The low C_T values indicate high amounts of *P. allii* DNA, consistent with a high number of *P. allii* cells in the sample. Interestingly, samples UG-2 (mean C_T = 12.50), UG-2a (mean C_T = 28.10), and UG-2b (mean C_T = 30.56) are replicates of the same diseased onion bulb but differ in their mean C_T values, suggesting differences in the amount of *P. allii* DNA in these replicates. This could indicate a nonuniform colonization of a diseased onion bulb by *P. allii*, which may be expected in samples from the field. Based on the standard curve (data not shown) derived from spiked onion tissues, the population densities on naturally infected onions ranged from 1.35×10^5 to 11.7×10^9 CFU/ml. Sample UG-2b had the lowest density of 1.35×10^5 CFU/ml. This density is close to the detection limit (10^5 CFU/ml) of the assay and thus is prone to some variability as a result of pipetting. This might explain why one of the replicates showed a slightly high C_T value. However, the C_T value of this replicate is still below the cutoff value for accurate and reliable *P. allii* detection using the assay. These data on differential C_T values indicate that the real-time PCR assay reported here could be used to determine the relative abundance of the pathogen

in plant tissues or the environment. The presence of viable *P. allii* cells in all of the diseased onions was confirmed by colony isolation and identification by DNA sequencing and detection by real-time PCR.

Testing of two *Pantoea*-like strains from diseased onion bulbs in Quebec did not yield a detectable fluorescence. Further taxonomic characterization based on 16S rRNA and *leuS* genes identified strain 2229 to be *P. ananatis*, whereas strain 2221 could be affiliated with *Kosakonia cowanii* with high confidence. The genus *Kosakonia* is one of the closest genera to genus *Pantoea*. This explains why these two strains did not exhibit a positive reaction with the real-time PCR assay reported here.

The TaqMan assay detected DNA of *P. allii* in all artificially inoculated green onions, 21 days after inoculation. The growth of bacteria ($>10^6$ CFU/g) on LB confirms the viability of *P. allii* cells in tissues of green onion leaves 21 days after inoculation, suggesting that the green onion tissues provide a stable environment for multiplication and disease induction. The mode of transmission of this pathogen is still to be unraveled. However, *P. allii*-contaminated onion seeds and soil could be potential sources of propagation of the disease. This TaqMan assay could be used to detect the presence of *P. allii* in diverse sample types.

The reported TaqMan assay targeting *leuS* is highly reproducible and compares favorably to the only conventional PCR assay reported by Figueiredo and Paccolla-Meirelles (2012). Our assay offers real-time monitoring of the targeted *leuS* amplicon with high specificity and no postamplification handling, thus minimizing sources of error and misidentification. In addition, the real-time PCR technique is robust and can be automated with potential high-throughput analysis of up to 96 samples (Tambong et al. 2008). Real-time PCR also offers the possibility of multiplexing for multiple pathogen detection in onions or *Allium* species. This study also showed, for the first time, that *P. allii* can cause yellow discolorations on green onion leaves. This could reduce the marketable value of the harvest, with potential income loss to onion growers. Because *P. allii*-incited diseases have not been reported in Canada, precautionary measures are needed to minimize the introduction of the pathogen through imported onions. The TaqMan assay validated in this study could be useful to regulatory agencies for screening onion imports for the presence of *P. allii*.

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Fig. 6. A, Sensitivity of detection of serially diluted *Pantoea allii* cultures and B, spiked onion tissues followed by total DNA extraction by TaqMan real-time assay targeting *leuS*. Mean viable plate counts were as follows: sample 1 = 10^8 CFU/ml, sample 2 = 10^7 CFU/ml, sample 3 = 10^6 CFU/ml, sample 4 = 10^5 CFU/ml, sample 5 = 10^4 CFU/ml, sample 6 = 10^3 CFU/ml, sample 7 = 10^2 CFU/ml, and sample 8 = negative control (autoclaved Milli-Q water) or unspiked onion tissue. Three microliters of the bacterial suspension or total DNA was assayed by TaqMan real-time PCR. The mean threshold values are given above the graph. The data are means of three independent runs.

Fig. 7. Detection of *Pantoea allii* LMG 24248^T in naturally infested onion bulb tissues by TaqMan real-time PCR targeting *leuS*. Lowercase letters indicate the following: a = infested onion samples UG-1, UG-1a, UG-1b, UG-2, UG-3a, and UG-3b; b = positive control (0.1 ng of *P. allii* DNA); c = infested onion sample UG-2a; d = infested onion sample UG-2b; and e = healthy onion and negative control (no *P. allii* DNA). The mean threshold values are given above the graph. The data are means of three independent runs.

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